Sarcolipin overexpression improves muscle energetics and reduces fatigue

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Sarcolipin overexpression improves muscle energetics and reduces fatigue. J Appl Physiol 118: 1050–1058, 2015. First published February 20, 2015; doi:10.1152/japplphysiol.01066.2014.—Sarcolipin (SLN) is a regulator of sarcoendoplasmic reticulum calcium ATPase in skeletal muscle. Recent studies using SLN-null mice have identified SLN as a key player in muscle thermogenesis and metabolism. In this study, we exploited a SLN overexpression (SlnOE) mouse model to determine whether increased SLN level affected muscle contractile properties, exercise capacity/fatigue, and metabolic rate in whole animals and isolated muscle. We found that SlnOE mice are more resistant to fatigue and can run significantly longer distances than wild-type (WT). Studies with isolated extensor digitorum longus (EDL) muscles showed that SlnOE EDL produced higher twitch force than WT. The force-frequency curves were not different between WT and SlnOE EDLs, but at lower frequencies the pyruvate-induced potentiation of force was significantly higher in SlnOE EDL. SLN overexpression did not alter the twitch and force-frequency curve in isolated soleus muscle. However, during a 10-min fatigue protocol, both EDL and soleus from SlnOE mice fatigued significantly less than WT muscles. Interestingly, SlnOE muscles showed higher carnitine palmitoyl transferase-1 protein expression, which could enhance fatty acid metabolism. In addition, lactate dehydrogenase expression was higher in SlnOE EDL, suggesting increased glycolytic capacity. We also found an increase in store-operated calcium entry (SOCE) in isolated flexor digitorum brevis fibers of SlnOE compared with WT mice. These data allow us to conclude that increased SLN expression improves skeletal muscle performance during prolonged muscle activity by increasing SOCE and muscle energetics.

Ca2+ ATPase; muscle fatigue; muscle metabolism

SKELETAL MUSCLE HAS AN ENORMOUS capacity to adapt to increased contractile demands, whether they are acute or prolonged, as found during endurance exercise. During acute exercise, this is accomplished by accelerating muscle metabolism via increased glucose (37) and fatty acid metabolism (12). On the other hand, during prolonged aerobic exercise training, as found in marathon runners, muscle is able to adapt by switching fiber type (52) and altering its metabolic phenotype with increased reliance on fatty acid as a substrate (22). Emerging data suggest that skeletal muscle is more than a contractile machine; it is a major determinant of basal metabolic rate (55) and can also play important roles in adaptive thermogenesis during cold exposure and diet overload (6, 9). Moreover, the failure of skeletal muscle to balance energy supply and demand can lead to fatigue (53), hypothermia, or even excess weight gain (48). Many studies have implicated that alteration in cytosolic calcium (Ca2+) (that increases to micromolar range) during muscle activation serves as the mediator between sarcoplasmic reticulum (SR) and mitochondria in coupling the energy (ATP) demand with its supply (7, 17–18, 21). The SR Ca2+ cycling thus has dual function; it not only regulates the amplitude and duration of muscle contraction, but also can serve as a signal to amplify metabolism. Inability to maintain this balance of supply and consumption of ATP leads to loss of force, which is perceived as fatigue. Another factor resulting in loss of force is the depletion of SR Ca2+, which occurs during repeated contractions. The depleting Ca2+ store in the SR is sensed by stromal interaction molecule (STIM) on the SR membrane, which then interacts with and opens sarcolemmal Ca2+ channels in a process called store-operated calcium entry (SOCE) (46, 54). It has been shown that SOCE increases cytosolic Ca2+ and is vital during intense physiological challenges (32), and any defect in this process makes the muscle highly susceptible to fatigue (24, 51). It is thus evident that alterations in SR Ca2+ handling can have profound effects on skeletal muscle function and metabolism.

The sarcoendoplasmic reticulum Ca2+ ATPase (SERCA) pump in muscle is responsible for refilling the SR and causing muscle relaxation. It is encoded by SERCA1 and SERCA2 genes (35); SERCA1 isoform is predominant in fast-twitch muscle, whereas SERCA2 is abundant in slow oxidative fibers (35). SERCA activity in muscle plays a crucial role, both in maintaining the resting Ca2+ concentration and the SR Ca2+ load needed for muscle contraction (26, 34). The SERCA pump activity is regulated by phospholamban (PLB) and sarcolipin (SLN) in muscle. The role of PLB as an affinity modulator of SERCA and its role in cardiac muscle physiology have been extensively investigated (27, 42). However, the functional relevance of SLN is only beginning to be understood. SLN is 31 amino acids long and is abundantly expressed in all skeletal muscles (30). We and others have recently shown that SLN interaction with SERCA is different from PLB; it interacts with SERCA even at high Ca2+ concentrations to inhibit maximal rate of Ca2+ uptake (3) and promotes uncoupling of Ca2+ uptake from ATP hydrolysis (28, 39). Smith et al. (44) showed that increasing the ratio of SLN to SERCA in a reconstituted SR vesicle system increases heat generation. We further investigated the role of SLN-mediated heat generation in muscle with the use of SLN-null (Sln−/−) mice. Our data showed that loss of SLN can...
lead to severe hypothermia when subjects are exposed to acute cold (6). Interestingly, mice lacking SLN became highly obese when fed a high-fat diet (6), which suggested that SLN-mediated uncoupling of SERCA also regulates muscle metabolism.

SLN can prolong cytosolic Ca\(^{2+}\) transient by uncoupling SERCA (3–5), and an increase in cytosolic Ca\(^{2+}\) has been shown to amplify mitochondrial metabolism (21). Because increased muscle metabolism is synonymous with repeated/prolonged muscle function, an inability to meet energy demand will lead to skeletal muscle fatigue (25). Thus the greater the ability of muscle to maintain ATP supply and SR Ca\(^{2+}\) store, the slower it will fatigue. Therefore, we tested the novel hypothesis that SLN, by uncoupling SERCA, can positively influence skeletal muscle energetics and function. Toward this goal, we studied prolonged exercise capacity, muscle contractility, fatigue resistance, and SOCE in a SLN overexpression (SlnOE) mouse model, which has higher SLN expression in all skeletal muscles than wild-type (WT) mice. Our data suggest that increased SLN expression is beneficial to muscle function during prolonged low-intensity exercise; it increases exercise capacity in mice and makes the muscle resistant to fatigue by enhancing SOCE and muscle energetics.

**MATERIALS AND METHODS**

**Ethical approval.** All study protocols were approved by the Ohio State University Institutional Animal Care and Use Committee. All of the animal procedures were carried out at our Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*.

**Generation of SlnOE mice.** The SLN overexpression mice (C57BL/6) were generated in collaboration with Dr. Jeffery Molkenstine. The overexpression of the NH\(_2\)-terminal flag-tagged SLN transgene in SlnOE mouse muscles was achieved using the human skeletal α-actin promoter, which drives expression of SLN transgene in all skeletal muscles but not in heart. The mice were housed at 23°C, up to five per cage, with a 12-h:12-h light/dark cycle, and had ad libitum access to regular chow diet (Harlan Laboratories) and water. Experiments were performed on male SlnOE and WT littermates between the ages of 3 and 6 mo.

**Gene expression profiling.** Total RNA was isolated from the extensor digitorum longus (EDL) from SlnOE and WT (n = 4 for each) using the TRIzol reagent in accordance with the manufacturer’s guidelines. RNA (0.5 μg) from EDL was reverse transcribed using the ThermoScript RT-PCR System (Life Technologies). Semiquantitative PCR was performed for myosin heavy chain (MHC) isoforms, using the following forward and reverse primers: MHCIIa: 5′-GAGGAGGATGAAAGGTGTTC-3′ and 5′-GAGATGGCTTGGAAAGAC-3′; MHCIIb: 5′-ACAAGCTGCGGTTGGAAGG-3′ and 5′-CGAGACGTACAAAGAAGCC-3′; MHCIId: 5′-CCAGAGTCAAGGAAATG-3′ and 5′-AGGAGACTGAGACCTG-3′; and MHCIIc: 5′-CAGAGGGCTTGAATGAGAGG-3′ and 5′-GCAAGAGTGCCCCACG-3′ (40).

**Protein quantification by Western blotting.** Western blotting was performed to determine the levels of SLN, SERCA1a, SERCA2a, calquestrin1 (CQ1), CQ2, carnitine palmitoyl transferase 1 (CPT1), lactate dehydrogenase (LDH), mitochondrial electron transport chain (ETC) subunit proteins, and citrate synthase (CS) in the muscles of SlnOE and WT mice (n = 4 for each). Tissue homogenates were separated using standard SDS-PAGE gels. The protein was immunoprobed, after transfer to nitrocellulose membrane, with custom-made primary antibodies, including SLN (1:500), SERCA1a (1:5,000), SERCA2a (1:5,000), CQ1 (1:3,000), and CQ2 (1:3,000), and commercial antibodies, including CPT1 (1:1,000; CPT1M11A; Alpha Diagnostics International), LDH (1:1,000; sc33781; Santa Cruz Biotechnology), mitochondrial ETC proteins (1:1,000; ab110413; Abcam), and CS (1:1,000; ab96600; Abcam), followed by horseradish peroxidase-conjugated secondary antibody. Signals were detected by WestDura substrate (Pierce) and quantified by densitometry (ImageJ 1.41o program).

**Measurement of basal metabolic parameters.** The basal metabolic parameters like oxygen consumption, respiratory exchange ratio (RER), and activity of the SlnOE mice and their WT littermates (n = 9 for each) were determined at 23°C using a Continuous Laboratory Animal Monitoring System (CLAMS) from Columbus Instruments, as described by Bal et al. (6). In brief, the mice were housed in individual cages with food and water, and oxygen consumed and carbon dioxide released were recorded over a 48-h period. The physical activity (horizontal and vertical) of each mouse was monitored using a multidimensional infrared light-detection system placed on the bottom and top levels of each individual cage of the CLAMS. A break in the infrared beam by mouse movement was counted as a single activity unit.

**Endurance running test.** WT (n = 15) and SlnOE mice (n = 16) were acclimatized to the treadmill (Columbus Instruments) during a 3-day running protocol. Mice ran at a speed of 10 m/min for 10 min with 0° incline on day 1, with 5° incline on day 2, and with 10° incline on day 3. On day 4 the endurance test was performed at 23°C, and the speed, incline, and time were changed as follows: 6 m/min, 0° incline for 8 min; 6–14 m/min, 0° incline, +1 m/min every min; 14 m/min, 5° incline for 30 min; 16 m/min, 10° incline for 30 min; and 18 m/min, 15° incline until exhaustion. The mice were made to run by mild shocks from a shock grid. Exhaustion was defined as 10 s on the shocker, at which point the run was terminated (19). The running protocol used is based on treadmill running endurance protocols for mice used by investigators in the field (11, 19, 33).

**Maximal oxygen consumption test.** The maximal rate of oxygen consumption was determined by treadmill running following the protocol by Ostler et al. (31), with certain modifications. Mice (n = 7) were placed on the treadmill, which was connected to the CLAMS setup for simultaneous measurement of oxygen and carbon dioxide (CO\(_2\)). The speed, duration, and incline were changed as follows: 0 m/min, 5° incline; 6 m/min, 0° incline; 6 m/min, 5 m/min, 0° incline; 7, 8, 9, and 10 m/min, 0° incline for 30 s each, 20° incline; and +1 m/min, each 1 min, 20° incline thereafter until exhaustion (5 s on shock grid). The maximal oxygen consumption (VO\(_2\) max) was determined by the peak oxygen consumption reached during the run when the respiratory exchange ratio was close to or above 1.0 (31).

**Isolated muscle contractile studies.** In vitro studies with isolated EDL (n = 8) and soleus (n = 6) muscles from WT and SlnOE mice were performed in a TIOX tissue bath system (Hugo Sachs Elektronik-Harvard Apparatus). EDL and soleus muscles were chosen as representative fast and slow muscles, respectively (41), to test whether SLN overexpression had modified the contractile properties and energetics of these two types of muscle. In addition to glucose, we also used pyruvate as a substrate for these studies. Pyruvate is a preferred substrate in slow oxidative muscle, and it is shown to potentiate force in slow oxidative muscle like soleus but to a lesser extent in fast glycolytic muscle like EDL (36). Thus we used pyruvate as a substrate to test whether overexpression of SLN in mouse EDL improved the oxidative capacity of the muscle. The in vitro experiments were performed at 30°C to ensure that optimal muscle function was maintained throughout the duration of the measurements. Although performing the experiments at higher, more physiological temperatures could affect the mechanical properties of muscle, this effect would be similar in WT and SlnOE muscles, and their differences would most likely persist. A square pulse electrical stimulator (Hugo Sachs Elektronik; type 263) was used for muscle stimulation, and a data acquisition platform (ADInstruments PowerLab 4/35 Data Acquisition System and LabChart 7 software) was used to record and
analyze muscle contractile responses. The $Sln^{OE}$ and WT mice were euthanized by CO$_2$ and EDL or soleus was immediately harvested and transferred to tyrode buffer (121.0 mM NaCl, 5.0 mM KCl, 1.8 mM CaCl$_2$, 0.4 mM Na$_2$HPO$_4$, 0.5 mM MgCl$_2$, 24.0 mM NaHCO$_3$, and 0.1 mM EDTA) with 10.0 mM glucose bubbled with 95% oxygen-5% CO$_2$. The muscle was tied at the myotendonous junction using 5/0 surgical silk and mounted in the TIOX tissue bath between a force transducer (Panlab TR1201) and a fixed hook (43), containing preoxygenated tyrode buffer with 10.0 mM pyruvate as substrate. The muscle was then performed, followed by 8 min of recovery. The muscle was then allowed to equilibrate for 30 min followed by a 300 for soleus) during the fatigue protocol.

The substrate was then changed to 10.0 mM glucose in tyrode buffer, containing 3 mg/ml collagenase A (Roche) for 45 min at 37°C, and fibers were isolated by titurating the muscle. The fibers were loaded with 5 μM fura-2 AM for 30 min, and then fura-2 AM was washed away. The fibers were then incubated with 60 μM N-Benzyl-p-toluene sulphonamide (BTS) (Sigma), a specific myosin II inhibitor, for 15 min to prevent contraction (15). The basal resting Ca$^{2+}$ and changes in intracellular Ca$^{2+}$ transients were recorded using dual-wavelength (excitation at 350 nm and 380 nm) PTI spectrofluorometer (Photon Technology International). The SR store was then depleted using 0 Ca$^{2+}$ buffer containing 0.5 mM EGTA, 20 mM caffeine, 1 μM ryanodine, 10 μM thapsigargin, and 60 μM BTS. After 30 min, when Ca$^{2+}$ had returned to basal levels, buffer containing 2.5 mM Ca$^{2+}$ was added, and fura-2 AM response was recorded. The difference between basal and peak fura-2 AM ratio (350/380 nm) was considered a SOCE response. Finally, 100 μM 2-aminoethoxydiphenyl borate (2-APB) was added to inhibit sarcolemmal Ca$^{2+}$ channels and confirm that Ca$^{2+}$ response seen was SOCE (10). The final values for calculations were obtained by subtracting background 350-nm and 380-nm fluorescence from that of the fiber.

Statistics. Data are presented as means ± SE. Statistical analysis was performed using the Prism 6.0 software. Student’s unpaired t-test was used to determine statistically significant differences. $P < 0.05$ was considered significant.

RESULTS

Overexpression of SLN did not affect the expression level of SERCA, CSQ, myosin isoforms, and muscle morphology. SLN overexpression in mouse skeletal muscle was achieved by the human skeletal α-actin promoter, which drives high-level SLN transgene expression in both fast- and slow-twitch muscle. In WT mice, SLN is expressed predominantly in slow oxidative muscles (soleus and diaphragm) and at very low levels in fast glycolytic muscles (quadriceps and EDL) (Fig. 1A). In $Sln^{OE}$ mice, however,
the expression of SLN is higher in fast-twitch muscle over slow-twitch muscle (Fig. 1A), which may be attributable to higher SR volume and SERCA expression, which are three- to fourfold higher in fast compared with slow muscle. Compared with WT littermates, the Sln\(^{OE}\) mice express higher levels of SLN protein in both fast- and slow-twitch skeletal muscles (Fig. 1A). Moreover, this particular line of Sln\(^{OE}\) mice was chosen for the present study because the pattern of SLN expression, higher in fast-twitch muscles and lower in slow-twitch muscles, is similar to that of larger mammals (2, 38). However, SLN expression was not altered in atria and ventricle of larger mammals (2, 38). However, SLN expression was not because the pattern of SLN expression, higher in fast-twitch muscles and lower in slow-twitch muscles, is similar to that of larger mammals (2, 38). However, SLN expression was not altered in atria and ventricle of Sln\(^{OE}\) mice (Fig. 1A) because the SLN transgene is driven by human skeletal α-actin promoter, which is specific to skeletal muscle. Importantly, SLN overexpression did not affect the expression of Ca\(^{2+}\)-handling proteins, including SERCA1α, SERCA2a, and CSQ1 in skeletal muscle (Fig. 1B). We further studied whether SLN overexpression had switched fiber type from fast to slow in fast-twitch muscle (EDL), but our analysis shows that the mRNA expression of myosin isoforms (I, IIa, IIb, and IId) remained unaltered in Sln\(^{OE}\) mouse EDL (Fig. 1C). In addition, the overexpression of SLN did not affect muscle morphology, as seen by hematoxylin and eosin staining of diaphragm, soleus, and quadriceps muscles (Fig. 1D).

Sln\(^{OE}\) mice run longer and have higher expression of CPT1 than WT control mice. We first tested whether higher expression of SLN had an effect on basal metabolic parameters using indirect calorimetry. We found that compared with WT mice, the expression of CPT1 in mouse (WT, 1.166 ± 0.79 m, n = 16; WT, 1.428 ± 61 m, n = 15, P = 0.029) (Fig. 3A). In a separate experiment, the maximal rate of oxygen consumption (\(\dot{V}O_{2\text{max}}\)) between WT and Sln\(^{OE}\) mice was found to be similar (WT, 119.5 ± 3.6 mL·kg\(^{-1}\)·min\(^{-1}\), n = 7; Sln\(^{OE}\), 116.6 ± 5.2 mL·kg\(^{-1}\)·min\(^{-1}\), n = 6, P = 0.648) (Fig. 3B), indicating that the improved endurance capacity was not a result of improved heart function.

To determine whether the improved muscle function was due to enhanced oxidative metabolism in muscle, we analyzed the expression levels of specific mitochondrial oxidative phosphorylation proteins in Sln\(^{OE}\) and WT skeletal muscles. We found that compared with WT mice, the expression of CPT1 protein (Fig. 3C), a transporter of long-chain fatty acids present on mitochondrial outer membrane, is significantly higher in Sln\(^{OE}\) fast glycolytic EDL (WT, 0.539 ± 0.173, n = 4; Sln\(^{OE}\), 1.569 ± 0.145, n = 4, P = 0.004) (Fig. 3D) and in slow oxidative soleus (WT, 0.559 ± 0.302, n = 4; Sln\(^{OE}\), 1.453 ± 0.023, n = 4, P = 0.025) (Fig. 3D). Analysis of mitochondrial electron transport chain proteins and CS showed no difference in their expression pattern between WT and Sln\(^{OE}\) soleus (Fig. 3, E and F) and EDL (data not shown) muscles. We also found that the expression level of LDH (Fig. 3G), an important regulator of muscle glycolytic rate and muscle fatigue, was significantly higher in Sln\(^{OE}\) EDL than WT EDL (WT, 0.358 ± 0.035, n = 4; Sln\(^{OE}\), 0.724 ± 0.016, n = 4, P < 0.0001) (Fig. 3H).

EDL of Sln\(^{OE}\) mouse produced a higher twitch force than WT. We studied both a fast glycolytic muscle (EDL) and a slow oxidative muscle (soleus) to understand whether higher expression of SLN altered the contractile properties and energetics of these two types of muscles. The contractile properties were studied in the presence of two different substrates, 10 mM glucose and 10 mM pyruvate. The EDL of Sln\(^{OE}\) produced a significantly higher twitch force than WT in pyruvate (WT, 3.447 ± 0.120 N/cm\(^2\), n = 8; Sln\(^{OE}\), 3.913 ± 0.108 N/cm\(^2\), n = 9, P = 0.011) but not in the presence of glucose (WT, 3.544 ± 0.145 N/cm\(^2\), n = 8; Sln\(^{OE}\), 3.893 ± 0.201 N/cm\(^2\), n = 9, P = 0.189) as a substrate (Fig. 4A and B).

The potentiation of force by pyruvate is higher in EDL overexpressing SLN. The force-frequency analysis of the EDL muscle showed that, compared with WT, higher expression of SLN did not affect the force-frequency curve of EDL, either in glucose or in pyruvate (n = 8) (Fig. 4C). However, at lower frequencies (30 and 50 Hz), the effect of pyruvate is amplified in Sln\(^{OE}\) soleus, as seen in the 50-Hz trace shown in Fig. 4D. Although WT EDL has a significantly higher average specific force than WT soleus (Sln\(^{OE}\), 0.212 N/cm\(^2\), n = 8, P = 0.026), this potentiation of force by pyruvate is greater in Sln\(^{OE}\) EDL (Sln\(^{OE}\) glucose, 3.975 ± 0.121 N/cm\(^2\), n = 8; Sln\(^{OE}\) pyruvate, 5.137 ± 0.21 N/cm\(^2\), n = 8, P = 0.0003) (Fig. 4E).

Higher SLN expression in soleus does not alter its contractile properties. Studies on isolated soleus muscle from WT and Sln\(^{OE}\) mice show that there is no difference in the twitch-force production in 10 mM glucose (WT, 6.359 ± 0.34 N/cm\(^2\), n = 6; Sln\(^{OE}\), 6.250 ± 0.272 N/cm\(^2\), n = 7, P = 0.805), and the effect of pyruvate is also similar in muscle from both groups (WT, 7.290 ± 0.432 N/cm\(^2\), n = 6; Sln\(^{OE}\), 7.182 ± 0.229 N/cm\(^2\), n = 6, P = 0.829) (Fig. 5A). The force-frequency curve shows that the potentiation of force by pyruvate persists equally, even at high frequencies, in both WT and Sln\(^{OE}\) mouse soleus muscles (n = 6) (Fig. 5B).

EDL and soleus muscles from Sln\(^{OE}\) mice are highly resistant to fatigue. We next wanted to investigate whether the fatigue resistance of the Sln\(^{OE}\) mice was also reflected at the individual muscle level. We found that both EDL (Fig. 6A) and

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**Fig. 2.** Overexpression of SLN does not affect the basal metabolic characteristics of Sln\(^{OE}\) mice. A: rate of O\(_2\) consumption of Sln\(^{OE}\) mice is not different from their WT littermates. B: respiratory exchange ratio (RER), which is the ratio of volume of carbon dioxide (V\(_{CO_2}\)) produced to volume of oxygen consumed (V\(_{O_2}\)), is also unchanged between the 2 genotypes. C: basal activity is similar in WT and Sln\(^{OE}\) mice (n = 9 for WT and Sln\(^{OE}\)).
soleus (Fig. 6B) muscles from WT produce significantly lower percentage of initial force than SlnOE muscle at various time points during a 10-min fatigue protocol [percentage of initial force at the 10th min for WT EDL, 33.30 ± 0.78%, n = 5; SlnOE EDL, 39.67 ± 1.59%, n = 6, P = 0.008 (Fig. 6A); percentage of initial force at the 10th min for WT soleus, 15.92 ± 2.44%, n = 5; SlnOE, 27.82 ± 2.50%, n = 6, P = 0.008 (Fig. 6B)]. Moreover, the SlnOE muscles have higher sum-of-average-specific forces than WT muscles over the course of 10 min (Fig. 6, C and D) (WT EDL, 1,246 ± 46 N/cm², n = 5; SlnOE EDL, 1,395 ± 29 N/cm², n = 6, P = 0.018) (WT soleus, 1,717 ± 80 N/cm², n = 5; SlnOE soleus, 2,190 ± 139 N/cm², n = 6, P = 0.021). This shows that SlnOE muscles produce more force but at the same time fatigue less than WT muscles.

FDB fibers from SlnOE mice show significantly higher SOCE activity. We studied SOCE activity using FDB fibers of WT and SlnOE mice. Our studies showed that, compared with WT, SOCE is significantly higher in SlnOE muscle (WT, 1.090 ± 0.072, n = 8; SlnOE, 1.379 ± 0.101, n = 8, P = 0.036) (Fig. 7, A and B).

Fig. 3. Overexpression of SLN improves muscle endurance exercise capacity and increases mitochondrial fatty acid transporter carnitine palmitoyl transferase 1 (CPT1) expression. A: SlnOE mice run significantly longer distances than the WT mice during a prolonged treadmill run. B: maximal oxygen consumption capacity (VO2 max) is similar for the 2 groups of mice. C: Western blot analysis of CPT1 protein showing that, compared with WT, its expression is higher in both EDL (fast glycolytic) and soleus (slow oxidative) muscles of SlnOE mice (EDL, 30 µg; soleus, 30 µg protein loaded). D: densitometric analyses of Western blot showing that CPT1 expression (normalized to GAPDH) is significantly higher in EDL and soleus muscles of SlnOE mice. AU, arbitrary units. E: Western blotting of specific subunit proteins of the mitochondrial electron transport chain complexes (CI, CII, CIII, CIV, and CV) shows that their expression is not altered in SlnOE mouse soleus (5 µg protein). F: expression of citrate synthase, a regulatory protein of Krebs cycle, is similar in WT and SlnOE mouse soleus (5 µg protein). GAPDH is the loading control. G: Western blot analysis showing protein expression of lactate dehydrogenase (LDH) in SlnOE and WT EDL (5 µg protein loaded). H: LDH expression is significantly higher in SlnOE EDL than in WT EDL. GAPDH is the loading control (n = 4 for all Western blot data) (*P < 0.05, **P < 0.01, and ****P < 0.0001).
**DISCUSSION**

SLN is a novel regulator of the SERCA pump in striated muscle, and its role in muscle physiology is not well understood. In a recently published study (6), we showed that loss of SLN in mice can lead to severe hypothermia during acute cold exposure but can be rescued by SLN overexpression. In addition, loss of SLN predisposed mice to develop diet-induced obesity, suggesting that SLN plays a role in muscle energy metabolism.

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**Fig. 4.** Overexpression of SLN increases twitch force and tetanus force (at lower frequencies) in isolated EDL. **A:** twitch profile of WT and SlnOE isolated EDL in buffer containing 10 mM pyruvate or 10 mM glucose as substrate. **B:** isolated EDL from SlnOE mouse produces significantly higher twitch force in 10 mM pyruvate than WT EDL. **C:** SLN overexpression does not affect the force-frequency curve at higher frequencies (>70 Hz), but it shifts the curve further to the left at lower frequencies (especially 30 and 50 Hz) in 10 mM pyruvate compared with 10 mM glucose. **D:** 50-Hz-specific force traces showing that the highest force is produced by SlnOE EDL in 10 mM pyruvate. **E:** SlnOE EDL has a significantly higher average specific force in pyruvate than in glucose at 50 Hz. The thick lines in A and D are the mean, and the thinner dashed lines are the range of SE (*P < 0.05, ***P < 0.001).

**Fig. 5.** The contractile properties of soleus are not altered in SlnOE mice. **A:** twitch force produced by WT and SlnOE mouse isolated soleus is similar in 10 mM glucose, and potentiation of force by pyruvate is also not different. **B:** overexpression of SLN does not affect the force-frequency curve of soleus, in both 10 mM glucose and 10 mM pyruvate, compared with WT soleus.
expenditure and metabolism. The main objective of this study was to determine how increasing SLN expression affects muscle contractile function and its ability to meet increased energy demand such as during exercise. A major finding of this study is that \textit{Sln} \textit{OE} mice show an increase in exercise endurance capacity compared with control littermates. Interestingly, isolated EDL and soleus muscles from \textit{Sln} \textit{OE} mice were more resistant to fatigue, and FDB fibers from \textit{Sln} \textit{OE} showed increased SOCE. We also document that a contributing factor to the increase in endurance capacity of \textit{Sln} \textit{OE} mice is likely an increased oxidative metabolism mediated by the increase in CPT1. CPT1 is thought to be the rate-limiting step for long-chain fatty acid entry into the mitochondria for \( \beta \)-oxidation (23). Moreover, a recent study (14) has shown that overexpression of CPT1 in muscle is sufficient for an increase in fatty acid oxidation and does not require a higher expression of mitochondrial ETC or Krebs cycle proteins, which is the case in \textit{Sln} \textit{OE} mice.

One of the key findings of isolated muscle studies is that, compared with WT, \textit{Sln} \textit{OE} mouse EDL produced higher twitch force and showed a greater pyruvate-induced potentiation of contractile force at lower frequencies. The increased twitch force is likely due to prolongation of calcium transient (attributable to uncoupling of SERCA by SLN) (3–5), resulting in higher activation of myosin cross bridges (47). The higher contractile force observed in the presence of pyruvate can be due to pyruvate-induced increase in myofilament Ca\(^{2+}\) sensitivity (49). This effect of pyruvate coupled with the prolongation of Ca\(^{2+}\) transient in the presence of SLN causes the peak force and force time integral at 50 Hz to be highest in \textit{Sln} \textit{OE} EDL. At higher frequencies, the effect of pyruvate and SLN is abolished, as Ca\(^{2+}\) reaches saturating concentrations in both

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Fig. 6. SLN overexpression significantly reduces fatigue in isolated EDL and soleus muscles. \(A\): during a 10-min fatigue, WT EDL has significantly higher reduction in % force than \textit{Sln} \textit{OE} muscle. \(B\): isolated soleus muscle from \textit{Sln} \textit{OE} mice produces significantly higher % force than WT soleus during a 10-min fatigue protocol. \(C\): sum of average specific forces over the 10-min fatigue is significantly greater for \textit{Sln} \textit{OE} EDL than WT EDL. \(D\): sum of average specific forces over the 10-min fatigue is significantly greater for \textit{Sln} \textit{OE} soleus than WT soleus (*\(P < 0.05\), **\(P < 0.01\)).

Fig. 7. Overexpression of SLN increases store-operated calcium entry (SOCE) in isolated flexor digitorum brevis (FDB) fibers. \(A\): representative trace of SOCE from WT (gray trace) and \textit{Sln} \textit{OE} (black trace) FDB fiber showing increased Ca\(^{2+}\) entry in fibers overexpressing SLN after depletion of SR stores. Black arrow shows addition of caffeine/ryanodine/thapsigargin buffer to deplete stores (WT has similar response as \textit{Sln} \textit{OE}). Gray arrow shows addition of Ca\(^{2+}\) buffer after SR store has been completely depleted. Inset: decline in SOCE after calcium release-activated Ca\(^{2+}\) channels have been blocked by addition of 2-aminoethoxydiphenyl borate (2-APB). \(B\): FDB fibers from \textit{Sln} \textit{OE} show significantly greater SOCE response compared with WT FDB fibers (\(n = 8\)) (*\(P < 0.05\)).
WT and SlnOE EDL and the myosin turnover rate is maximal, resulting in similar peak force in both mouse EDLs regardless of substrate. On the other hand, studies with isolated WT and SlnOE soleus suggest that expression of SLN beyond a certain level does not affect force during a single twitch or single contraction. The reason why potentiation of force by pyruvate is seen even at high frequencies in soleus is because it reduces free phosphate levels in the soleus (16, 36), which in turn can increase the rate of myosin turnover (1), generating more force.

Isolated muscle is an ideal model for studying peripheral fatigue, as it is free of central regulation. An interesting observation of our studies is that both SlnOE EDL and soleus start producing higher force than WT muscle within the first few minutes of fatigue protocol and continue to do so until the end. This suggests that SlnOE muscles are better equipped to supply Ca\(^{2+}\) and generate ATP than WT muscles during repeated contractions. One factor delaying fatigue in SlnOE EDL could be the higher LDH expression. LDH catalyzes the conversion of pyruvate to lactate, which regenerates NAD\(^+\) that is required for continuous GAPDH activity, thus promoting glycolysis and ATP generation (45). The SlnOE EDL would require an increased glycolytic rate to match the higher ATP consumption compared with WT. Another mechanism contributing to the enhanced fatigue resistance seen in SlnOE muscle can be its increased SOCE (51). The increased SOCE seen in the SlnOE mouse muscle could be a compensatory response to the slower refilling of SR Ca\(^{2+}\) in the presence of increased SLN expression. As a result, there could be greater Ca\(^{2+}\) entering the cytosol in SlnOE muscle for similar contractile stimulation. This is particularly important during prolonged muscle function because higher cytosolic Ca\(^{2+}\) in SlnOE muscles can maintain SR Ca\(^{2+}\) load and force production. In addition, higher cytosolic Ca\(^{2+}\) can serve as a metabolic signal and increase oxidative capacity through activation of metabolic enzymes that are Ca\(^{2+}\) sensitive, and this can boost ATP synthesis during high demand such as prolonged exercise (20).

Collectively, our studies suggest that SLN overexpression is beneficial to muscle physiology, as it can enhance whole-animal endurance capacity and energetics. Our studies show that SLN overexpression enhances fatigue resistance in fast and slow isolated muscles without compromising force during isometric conditions. However, as muscles are often undergoing length change during locomotion, additional studies will be required to understand how SLN affects muscle function during more dynamic activities. Moreover, this is a novel finding because SLN overexpression has primed the muscle to respond to increased metabolic demand without causing a switch in muscle fiber type. Further work on this molecule is needed to reveal the intricate details of how SLN orchestrates improved muscle function. In conclusion, we propose that SLN-SERCA interaction is a novel target to increase muscle metabolism to combat metabolic disorders like obesity and type II diabetes.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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