Conditional knockout of Mn-SOD targeted to type IIB skeletal muscle fibers increases oxidative stress and is sufficient to alter aerobic exercise capacity

Michael S. Lustgarten,1 Youngmok C. Jang,2 Yuhong Liu,4 Florian L. Muller,3,4 Wenbo Qi,4 Mark Steinhelper,1 Susan V. Brooks,5 Lisa Larkin,5 Takahiko Shimizu,6 Takuji Shirasawa,6 Linda M. McManus,3,4 Arunabh Bhattacharya,2,4 Arlan Richardson,2,4 and Holly Van Remmen2,4

Departments of 1Physiology, 2Cellular and Structural Biology, and 3Pathology, and 4Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center at San Antonio, San Antonio, Texas; 5Molecular and Integrative Physiology and Biomedical Engineering, University of Michigan, Ann Arbor, Michigan; and 6Molecular Gerontology-Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

Submitted 14 August 2009; accepted in final form 22 September 2009

SKELETAL MUSCLE FATIGUE has been defined as the decline in muscle performance associated with muscle activity (1). An association between oxidative stress and skeletal muscle fatigue was first shown by Davies et al. (10), who measured a twofold increase in carbon-centered free radicals following exercise in rats. This finding was confirmed in rats and subsequently verified in mice and humans (25) by identification of a 70% increase in muscle free radical content following contractile activity. Increases in free radical content following muscle contractile activity are also associated with increased oxidative damage to lipid (11, 12, 49), protein (29, 55), and DNA (36, 52).

Whether increased free radicals and oxidative damage are a cause or a consequence of fatiguing contractile activity has been studied using antioxidant supplementation. Intravenous injection of N-acetylcysteine (NAC), a reduced thiol donor that has general antioxidant properties (4), into anesthetized rabbits reduced diaphragm fatigue by 25% during a protocol of repetitive contractions, a finding that directly link oxidative stress to muscle fatigue (46). In addition, incubation of rat diaphragm muscle fibers with NAC in vitro has been shown to reduce fatigue, eliminating the potential role of NAC in nonmuscle targets (26). Addition of antioxidants such as Cu/Zn-SOD, catalase, and DMSO to isolated muscle fiber bundles has also been shown to reduce muscle fatigue during contractile activity (39). Cu/Zn-SOD and catalase are membrane impermeable, and reduction of fatigue in their presence indicates that extracellular oxidative stress contributes to reduced muscle function. On the other hand, NAC and DMSO are membrane permeable, which suggests that intracellular oxidative stress may limit muscle function. Incubation of skeletal muscle with the membrane-permeable oxidant H2O2 has also been shown to reduce muscle fatigue (39). Cu/Zn-SOD and catalase are membrane impermeable, and reduction of fatigue in their presence indicates that extracellular oxidative stress contributes to reduced muscle function. On the other hand, NAC and DMSO are membrane permeable, which suggests that intracellular oxidative stress may limit muscle function. Incubation of skeletal muscle with the membrane-permeable oxidant H2O2 has also been shown to reduce muscle fatigue (39). Cu/Zn-SOD and catalase are membrane impermeable, and reduction of fatigue in their presence indicates that extracellular oxidative stress contributes to reduced muscle function.

Mitochondria are a possible source of the oxidants that limit contractile activity. A role for mitochondria-generated oxidants is supported by the observation that mitochondrial antioxidant defense was decreased (53), muscle mitochondrial oxidative stress was elevated (30, 57), and treadmill endurance capacity was reduced (28) in mice heterozygous for Sod2 (Sod2+/−). However, the tissue that is limiting with respect to oxidative stress and exercise is not known, inasmuch as all tissues in Sod2−/− mice are reduced in terms of Sod2 content. The purpose of this study was to determine whether skeletal muscle-specific mitochondrial oxidative stress is sufficient to limit muscle function.
Skeletal muscle is not homogeneous with respect to fiber type. Individual skeletal muscle fibers contain myosin heavy chain type I, IIA, IIX, or IIB (42, 43) and are different in terms of oxidative and glycolytic enzyme content. Oxidative enzymes and mitochondria are most abundant in type I muscle fibers, but glycolytic protein is less abundant in type I than in type IIA, IIB, or IIX fibers. Levels of oxidative enzymes and mitochondria are low, but abundance of glycolytic proteins is high, in type IIB muscle fibers (37). Type IIA and IIX fibers are intermediate in terms of combined oxidative and glycolytic capacity. Mitochondria from type I and type II skeletal muscle have been shown to differentially produce reactive oxygen species. Mitochondrial free radical leak was reported to be greater in white gastrocnemius (a muscle rich in type IIB fiber) than in muscles consisting mostly of type IIA (red gastrocnemius) or type I (soleus) fibers (2, 38). In support of these data, mitochondrial protein oxidative damage has been shown to be greater in muscles consisting mostly of type II fibers [tibialis anterior (TA), extensor digitorum longus (EDL), and plantaris] than in the soleus, a muscle enriched with type I muscle fibers (14). The goal of the present report was to investigate the direct role of elevated type II skeletal muscle-specific mitochondrial oxidative stress in muscle function. To address our hypothesis, we used young TnIFastCreSod2fl/fl mice, in which Mn-SOD is reduced by 70% only in type IIB skeletal muscle.

**MATERIALS AND METHODS**

*Creation of TnIFastCreSod2fl/fl mice.* A Cre recombinase-LoxP approach (44) was used to reduce Mn-SOD content specifically in type IIB skeletal muscle fibers. Cre activity is dependent on the activity of the promoter to which it is coupled. The promoter gC1012 [from quail, as prepared by Yutzey et al. (58)] for the inhibitory subunit of troponin (TnIFast) was used to drive Cre recombinase expression. Expression of the TnIFast promoter has been shown to be fourfold greater in type IIB than in type I, IIA, and IIX skeletal muscle fibers (21, 22). An improved version of Cre recombinase, known as iCre (45), has been shown to have greater recombinatorial efficiency in mammalian systems than the Cre as described by Schwenk et al. (44) and was used to drive Cre expression. To create the TnIFastCre construct, we discarded all DNA downstream of the TnIFast ATG initiation codon in exon 2. Upstream of exon 2, the TnIFast promoter contains 530 bp of 5-flanking DNA, exon 1, intron 1, the IRE enhancer (22) and the first (untranslated) part of exon 2 of the quail TnIFast gene. The TnIFastCre junction exists within the TnIFast ATG initiation codon. For creation of the TnIFastCre construct, TnIFast protein-coding sequences upstream of exon 2 were replaced with iCre coding sequences and linked to SV-40 splicing and polyadenylation signals (Fig. 1A). Sod2fl/fl mice (24) were then bred to mice containing TnIFastCre to produce TnIFastCreSod2fl/fl. All mice were from a C57/BL6 background. PCR was used to identify mice carrying the Cre and Flax constructs. For the Cre PCR, 5'-ACTACCTCC TGTACC TGAAGC-3' and 5'-GGAGATGTCCTTCACTCTG- ATT-3' were used as Cre-forward and Cre-reverse primers, respectively. A band at 350 bp is indicative of the presence of the Cre construct. For the Flax PCR, three primers were used: Flax P1 (5'-CGAGGGCATCTAGT GGAAGA-3'), Flax P2 (5'-TTAGGGCCTGAGTTTGTCCTGAGA-3'), and Flax P4 (5'-AGC TTGGCGTGCACTGAA-3') (24). An iCre mouse was identified by the presence of a single band at 358 bp. Mn-SOD activity assays on glycolytic (white gastrocnemius and quadriceps) and oxidative (soleus) muscle homogenates were performed according to the method of Beauchamp and Fridovich (5) after PCR identification of mice containing the Cre and Sod2fl/fl constructs (TnIFastCreSod2fl/fl). All mice were housed under specific pathogen-free barrier conditions. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and the University of Michigan (for contraction studies).

*Animals.* Young (5- to 8-mo-old) female TnIFastCreSod2fl/fl and wild-type mice were used for all experiments, except assays involving the rate of mitochondrial H$_2$O$_2$ and F$_2$-isoprostanes, in which young (3- to 4-mo-old) male mice were used. An animal was considered wild-type in the absence of TnIFastCre and in the presence of Sod2fl/fl.

*Measurement of lean body mass and body fat percentages.* Quantitative magnetic resonance imaging (qMRI) was used to determine the percentages of body fat and lean mass. qMRI measurements of body fat and lean mass provide increased precision, accuracy, speed of results, and ease of use compared with dual-energy X-ray absorptiometry or chemical methods (50, 51). For quantitative magnetic resonance measurements, live mice were placed into a thin-walled plastic cylinder (4.7 cm ID, 0.15 cm thick), where they were free to turn around but were limited to ~4-cm vertical movements by a plastic insert. The plastic cylinder containing the live mice was then placed into the qMRI machine (EchoMRI, Echo Medical Systems, Houston, TX) for measurement of lean and fat mass. Measurements were completed after 2 min, and the mice were returned to their home cage (51).

*Mn-SOD and Cu,Zn-SOD activity.* Mn-SOD and Cu,Zn-SOD activity in tissue homogenates of liver, heart, brain, kidney, lung, and skeletal muscle isolated from wild-type and TnIFastCreSod2fl/fl mice was measured using native gels, as previously described (5). White portions of gastrocnemius and quadriceps muscles were used to
evaluate Mn-SOD activity in mainly glycolytic skeletal muscle. Glycogen 
thomogenates were prepared with a Potter-Elvejhem homogenizer. An equivalent volume of buffer B (buffer A + 0.2% BSA with 1 mM EGTA) was added, and the sample was centrifuged at 12,000 g for 10 min at 4°C. The pellet was resuspended in buffer B and then homogenized. The mitochondrial-rich supernatant was obtained by centrifugation at 600 g. Skeletal muscle mitochondria were pelleted by centrifugation at 7,000 g for 15 min at 4°C. Mitochondria were washed with 2.5 ml of buffer B and centrifuged again at 7,000 g for 15 min at 4°C, the supernatant was removed, and skeletal muscle mitochondria were resuspended in 1 ml of KME (100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA) and centrifuged at 7,000 g for 15 min at 4°C. The resulting supernatant was discarded, and the pellet containing mitochondria was used immediately. Mitochondrial protein concentration was measured using the Bradford method.

Measurement of the rate of mitochondrial H$_2$O$_2$ production. 
Mitochondrial H$_2$O$_2$ release was measured using Amplex Red (N-acetyl-3,7-dihydroxyphenoxazone; Molecular Probes, Eugene, OR)-horse-radish peroxidase (HRP) (59). The assay utilizes HRP to catalyze the H$_2$O$_2$-dependent oxidation of the nonfluorescent compound Amplex Red to the fluorescent resorufin and detects only H$_2$O$_2$ that has been released from the mitochondria, inasmuch as the size of HRP prevents its entry into the mitochondria. The rate of mitochondrial H$_2$O$_2$ production was measured as previously described (33). Briefly, 80 nM Amplex Red reagent and 1 U/ml HRP were added to the mitochondria (40 μg protein per sample) or to the H$_2$O$_2$ standard solution in 100 μl of reaction buffer: 125 mM KCl, 10 mM MOPS, 5 mM MgCl$_2$, and 2 mM K$_2$HPO$_4$ (pH 7.44). Glutamate + malate (2.5 mM) was used to stimulate respiration through complex I, III, and IV. Succinate (5 mM) + rotenone (0.5 μM) was added to drive respiration through complex II, III, and IV. Antimycin A (AA, 0.5 μM), an inhibitor specific for the Q site of complex III, was added to succinate + rotenone-supported respiration to examine the maximal rate of mitochondrial H$_2$O$_2$ production. Cu,Zn-SOD (100 U/ml) was added to convert any O$_2^{**-}$ present to H$_2$O$_2$, preventing interaction of the superoxide with the HRP directly. Fluorescence was followed at 530-nm excitation and 590-nm emission for 10 min in a automatic microplate reader (Labsystems, Helsinki, Finland) equipped with a thermally controlled compartment.

Measurement of mitochondrial superoxide release. Mitochondrial superoxide release was directly measured by electron paramagnetic resonance (EPR) with use of the spin trap 5-disopropoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMP) (9). EPR measurements were performed using an X-band MS200 spectrometer (Magnettech, Berlin, Germany) following the general outline of the methodology of Bhattacharya et al. (6). Mitochondria (20 μg) isolated from glycolytic skeletal muscle in young mice were incubated for 30 min at 37°C in buffer containing 125 mM KCl, 10 mM MOPS, 5 mM MgCl$_2$, 2 mM K$_2$HPO$_4$, 2 mM diethylenetriamine pentaacetic acid, 50 mM DIPPMPO, a 1:1,000 dilution of catalase (to eliminate potential hydroxyl radical generation from H$_2$O$_2$), and either complex I (glutamate + malate, 24 mM) or complex II substrate (24 mM succinate + 2.4 μM rotenone), pH 7.44. Rotenone was added to minimize
reverse electron transfer through complex I while mitochondria re-
spired on succinate. EPR measurements were begun by the addition of
40 μl of this mixture to a 50-μl capillary tube at 37°C with the
following settings: 5 × 10^5 receiver gain, 20 mW microwave power,
9.55 GHz microwave frequency, 2 G modulation amplitude, 40 s scan
time, and 100 G scan width, with an accumulation of 10 scans. The
intensity of the EPR spectra corresponds to the amount of DIPPMPO
bound to superoxide, inasmuch as the addition of Cu,Zn-SOD (1
U/μl) completely abolished the EPR signal. EPR data are expressed as
relative intensity units per 20 μg of mitochondrial protein.

Measurement of isoprostanes. Levels of F2-isoprostanes in TA
muscles were determined as described by Ward et al. (56). Briefly,
F2-isoprostanes were extracted and quantified by gas chromatogra-
ph-mass spectrometry using the internal standard [3H]8-IsopGF2α,
which was added to the samples at the beginning of extraction to
correct yield of the extraction process. The amount of F2-isoprostanes
in TA muscles was expressed as picograms of 8-IsopGF2α per
milligram of total TA protein.

Skeletal muscle fatigueability. Mice were anesthetized with initial
intraperitoneal injection of tribromoethanol (400 mg/kg) and supple-
mental injections to maintain an adequate level of anesthesia during
the procedure. Gastrocnemius muscle contractile properties were
measured in situ (31). In anesthetized mice, the whole gastrocnemius
muscle was isolated from surrounding muscle and connective tissue,
and the distal tendon was severed and secured to the lever arm of a
servomotor (model 305B, Aurora Scientific). The muscle was simul-
ated via the tibial nerve using a bipolar platinum wire electrode.
Stimulation voltage (typically 5–10 V) and then muscle length were
adjusted to give maximum twitch force (P0). With muscles held at
optimal muscle length, trains of 0.2-msec stimulus pulses were applied
at increasing frequencies until a maximum P0 was reached. For the
fatigue test, muscles were stimulated with 100-Hz trains of 0.5-s
durations, once each 5 s for 15 min. For soleus and EDL muscles, the
contraction protocol was identical to that described for gastrocnemius
muscles, except measurements were made in vitro and the duration of
the fatigue protocol was 5 min, rather than 15 min. For in vitro
contractile properties, each muscle was removed from the animal and
placed in a horizontal bath containing buffered mammalian Ringer
solution (in mM: 137 NaCl, 24 NaHCO3, 11 glucose, 5 KCl, 2 CaCl2,
1 MgSO4, 1 NaH2PO4, and 0.025 turbocurarine chloride) maintained
at 25°C and bubbled with 95% O2-5% CO2 to stabilize pH at 7.4. One
tendon was tied to a force transducer (model BG-50, Kulite Semi-
condtor Products) and the other tendon to a fixed post. Muscles were
stimulated between two stainless steel plate electrodes. After all force
measurements, muscles were blotted and weighed, and total fiber
cross-sectional area was calculated by dividing the muscle mass by
the product of fiber length (determined from previously established
muscle length-to-fiber length ratios) and muscle density, 1.06 g/cm2.
Specific P0 (kN/m2) was calculated for each muscle by dividing P0 by
the fatigue protocol was 5 min, rather than 15 min. For in vitro
contractile properties, each muscle was removed from the animal and
placed in a horizontal bath containing buffered mammalian Ringer
solution (in mM: 137 NaCl, 24 NaHCO3, 11 glucose, 5 KCl, 2 CaCl2,
1 MgSO4, 1 NaH2PO4, and 0.025 turbocurarine chloride) maintained
at 25°C and bubbled with 95% O2-5% CO2 to stabilize pH at 7.4. One
tendon was tied to a force transducer (model BG-50, Kulite Semicon-
ductor Products) and the other tendon to a fixed post. Muscles were
stimulated between two stainless steel plate electrodes. After all force
measurements, muscles were blotted and weighed, and total fiber
cross-sectional area was calculated by dividing the muscle mass by
the product of fiber length (determined from previously established
muscle length-to-fiber length ratios) and muscle density, 1.06 g/cm2.
Specific P0 (kN/m2) was calculated for each muscle by dividing P0 by

Statistics. Unpaired Student’s t-test was used for all analyses,
unless otherwise indicated.

Chemicals. All chemicals were purchased from Sigma Chemical,
unless otherwise indicated.

RESULTS

Young TnIFastCreSod2fl/fl mice appear phenotypically nor-
mal. To identify whether a glycolytic skeletal muscle-specific
reduction of Mn-SOD would alter gross phenotype, we mea-
sured body mass, the percentages of lean and fat mass, and
muscle mass in young female (5- to 8-mo-old) and male (3- to
4-mo-old) TnIFastCreSod2fl/fl and wild-type mice. No difference
in body mass was observed in female or male TnIFastCreSod2fl/fl
mice compared with wild-type mice. Similarly, lean body mass,
body fat, and gastrocnemius and soleus muscle mass in
males and females were not significantly different from
the corresponding values for wild-type mice (Table 1). In addition,
there were no differences in muscle fiber morphology (Fig. 1B)
or in the number of centrally located myofiber nuclei (indicative
of muscle fiber regeneration) in sections of gastrocnemius
from wild-type mice compared with sections of gastrocnemius
from TnIFastCreSod2fl/fl mice.

Mn-SOD is reduced selectively only in glycolytic skeletal
muscle isolated from TnIFastCreSod2fl/fl mice. To verify the
tissue specificity of TnIFastCre, Mn-SOD activity was mea-
sured in muscles consisting mostly of type IIB fibers, white
gastrocnemius and quadriceps (“glycolytic muscle”), and a
muscle enriched in type I + IIA fibers, soleus (8). Mn-SOD
activity was reduced 82% in homogenates of mainly glycolytic
skeletal muscle (Fig. 2, A and B) but was not significantly
different in homogenates of soleus muscle (Fig. 2, A and C)
isolated from young female TnIFastCreSod2fl/fl mice relative to
soleus muscle homogenates isolated from wild-type mice.
Mn-SOD activity was unchanged in all other tissues (Fig. 2D,
Table 2). Cu,Zn-SOD activity was also measured to determine
whether there was a potential upregulation of this form of
SOD. Cu,Zn-SOD activity in homogenates of skeletal muscle
containing glycolytic or oxidative fibers was not significantly
different between wild-type and TnIFastCreSod2fl/fl mice,
which indicates that the other mitochondrial isoform of SOD is
not upregulated in response to a reduction in mitochondrial

EXHAUSTION TESTS

Treadmill endurance capacity test. Young female mice were run on
a treadmill (Exer-6, Columbus Instruments, Columbus, OH) on a 15%
incline. The first 5 min were considered exercise acclimatization,
as mice were run at 7 m/min for 5 min. Mice were then run at 12 m/min
for 120 min. Treadmill speed was increased to 17 m/min for 10 min
if an animal reached 120 min and then increased to 22 m/min until
exhaustion. Exhaustion was determined by a failure to engage the
treadmill in the presence of a mild shock and by physical prodding.
Exhaustion was also defined biochemically, with blood glucose
reaired at the indicated time points (i.e., at 0,
20, and 40 min of running and at exhaustion) with use of the glucose
and lactate meters, respectively.

Western blotting. Skeletal muscle homogenates were prepared in
assay buffer containing 50 mM Tris-HCl buffer with 150 mM NaCl, 1%
Nonidet P-40, 0.25% sodium deoxycholate, and 1× protease
inhibitor. Equivalent amounts of protein (40–80 μg) for each sample
were resolved in 4–20% Tris-HCl-SDS-polyacrylamide gels in trip-
llicate. After electrophoresis, the proteins were transferred to a poly-
vinyldene difluoride membrane. The membrane was incubated in
Tris-buffered saline, pH 7.4, with 0.05% Tween 20 (TBS-T) contain-
ing 10% nonfat milk for 1 h at room temperature. The blots were
reacted with mouse aconitase (1:2,000 dilution, a kind gift from
Luke Szwedaw, Oklahoma Medical Research Foundation, Oklahoma City,
OK) and succinate dehydrogenase complex A (1:1,000 dilution;
Invitrogen) and succinate dehydrogenase complex B (1:500 dilution;
Invitrogen) antibodies at 4°C overnight. The blots were washed with
TBS-T and then incubated with goat anti-rabbit IgG-HRP or goat
anti-mouse IgG-HRP (1:1,000 dilution; Sigma) for 2 h at room
temperature. The blots were washed five times with TBS-T, and the
bands corresponding to protein content were visualized using chemi-
oluminescent detection reagents (Amer sham Pharmacia Biotech).

AJP-Cell Physiol • VOL 297 • DECEMBER 2009 • www.ajpcell.org

C1523
matrix Mn-SOD (Fig. 2E). Cu,Zn-SOD activity was also unchanged in all other tissues from TnIFastCreSod2fl/fl relative to wild-type mice (Fig. 2E, Table 2).

Mn-SOD is specifically reduced in type IIB skeletal muscle. Measurement of Mn-SOD activity in muscle homogenates does not indicate the skeletal muscle fiber type in which Mn-SOD is specifically reduced. Using fluorescence-tagged antibodies for myosin heavy chain type I, IIA, and IIB and Mn-SOD, we measured the colocalization of immunofluorescence for myosin heavy chain type I, IIA, and IIB and Mn-SOD on cryostat sections of gastrocnemius with attached soleus (Fig. 3A). Types I, IIA, IIX, and IIB and Mn-SOD were analyzed by comparison of the relative fluorescence intensity of Mn-SOD followed by division by muscle fiber type area (Mn-SOD intensity/fiber type area). Total Mn-SOD intensity/fiber area was examined in sections from seven wild-type and four TnIFastCreSod2fl/fl mice (Fig. 3B). Expression of the TnIFast promoter has been shown to be more than fourfold higher in type IIB than type I + IIA and type IIX skeletal muscle fibers (22). Therefore, the greatest reduction in Mn-SOD content (as a result of TnIFastCre-mediated deletion) was expected in type IIB muscle fibers. The Mn-SOD intensity/fiber type area for Mn-SOD in type I + IIA skeletal muscle fibers from TnIFastCreSod2fl/fl mice was reduced by 13% relative to wild-type mice, but the difference was not statistically significant. Mn-SOD intensity/fiber type area for type IIX fibers from TnIFastCreSod2fl/fl mice was reduced by 14%, but this value was not significantly different from wild-type mice. The Mn-SOD intensity/fiber type area in type IIB fibers from

### Table 1. Phenotypic data in young TnIFastCreSod2fl/fl mice

<table>
<thead>
<tr>
<th></th>
<th>Body Mass, g</th>
<th>Lean Mass, %</th>
<th>Body Fat, %</th>
<th>Muscle Mass/Body Mass, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Soleus</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>26.3 ± 1.2 (5)</td>
<td>81.1 ± 1.3 (5)</td>
<td>18.6 ± 1.2 (5)</td>
<td>0.4 ± 0.03 (5)</td>
</tr>
<tr>
<td>TnIFastCreSod2fl/fl</td>
<td>27.3 ± 1.9 (5)</td>
<td>82.3 ± 0.8 (5)</td>
<td>17.3 ± 0.9 (5)</td>
<td>0.4 ± 0.02 (5)</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>21.3 ± 0.3 (14)</td>
<td>83.2 ± 0.6 (23)</td>
<td>16.5 ± 0.6 (23)</td>
<td>0.32 ± 0.01 (14)</td>
</tr>
<tr>
<td>TnIFastCreSod2fl/fl</td>
<td>20.9 ± 0.3 (14)</td>
<td>81.7 ± 0.6 (29)</td>
<td>17.8 ± 0.6 (29)</td>
<td>0.33 ± 0.02 (14)</td>
</tr>
</tbody>
</table>

Values are means ± SE of number of mice in parentheses. Female (5–8 mo old) and male (3–4 mo old) TnIFastCreSod2fl/fl mice were phenotypically normal compared with wild-type (WT) mice. No significant difference was observed for body mass, percent lean mass or percent body fat, or muscle mass normalized to body mass for soleus or gastrocnemius muscle.

Fig. 2. Mn-SOD and Cu,Zn-SOD activity in young female TnIFastCreSod2fl/fl mice. A: glycolytic and oxidative skeletal muscle Mn-SOD activity. Protein extracts from 3 wild-type and 3 TnIFastCreSod2fl/fl mice were analyzed using activity gels. B and C: quantification of Mn-SOD activity in glycolytic and oxidative skeletal muscle, respectively. Values are means ± SE. ***Significantly different (P < 0.001) from WT. D and E: Mn-SOD and Cu,Zn-SOD activity, respectively. Cu,Zn-SOD activity was not different in glycolytic or oxidative skeletal muscle and various other tissues. Protein extracts from 3 wild-type and 3 TnIFastCreSod2fl/fl mice were analyzed using activity gels.
TnFastCreSod2fl/fl mice was significantly reduced by 70% relative to wild-type mice (Fig. 3B).

Aconitase activity and content are reduced in only glycolytic skeletal muscle isolated from young TnFastCreSod2fl/fl mice. Aconitase activity has been shown to be sensitive to inactivation by superoxide (16–19). We hypothesized that Mn-SOD reduction would increase mitochondrial matrix superoxide content and decrease aconitase activity. Aconitase activity in homogenates of glycolytic skeletal muscle was reduced by 56% (Fig. 4A) and 52% (data not shown) in young male and female TnFastCreSod2fl/fl mice, respectively, compared with aconitase activity in homogenates of glycolytic muscle isolated from wild-type mice. Aconitase protein content (Fig. 4, C and D) was reduced by 50% in homogenates of glycolytic skeletal muscle isolated from young female TnFastCreSod2fl/fl mice. Aconitase activity (Fig. 4B) and protein content (Fig. 4, C and E) in homogenates of soleus muscles were not significantly different relative to these levels in young TnFastCreSod2fl/fl and wild-type mice. Soleus muscle consists exclusively of myosin heavy chain type I and IIA fibers (8). The absence of a reduction in aconitase activity in soleus muscles indicates that mitochondrial matrix superoxide content is not elevated in type I and IIA muscle fibers in TnFastCreSod2fl/fl mice.

Mitochondria isolated from glycolytic skeletal muscle in young TnFastCreSod2fl/fl mice release greater than twofold more superoxide than mitochondria isolated from glycolytic skeletal muscle in wild-type mice. To directly measure mitochondrial superoxide content, we used EPR in conjunction with the superoxide-specific spin trap DIPPMPO. Mitochondria isolated from glycolytic skeletal muscle in young female TnFastCreSod2fl/fl mice release 2.1- and 2.3-fold more superoxide with complex I-linked substrate (Fig. 4, F and H) and complex II-linked substrate (Fig. 4, G and I), respectively, than mitochondria isolated from glycolytic skeletal muscle in wild-type mice. Addition of Cu,Zn-SOD completely abolished the superoxide-derived EPR signal for complex I- and II-linked substrates (Fig. 4, F and G). Because exogenously added Cu,Zn-SOD is too large to cross the mitochondrial membrane(s), DIPPMPO detects superoxide that has been released from the mitochondria.

Rate of mitochondrial H2O2 production is reduced with complex II-linked substrate but increased in the presence of an inhibitor for complex III in young TnFastCreSod2fl/fl mice. The rate of mitochondrial H2O2 production was measured to further examine the oxidant burden in young TnFastCreSod2fl/fl mice. The rate of mitochondrial H2O2 production with the complex I-linked substrate glutamate + malate in mitochondria isolated from glycolytic skeletal muscle in young TnFastCreSod2fl/fl mice was not different from that in wild-type mice (Fig. 5A). The rate of mitochondrial H2O2 production with the complex II-linked substrate succinate + rotenone was significantly reduced by 33% in mitochondria isolated from glycolytic skeletal muscle in TnFastCreSod2fl/fl mice relative to wild-type mice (Fig. 5A). However, succinate + rotenone + an inhibitor of the Q, site of complex III (AA) significantly increased the rate of mitochondrial H2O2 production by 56% in TnFastCreSod2fl/fl mice relative to wild-type mice (Fig. 5B).

Lipid oxidative damage is elevated in TA muscles isolated from young TnFastCreSod2fl/fl mice. To determine whether a reduction in Mn-SOD content only in type IIB skeletal muscle would increase oxidative damage, we measured lipid peroxidation in TA muscles isolated from young TnFastCreSod2fl/fl mice. TA muscle is composed of ~65% myosin heavy chain type IIB (8). Lipid peroxidation was assessed by measurement of F2-isoprostanes, which are stable, prostaglandin-like products formed nonenzymatically in vivo by the free radical-catalyzed peroxidation of arachidonic acid (34, 56). F2-isoprostanes increased significantly by 36% in TA muscles isolated from young TnFastCreSod2fl/fl mice compared with wild-type mice (Fig. 5C).

Glycylcine muscle function and running capacity are reduced in young TnFastCreSod2fl/fl mice. We hypothesized that the type IIB skeletal muscle-specific reduction in Mn-SOD content would alter contractile function only in glycolytic protein-containing muscles in TnFastCreSod2fl/fl mice. To test this hypothesis, we measured contractile force production in glycolytic (EDL and gastrocnemius) and oxidative (soleus) muscles. No significant difference in maximum specific isometric force was found in EDL, gastrocnemius, or soleus muscles from TnFastCreSod2fl/fl mice compared with wild-type mice (Fig. 6A).

The decline in force during repeated contractions by muscles from TnFastCreSod2fl/fl and wild-type mice was compared by two-factor repeated-measures ANOVA. For EDL muscles, this analysis showed a significant (P < 0.001) decrease in force for TnFastCreSod2fl/fl and wild-type mice but a significant (P < 0.001) interaction between genotype and contraction number; that is, the effect of the repeated contractions on force was different between the genotypes. For EDL muscles of wild-type mice, force decreased until contraction 27, after which the ANOVA indicated no further statistically significant decline. In contrast, force production by EDL muscles isolated from TnFastCreSod2fl/fl mice continued to decline until contraction 34 (Fig. 6B). Force production was significantly (P < 0.05) different between EDL muscles isolated from TnFastCreSod2fl/fl and wild-type mice starting at contraction 26. The average minimum force produced by EDL

### Table 2. Quantification of Mn-SOD and Cu,Zn-SOD activity in tissues isolated from wild-type and TnFastCreSod2fl/fl mice

<table>
<thead>
<tr>
<th>Mn-SOD activity</th>
<th>Glycolytic Muscle</th>
<th>Soleus</th>
<th>Heart</th>
<th>Brain</th>
<th>Lung</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>20.0±0.8</td>
<td>160.5±18.6</td>
<td>324.0±28.5</td>
<td>148.9±11.1</td>
<td>74.9±15.2</td>
<td>77.1±1.2</td>
<td>269.6±5.0</td>
</tr>
<tr>
<td>TnFastCreSod2fl/fl</td>
<td>3.6±1.7*</td>
<td>155.3±10.4</td>
<td>337.4±20.2</td>
<td>170.1±12.9</td>
<td>86.0±8.5</td>
<td>78.5±2.4</td>
<td>334.8±35.3</td>
</tr>
</tbody>
</table>

Cu,Zn-SOD activity

| WT             | 43.4±3.5         | 166.4±9.2   | 249.0±31.2  | 362.9±7.5  | 96.7±16.6 | 168.7±3.3 | 532.9±5.3 |
| TnFastCreSod2fl/fl | 49.2±1.0        | 182.3±6.4   | 304.3±12.3  | 403.9±6.2  | 137.4±2.8 | 173.8±1.3 | 575.1±19.7 |

Values (means ± SE for 3 samples) are expressed as arbitrary units, relative to equal amounts of protein for wild-type and TnFastCreSod2fl/fl (25–80 μg, depending on the tissue). Mn-SOD and Cu,Zn-SOD activities were determined using activity gels. *Significantly different (P < 0.001) from WT.
muscles from *TnIFastCreSod2fl/fl* mice was 7% less than that produced by EDL muscles from wild-type mice (Fig. 6C).

A similar analysis showed that force production declined for gastrocnemius muscles of *TnIFastCreSod2fl/fl* and wild-type mice (*P* < 0.001), with force levels leveling off after *contraction 39* for muscles of wild-type mice, but only after *contraction 66* for muscles of *TnIFastCreSod2fl/fl* mice (Fig. 6D). Force production was significantly (*P* < 0.05) different between gastrocnemius muscles isolated from *TnIFastCreSod2fl/fl* and wild-type mice starting at *contraction 103*. The average minimum force produced by gastrocnemius muscles in *TnIFastCreSod2fl/fl* mice was 10% less than that produced by gastrocnemius muscles in wild-type mice (Fig. 6E).

Although soleus muscles from *TnIFastCreSod2fl/fl* and wild-type mice showed decreases (*P* < 0.001) in force during repeated contractions, the ability to produce force as a function of time was not different (*P* = 0.43) in soleus muscles isolated from *TnIFastCreSod2fl/fl* mice compared with wild-type mice (Fig. 6F). The average minimum force produced by soleus muscles isolated from *TnIFastCreSod2fl/fl* mice was not significantly different from that produced by soleus muscles isolated from wild-type mice (Fig. 6G).
Muscle fatigue is defined as the decline in performance during contractile activity or exercise (13). To examine the in vivo significance of a glycolytic skeletal muscle-specific reduction of Mn-SOD content on muscle fatigue during exercise, young TnIFastCreSod2fl/fl and wild-type mice were run to exhaustion on a treadmill. For the TnIFastCreSod2fl/fl mice, the average time of exhaustion was 62.8/110.0 min. Wild-type mice ran for 138.8/114.9 min during treadmill testing (Fig. 6H). In addition, the distance run by the TnIFastCreSod2fl/fl mice was 55% less than the distance run by the wild-type animals (754.0 ± 129.4 vs. 1,807 ± 103.1 m, P < 0.001).

Changes in blood glucose and lactate are observed only during exercise in TnIFastCreSod2fl/fl mice. During submaximal exercise, mitochondrial dysfunction may lead to a shift from oxidative to glycolytic energy production, resulting in an elevation in serum lactate (15, 23). Blood lactate and glucose were measured before and during exercise to indirectly investigate mitochondrial function. No significant differences in fasting levels of blood glucose and lactate (data not shown) were identified in TnIFastCreSod2fl/fl mice relative to wild-type mice. Nonfasted, preexercise levels of blood glucose (Fig. 7A) and lactate (Fig. 7B) were not different between TnIFastCreSod2fl/fl and wild-type mice. Blood lactate levels in
TnIFastCreSod2fl/fl mice were elevated by 61% after 20 min of running, although the difference was just below the level of statistical significance. Lactate levels in TnIFastCreSod2fl/fl mice became significantly different from wild-type mice after 40 min of running. In contrast, blood glucose levels in TnIFastCreSod2fl/fl mice were not different from those in wild-type mice up to 40 min of running. However, TnIFastCreSod2fl/fl mice utilized glucose at a fourfold faster rate than wild-type mice from 40 min of running until exhaustion (Fig. 7C). The faster disappearance of glucose from the blood of TnIFastCreSod2fl/fl mice during exercise suggests that mitochondria from TnIFastCreSod2fl/fl mice are not able to meet the aerobic energy demand, thereby leading to an increased reliance on glycolysis.

DISCUSSION

The main finding from the present study is that a reduction in Mn-SOD content in type IIB skeletal muscle is sufficient to increase glycolytic muscle mitochondrial oxidative stress and reduce glycolytic muscle contractile function and aerobic exercise capacity. Using TnIFastCreSod2fl/fl mice, we found an 82% reduction in Mn-SOD activity in white gastrocnemius and quadriceps (red fibers removed), muscles known to consist mainly of type IIB fibers (8). Mn-SOD activity in soleus muscle was not reduced in TnIFastCreSod2fl/fl mice compared with wild-type mice, inasmuch as soleus muscle consists exclusively of type I and IIA fibers (8). From these data, it can be concluded that the specificity of the Mn-SOD reduction does not include type I or IIA muscle fibers. To measure Mn-SOD content in type IIX or IIB fibers, we performed immunohistochemistry on gastrocnemius (with attached soleus) muscle sections. Immunofluorescent analysis indicated that Mn-SOD content was significantly reduced by 70% only in type IIB muscle from TnIFastCreSod2fl/fl mice relative to wild-type mice. No significant difference was observed for type I or IIA Mn-SOD content between muscle sections from TnIFastCreSod2fl/fl and wild-type mice.

The 70% reduction in Mn-SOD content in type IIB skeletal muscle increased mitochondrial oxidative stress in muscles composed primarily of type IIB fibers, white gastrocnemius and quadriceps. A reduction in Mn-SOD would be expected to result in an increase in superoxide anion, the substrate for the enzyme. To determine whether levels of superoxide anion are increased in the mitochondrial matrix, we measured the activity of aconitase. At the active site of aconitase is a cubane [4Fe-4S]2+ cluster, in which three of the four irons are ligated to cysteine residues. The fourth iron is exposed to the aqueous media of the mitochondrial matrix and is open to attack from superoxide. Superoxide causes a one-electron oxidation of the iron-sulfur cluster, releasing the exposed iron in the ferrous state and inactivating the enzyme (16–19). Reduced aconitase activity has been observed during certain physiological and pathophysiological processes associated with increased generation of oxygen radicals (7, 40, 41, 48). Inhibition of aconitase may serve to reduce the supply of NADH for electron transport, thereby limiting the production of free radical species (47). Aconitase is protected from inactivation when Mn-SOD is present (18). We found a significant reduction in aconitase activity only in homogenates of glycolytic muscle isolated from young TnIFastCreSod2fl/fl mice. No significant difference in aconitase activity was found in homogenates of soleus muscles isolated from TnIFastCreSod2fl/fl mice compared with wild-type mice.
Fig. 6. Muscle function in young TnIFastCreSod2fl/fl mice. A: maximum isometric specific force. Extensor digitorum longus (EDL), gastrocnemius, and soleus muscles were maximally activated, and force production was measured. Values are means ± SE for 5 EDL samples for wild-type and 8 EDL samples for TnIFastCreSod2fl/fl mice, 5 gastrocnemius samples for wild-type and 6 gastrocnemius samples for TnIFastCreSod2fl/fl mice, and 4 soleus samples for wild-type and 8 soleus samples for TnIFastCreSod2fl/fl mice. B: force production as a function of time for EDL muscles isolated from TnIFastCreSod2fl/fl mice. Every 0.5 s, at a frequency of 100 Hz, the muscle was stimulated, such that there was 1 contraction every 5 s; force production was then measured. Starting with contraction 26, EDL muscles isolated from TnIFastCreSod2fl/fl mice produced significantly less force than EDL muscles isolated from wild-type mice. *Significantly different (P < 0.05) from WT. C: force level at the end of the 5-min contraction protocol expressed as percentage of initial force for EDL muscles isolated from TnIFastCreSod2fl/fl and wild-type mice. *Significantly different (P < 0.05) from WT. D: force production as a function of time for gastrocnemius (GTN) muscles in TnIFastCreSod2fl/fl and wild-type mice. Starting with contraction 103, gastrocnemius muscles in TnIFastCreSod2fl/fl mice produced significantly less force than gastrocnemius muscles in wild-type mice. *Significantly different (P < 0.05) from WT. E: force level at the end of the 15-min contraction protocol expressed as percentage of initial force for gastrocnemius muscles in TnIFastCreSod2fl/fl and wild-type mice. *Significantly different (P < 0.05) from WT. F: force production by soleus muscles isolated from TnIFastCreSod2fl/fl and wild-type mice was not different at any time during the contraction protocol. G: force level at the end of the 5-min contraction protocol expressed as percentage of initial force for soleus muscles isolated from TnIFastCreSod2fl/fl and wild-type mice. H: maximum endurance capacity test. TnIFastCreSod2fl/fl and wild-type mice were run on a treadmill at a 12% grade at 12 m/min for 120 min, then speed was increased to 17 m/min for 10 min and, finally, to 22 m/min until exhaustion. Exhaustion was also defined biochemically, via blood glucose levels <60 mg/dl or blood lactate levels >12 mM. Values are means ± SE for 6 samples. ***Significantly different (P < 0.001) from WT.
homogenates of soleus muscles isolated from wild-type mice. It is important to note that aconitase protein content in homogenates of glycolytic skeletal muscle isolated from young TnIFastCreSod2\textsuperscript{fl/fl} mice was reduced by \( \sim 50\% \). No significant difference in aconitase protein content was found in homogenates of soleus muscles isolated from TnIFastCreSod2\textsuperscript{fl/fl} mice compared with homogenates of soleus muscles isolated from wild-type mice. Aconitase activity has been reported to be indicative of mitochondrial matrix superoxide content, but, with a corresponding decrease in aconitase protein content, the decrease in aconitase activity only in glycolytic skeletal muscle isolated from TnIFastCreSod2\textsuperscript{fl/fl} mice may be reflective of chronic mitochondrial matrix oxidative stress. The metabolic fate of aconitase under such conditions has been reported to be reversible posttranslational inactivation, release of a labile iron from the [4Fe-4S]\textsuperscript{2+} cluster, disassembly of the [4Fe-4S]\textsuperscript{2+} cluster, carboxylation, and protein degradation (7).

We then asked whether the decrease in Mn-SOD content in glycolytic muscle would result in changes in mitochondrial reactive oxygen species generation as measured indirectly as H\(_2\)O\(_2\) and also directly as mitochondrial superoxide release (via EPR). Interestingly, the rate of mitochondrial H\(_2\)O\(_2\) production was reduced, but only with complex II substrate in young TnIFastCreSod2\textsuperscript{fl/fl} mice. In contrast, addition of an inhibitor for complex III, AA, in the presence of complex II substrate increased the rate of mitochondrial H\(_2\)O\(_2\) production by 56\% in young TnIFastCreSod2\textsuperscript{fl/fl} mice relative to wild-type mice. An increased rate of mitochondrial H\(_2\)O\(_2\) production has been observed in the presence of complex II substrate + AA when complex II is partially inhibited (12). Future experiments will address this issue. Furthermore, mitochondrial superoxide release was elevated more than twofold by mitochondria with use of complex I or complex II substrate in young TnIFastCreSod2\textsuperscript{fl/fl} mice compared with mitochondria isolated from glycolytic muscle in wild-type mice. The net effect of elevated oxidative stress in young TnIFastCreSod2\textsuperscript{fl/fl} mice is a 36\% increase in lipid oxidative damage. These data are in agreement with previous studies in conditional knockout mice lacking Mn-SOD in heart and skeletal muscle (H/M-Sod2\textsuperscript{–/–}) (35). Mitochondria isolated from TA muscles in H/M-Sod2\textsuperscript{–/–} mice produce fivefold more superoxide but 52\% less H\(_2\)O\(_2\), a result likely due to the reduction of Mn-SOD. In addition, lipid oxidative damage was found to be elevated in heart isolated from H/M-Sod2\textsuperscript{–/–} mice (35).

Contractile muscle function was adversely affected in EDL and gastrocnemius muscles from young TnIFastCreSod2\textsuperscript{fl/fl} mice, whereas no differences were identified in contractile force production by soleus muscles isolated from TnIFastCreSod2\textsuperscript{fl/fl} compared with soleus muscles isolated from wild-type mice. Mn-SOD and aconitase activity in homogenates of soleus muscles isolated from wild-type mice were not different from Mn-SOD and aconitase activity in homogenates of soleus muscles isolated from TnIFastCreSod2\textsuperscript{fl/fl} mice. Collectively, these data suggest that type I + IIA muscle fibers (soleus) were unaffected in terms of oxidative stress, and, as a result, contractile function in soleus muscle was not altered. EDL muscles are composed of \( \sim 50\% \) type IIA and 50\% IIB muscle fibers, gastrocnemius muscles are composed of \( \sim 30\% \) type IIA and 70\% type IIB muscle fibers, and soleus muscles are composed exclusively of type I + IIA muscle fibers (8). In contrast, in muscles (EDL and gastrocnemius) that are composed of large numbers of type IIB muscle fibers, oxidative stress and oxidative damage were elevated and contractile function was reduced in TnIFastCreSod2\textsuperscript{fl/fl} mice compared with wild-type.
mice. Overall, these findings are consistent with our hypothesis that mitochondrial oxidative stress in glycolytic skeletal muscle is associated with deficits in contractile muscle function.

To investigate the in vivo significance of an Mn-SOD reduction only in type IIB skeletal muscle, treadmill testing was performed. Young TnI Fast Cre Sod2<sup>fl/fl</sup> mice were found to be exercise intolerant, inasmuch as they ran a shorter distance and for less time than wild-type mice. These data implicate a causative role for skeletal muscle fiber type IIB-specific mitochondrial oxidative stress in aerobic exercise capacity.

Mitochondrial dysfunction is one possible explanation for the reduction in muscle function in TnI Fast Cre Sod2<sup>fl/fl</sup> mice. Elevated serum lactate during exercise has been shown to be demonstrative of mitochondrial dysfunction (15, 23). Significantly more lactate was present in the blood of TnI Fast Cre Sod2<sup>fl/fl</sup> mice after 40 min of running and at exhaustion. The blood lactate concentration in TnI Fast Cre Sod2<sup>fl/fl</sup> mice after 20 min of running was elevated by 61%, but significance was borderline. Blood glucose levels were not significantly different between TnI Fast Cre Sod2<sup>fl/fl</sup> and wild-type mice from 0 to 40 min of running. However, a fourfold greater rate of glucose utilization was measured from 40 min of running until exhaustion in TnI Fast Cre Sod2<sup>fl/fl</sup> mice. These data indicate that a critical point is reached at which mitochondrial metabolism is no longer able to maintain the ATP demand of submaximal exercise, and a shift from aerobic to anaerobic metabolism occurs. A greater reliance on glycolysis to meet the ATP demands of the cell will produce an increase in pyruvate content if mitochondrial dysfunction is present (15). Pyruvate enters the mitochondria, where it is oxidized by the tricarboxylic acid (citric acid) cycle. However, in the presence of mitochondrial dysfunction, elevated pyruvate may not be oxidized at the necessary rate by the tricarboxylic acid cycle and, via mass action, will form lactate. Cumulatively, these data suggest mitochondrial dysfunction in TnI Fast Cre Sod2<sup>fl/fl</sup> mice. Future experiments will address the effect of a type IIB skeletal muscle-specific reduction of Mn-SOD on glycolytic muscle mitochondrial function.

Our results are consistent with previous studies in mice heterozygous for Sod2 (Sod2<sup>+/−</sup>) (30, 53, 54) and mice lacking Mn-SOD in both heart and skeletal muscle (H/M-Sod2<sup>−/−</sup>) (35). An association between increased mitochondrial oxidative stress and decreased exercise capacity has been shown for Sod2<sup>+/−</sup> and H/M-Sod2<sup>−/−</sup> mice. However, the tissue that is limiting with respect to muscle function in either animal model is unknown. In vitro studies of isolated skeletal muscle have shown that oxidative stress is limiting with respect to contractile function (3, 26, 32, 39, 46). In addition, mitochondrial oxidative stress and oxidative damage have been shown to be elevated in type II relative to type I skeletal muscle (2, 14, 38). These data suggest an association between type II skeletal muscle mitochondrial oxidative stress, oxidative damage, and reduced muscle function. Our results indicate that a type IIB skeletal muscle-specific reduction in Mn-SOD is sufficient to increase glycolytic muscle mitochondrial oxidative stress and is directly associated with reductions in contractile muscle function and aerobic exercise capacity.

ACKNOWLEDGMENTS
We thank Corinne Price for editing the manuscript.

REFERENCES
OXIDATIVE STRESS AND AEROBIC EXERCISE CAPACITY


