Type II skeletal myofibers possess unique properties that potentiate mitochondrial H$_2$O$_2$ generation

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Anderson, Ethan J., and P. Darrell Neufer. Type II skeletal myofibers possess unique properties that potentiate mitochondrial H$_2$O$_2$ generation. Am J Physiol Cell Physiol 290: C844–C851, 2006. First published October 26, 2005; doi:10.1152/ajpcell.00402.2005.—Mitochondrial dysfunction is implicated in a number of skeletal muscle pathologies, most notably aging-induced atrophy and loss of type II myofibers. Although oxygen-derived free radicals are thought to be a primary cause of mitochondrial dysfunction, the underlying factors governing mitochondrial superoxide production in different skeletal myofiber types is unknown. Using a novel in situ approach to measure H$_2$O$_2$ production (indicator of superoxide formation) in permeabilized rat skeletal muscle fiber bundles, we found that mitochondrial free radical leak (H$_2$O$_2$ produced/O$_2$ consumed) is two- to threefold higher ($P < 0.05$) in white (WG, primarily type IIB fibers) than in red (RG, type IIA) gastrocnemius or soleus (type I) myofibers during basal respiration supported by complex I (pyruvate + malate) or complex II (succinate) substrates. In the presence of respiratory inhibitors, maximal rates of superoxide produced at both complex I and III have been identified as sites of O$_2$ production in mitochondria isolated from rat skeletal muscle (1, 6, 30). However, mitochondrial in skeletal muscle are morphologically and functionally heterogeneous, reflecting differences in metabolic demand associated with each specific fiber type as well as local energy demands associated with particular subcellular regions within each myofiber (e.g., subsarcolemmal and intramyofibrillar mitochondria) (19). Moreover, skeletal muscle mitochondria in vivo are arranged in a highly organized, interconnected reticulum or “crystallike” pattern (32, 49), are dependent on cytoskeletal interactions to maintain proper function (53), and, in oxidative fibers, are thought to interact functionally with myofibrils and the sarcoplasmic reticulum, forming energy transfer systems (creatine kinase, adenylate kinase, hexokinase II) that channel ADP to the mitochondria to more efficiently couple energy utilization and oxidative phosphorylation (38).

The extent to which morphological or metabolic properties intrinsic to the three main skeletal muscle fiber types may influence mitochondrial O$_2$ production has not been explored previously. To this end, we developed a novel in situ approach to examine the factors governing mitochondrial O$_2$ production under conditions in which the cellular architecture of the myofibers and the structural integrity of the mitochondrial reticulum are maintained in their native state (35). The aim of the present study was to determine whether mitochondrial O$_2$ production varies among the three major types of myofibers by determining the sites and topology of mitochondrial O$_2$ production in permeabilized fiber bundles from rat soleus (predominantly type I oxidative fibers), red gastrocnemius (RG, predominantly type IIA oxidative/glycolytic fibers), and white gastrocnemius (WG, predominantly type IIB glycolytic fibers) muscle. Our findings reveal that the rate of mitochondrial free radical leak under both basal and elevated redox state conditions is substantially greater in type II, particularly type IIB, than in type I myofibers, providing a potential mechanistic basis for the mitochondrial dysfunction, atrophy, and loss of type II myofibers that develop with aging (28, 45, 52).

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MATERIALS AND METHODS

Animals and reagents. Male Sprague-Dawley rats were bred in house or purchased from Charles River Laboratory (Wilmington, MA). All rats were housed in a temperature (22°C) - and light-controlled room and were given free access to food and water. At the time of the experiments, rats were 7–8 wk of age and weighed 250–350 g. Skeletal muscle was obtained from anesthetized animals (100 mg/kg ip ketamine-xylazine). After surgery, animals were killed by cervical dislocation while anesthetized. The Pierce Laboratory is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institute, and all procedures were approved by the Pierce Animal Care and Use Committee. Amplex Red Ultra reagent was obtained from Molecular Probes. Stigmatellin and horse-radish peroxidase (HRP) were obtained from Fluka Biochemika, and all other chemicals were purchased from Sigma-Aldrich.

Preparation of permeabilized muscle fibers. The technique is partially adapted from previous methods (24, 44). Briefly, small portions (~25 mg) of soleus, RG, and WG muscle were dissected and placed in ice-cold buffer X, containing (in mM) 60 K-MES, 35 KCl, 7.23 K2EGTA, 2.77 CaK2EGTA, 20 imidazole, 0.5 DTT, 20 taurine, 5.7 ATP, 15 PCR, and 6.56 MgCl2·6 H2O (pH 7.1, 295 mosmol/kgH2O). The muscle was trimmed of connective tissue and cut down to fiber bundles (~2 × 7 mm, 4–8 mg wet wt). With a pair of needle-tipped forceps under a dissecting microscope, fibers were gently separated from one another to maximize surface area of the fiber bundle, leaving only small regions of contact. To permeabilize the myofibers, each fiber bundle was placed in ice-cold buffer X containing 50 µg/ml saponin and incubated on a rotator for 30 min at 4°C. The permeabilized bundles were then placed in ice-cold buffer Z containing (in mM) 110 K-MES, 35 KCl, 1 EGTA, 5 K2HPO4, and 3 MgCl2·6 H2O, 0.05 pyruvate, and 0.02 malate with 0.5 mg/ml BSA (pH 7.1, 295 mosmol/kgH2O). Permeabilized fibers remained in buffer Z on a rotator at 4°C until analysis (~90 min) without any deterioration in mitochondrial function [i.e., respiratory control index (RCI), see below].

H2O2 production in permeabilized fibers. H2O2 production was measured with Amplex Red reagent, which reacts with H2O2 in a 1:1 stoichiometry catalyzed by HRP to yield the fluorescent compound resorufin and molar equivalent O2. Resorufin has excitation/emission characteristics of 563 nm/587 nm and is extremely stable once formed. Fluorescence was measured continuously [change in fluorescence (ΔF/min)] with a Spex Fluoromax 3 (Jobin Yvon) spectrofluorometer with temperature control and magnetic stirring. After baseline ΔF (reactants only) was established, the reaction was initiated by addition of a permeabilized fiber bundle to 300 µl of buffer Z containing 5 µM Amplex Red and 0.5 U/ml HRP, with 5 mM pyruvate and 2 mM malate for complex I substrate or 3 mM succinate for complex II substrate. The rate of O2 consumption was measured in 2–min increments and expressed as nanomoles of O2 consumed per minute per milligram of dry weight. Basal respiration (state 4; VO2) was determined in the presence of 10 µg/ml oligomycin to inhibit ATP synthesis. Maximal respiration (state 3; VO2 max) was determined in parallel fiber bundles in the presence of 330 µM ADP. The RCI was calculated as VO2 max/VO2 (35). RCI values averaged ~4.0 for all three muscle types (Table 1), indicating a similar high degree of coupling of respiration to phosphorylation. The percent free radical leak was calculated as the rate of H2O2 production divided by two times the rate of O2 consumption and multiplied by 100 (36).

Mitochondrial H2O2 scavenging in permeabilized fibers. To compare the mitochondrial H2O2 scavenging capacity in the different muscle types, permeabilized fiber bundles from soleus, RG, and WG muscles were placed in 500 µl of buffer Z containing 40 mM H2O2 and 50 µM pyruvate-20 µM malate in a thermally controlled chamber set at 37°C with rapid stirring. Aliquots were removed every 20 s and placed on ice. The H2O2 content in each aliquot was measured with 10 µM Amplex Red and 0.5 U/ml HRP in a 96-well plate and a Perkin Elmer plate-reading fluorometer (excitation 540/emission 595 nm). The total amount of H2O2 remaining at each time point was calculated from a standard curve and corrected for background loss. H2O2 scavenging rate was calculated within the linear portion of the decay curve (0–60 s) for each of the three types of muscle and expressed as micromoles of H2O2 scavenged per minute per milligram of dry weight. Stoichiometric scavenging of H2O2 by Cu,Zn-SOD (40 U/ml) was determined in the presence of 10 µg/ml oligomycin to inhibit ATP synthesis. Stoichiometric scavenging of H2O2 by Cu,Zn-SOD was determined in the presence of substrate + ADP (330 µm). Stoichiometric scavenging rate was calculated within the linear portion of the decay curve (0–60 s) for each of the three types of muscle and expressed as micromoles of H2O2 scavenged per minute per milligram of dry weight.

Table 1. Respiratory characteristics of permeabilized rat soleus and red and white gastrocnemius muscle

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Muscle</th>
<th>Major Fiber Type</th>
<th>Respiration, nmol O2-min⁻¹·mg dry wt⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>State 4</td>
</tr>
<tr>
<td>Pyruvate-malate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>Type I</td>
<td>2.44±0.39*</td>
<td>9.87±1.24*</td>
</tr>
<tr>
<td>RG</td>
<td>Type IIA</td>
<td>2.80±0.50*</td>
<td>11.56±1.53*</td>
</tr>
<tr>
<td>WG</td>
<td>Type IIB</td>
<td>1.45±0.09</td>
<td>5.70±0.79</td>
</tr>
<tr>
<td>Succinate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>Type I</td>
<td>10.01±0.32*</td>
<td>ND</td>
</tr>
<tr>
<td>RG</td>
<td>Type IIA</td>
<td>15.24±0.58*</td>
<td>ND</td>
</tr>
<tr>
<td>WG</td>
<td>Type IIB</td>
<td>5.54±0.41</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3 or 4/condition. RG, red gastrocnemius; WG, white gastrocnemius; ND, not determined. O2 consumption was measured at 37°C in a water-jacketed cell. State 4 (basal respiration, VO2) was measured in the presence of substrate + oligomycin. State 3 (maximal respiration, VO2 max) was measured in the presence of substrate + ADP (330 µm). Respiratory control index (RCI) is calculated as VO2 max/VO2. *Significantly different from WG (P < 0.05).
Mitochondrial glutathione peroxidase and citrate synthase activity. After the H$_2$O$_2$ scavenging experiments, permeabilized fiber bundles were placed in 300 μl of standard TE buffer (in mM: 10 Tris and 1 EDTA) + 1% Tween 20 and protease inhibitor cocktail and allowed to chill on ice for ~30 min. The solutions containing the fiber bundles were then freeze-thawed in liquid N$_2$ three times to fully fracture and liberate all remaining membrane-bound compartments. After brief centrifugation, the supernatants were collected and assayed for glutathione peroxidase (Northwest Life Science Specialties, Vancouver, WA) and citrate synthase (39) activity. The fiber bundles remaining were rinsed in H$_2$O, dried, and weighed as described above.

Statistical analysis. Statistical analyses were performed using two-way ANOVA, with all pairwise multiple comparisons among conditions performed using the Student-Newman-Keuls method. The level of significance was set at $P < 0.05$.

RESULTS

Titration of saponin permeabilization. Parallel fiber bundles were prepared from rat RG muscle and subjected to permeabilization with increasing concentrations of saponin (0–100 μg/ml) for 30 min. Maximal ADP-stimulated respiration (330 μM ADP) supported by pyruvate-malate (5 mM-2 mM) progressively increased with exposure to increasing concentrations of saponin, peaking at 50 μg/ml (Fig. 1A). Similarly to previous reports (35), exposure to higher concentrations of saponin (e.g., ≥100 μg/ml) reduced maximal ADP-stimulated respiration, likely reflecting loss of nucleotide sensitivity at the outer mitochondrial membrane. Detection of basal H$_2$O$_2$ production during state 4 respiration supported by pyruvate-malate also reached a maximum at 50 μg/ml saponin (Fig. 1B), confirming full permeabilization at this concentration. A low level of H$_2$O$_2$ production was observed in deenergized (no substrate) nonpermeabilized fiber bundles, presumably originating from metabolism of endogenous intracellular substrates.

Mitochondrial respiration. Pyruvate-malate-supported state 4 (non-ADP stimulated) and state 3 (saturating ADP concentration) respiration rates were approximately twofold greater in permeabilized soleus and RG than WG muscle (Table 1), consistent with the well-established differences in mitochondrial density in type I and type IIA vs. type IIB muscle fibers (9, 32, 49). State 4 respiration supported by succinate was also greater in soleus and RG than in WG muscle. Succinate-supported state 4 respiration was markedly higher than pyruvate-malate-supported respiration, most likely because of the large reverse electron flow and O$_2^-$ production generated under these conditions (see below) (31).

Mitochondrial H$_2$O$_2$ production during pyruvate-malate-supported respiration. Shown in Fig. 2A are representative traces from experiments with pyruvate-malate-supported state 4 respiration from permeabilized soleus, RG, and WG fiber bundles. The average basal rate of mitochondrial H$_2$O$_2$ production (addition of fiber bundle, no inhibitors present) was highest ($P < 0.05$) in WG, followed by RG and then soleus muscle (Fig. 2B). In isolated mitochondria, the rate of mitochondrial O$_2^-$ formation is directly governed by both membrane potential ($\Delta\Psi$) and pH gradient across the inner membrane (5) and thus is favored only under state 4 conditions. In the present study, addition of ADP or FCCP (uncoupler; data not shown) completely eliminated the basal level H$_2$O$_2$ production, consistent with the fall in $\Delta\Psi$ that occurs with transition to state 3 respiration or mild uncoupling and confirming that mitochondria are the source of H$_2$O$_2$ in permeabilized fibers (Fig. 2B).

Mitochondrial H$_2$O$_2$ production rates also varied dramatically among the three muscle types in response to addition of respiratory inhibitors (Fig. 2C). In the presence of exogenous SOD, soleus muscle again had the lowest, and RG and WG muscle displayed the highest, overall rates of mitochondrial H$_2$O$_2$ production. Within each specific muscle type, the highest rate of pyruvate-malate-supported H$_2$O$_2$ production was observed in the presence of antimycin A, an inhibitor that prevents electron flow through the cytochrome b proteins of complex III. Subsequent addition of stigmatellin, which blocks electron entry into complex III, reduced antimycin A-stimulated H$_2$O$_2$ production by ~50–75% in all three muscle types, confirming that complex III is an extremely active site of O$_2^-$ generation in permeabilized fibers when respiration is inhibited by antimycin A. In separate experiments, addition of rotenone to permeabilized fibers respiring on pyruvate-malate also induced H$_2$O$_2$ production, confirming complex I as a site of O$_2^-$ formation. Interestingly, the level of H$_2$O$_2$ production remaining after addition of stigmatellin was similar in all three muscles to the rate generated at complex I in the presence of rotenone, suggesting that inhibition of electron entry at complex III slows electron flow at complex I, elevating the redox state (i.e., similar to rotenone) and accelerating O$_2^-$ formation at complex I.

Mitochondrial H$_2$O$_2$ production during succinate-supported respiration. Respiration supported by the complex II substrate succinate generated extremely high rates of H$_2$O$_2$ production.
under basal state 4 conditions (no inhibitors; Fig. 3). Transition to state 3 respiration by addition of ADP completely eliminated H$_2$O$_2$ production (Fig. 3, A, inset, and B), again confirming mitochondria as the source of H$_2$O$_2$. Both under basal conditions (Fig. 3 B) and in the presence of inhibitors (Fig. 3 C), mitochondrial H$_2$O$_2$ production was highest in both RG and WG muscle, whereas soleus muscle displayed the lowest overall responses. In all three muscle types, addition of rotenone reduced H$_2$O$_2$ production by $\frac{70}{110}$ (Fig. 3, A, inset, and C), indicating that complex I is the major site of O$_2$•− generation during respiration supported exclusively by succinate because of reverse electron flow from complex II to complex I. As anticipated, subsequent addition of antimycin A in fibers supported by succinate/rotenone elicited a marked increase in O$_2$•− production at complex III that, in turn, was blocked by addition of stigmatellin.

**Topology of mitochondrial O$_2$•− production.** To evaluate the “sidedness” or topology of O$_2$•− release among the three muscle types, both pyruvate-malate- and succinate-supported experiments were repeated without SOD added to the reaction buffer, minimizing detection of O$_2$•− released to the cytoplasmic side of the inner membrane. Mitochondrial H$_2$O$_2$ production responses were nearly identical in the presence or absence of exogenous SOD in soleus and RG fibers under all experimental conditions. In RG fibers, small but significantly lower H$_2$O$_2$ production rates were observed in the presence of rotenone during respiration supported by pyruvate-malate (Fig. 2 C) and under basal conditions during respiration supported by succinate.

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**Fig. 2.** Rates of pyruvate-malate-supported H$_2$O$_2$ production in permeabilized fiber bundles (FB) from rat soleus (Sol), red gastrocnemius (RG), and white gastrocnemius (WG) muscle. A: representative real-time traces showing the rate of change in Amplex Red fluorescence (arbitrary units (AU)) in permeabilized fiber bundles from Sol, RG, and WG. At the points indicated, 10 μM antimycin A (AA) and 1 μM stigmatellin (Stig) were added. Inset: representative traces showing the effect of 5 μM rotenone (Rot) vs. 330 μM ADP on the basal rate of H$_2$O$_2$ production. B: quantified basal rates of H$_2$O$_2$ production (J; absence of inhibitors) and effect of ADP. C: quantified H$_2$O$_2$ production rates (mean ± SE; n = 4 or 5) across all 3 muscle types in the absence/presence of inhibitors and ±exogenous SOD. *Significantly (P < 0.001) different from basal; †significantly (P < 0.001) different from +SOD condition.

**Fig. 3.** Rates of succinate-supported H$_2$O$_2$ production in permeabilized fiber bundles from rat Sol, RG, and WG muscle. A: representative real-time traces showing the rate of change in Amplex Red fluorescence (arbitrary units) in permeabilized fiber bundles from Sol, RG, and WG. At the points indicated, 10 μM antimycin A and 1 μM stigmatellin were added. Inset: representative traces showing the effect of 5 μM rotenone vs. 330 μM ADP. B: quantified basal rates of H$_2$O$_2$ production (absence of inhibitors) and effect of ADP. C: quantified H$_2$O$_2$ production rates (mean ± SE; n = 4 or 5) across all 3 muscle types in the absence/presence of inhibitors and ±exogenous SOD. *Significantly (P < 0.001) different from basal; †significantly (P < 0.001) different from +SOD condition.
nate (Fig. 3C). In WG fibers, however, the absence of added SOD decreased H$_2$O$_2$ detection by 65–90% under each experimental condition in both pyruvate-malate- and succinate-supported respiration.

**Mitochondrial free radical leak.** Depicted in Fig. 4 is the fraction of molecules of O$_2$ consumed that give rise to H$_2$O$_2$ released by mitochondria (free radical leak) during either pyruvate-malate- or succinate-supported state 4 respiration in each of the three muscle types. Free radical leak was significantly ($P < 0.05$) higher in WG than in either RG or soleus muscle, providing evidence that type IIB myofibers possess an inherently greater propensity to generate/release O$_2^-$-derived free radicals under state 4 conditions.

**H$_2$O$_2$ scavenging capacity.** To compare the endogenous H$_2$O$_2$ removal capacity among the three muscle types, the rate of clearance of exogenous H$_2$O$_2$ added to permeabilized fiber bundles respiring on pyruvate-malate was measured in the three muscle types. Shown in Fig. 5A is a representative H$_2$O$_2$ scavenging experiment from each muscle type. In soleus myofibers, the overall H$_2$O$_2$ scavenging rate was ~1.5- and 3.0-fold greater ($P < 0.05$) than rates obtained from RG and WG fibers, respectively (Fig. 5B, left). The H$_2$O$_2$ scavenging rate was also ~1.5-fold greater ($P < 0.05$) in RG than in WG fibers. Even after normalization for mitochondrial content, overall H$_2$O$_2$ scavenging rate (nmol·min$^{-1}$·citrate synthase activity$^{-1}$) was still significantly greater in soleus than in RG and WG muscle, whereas rates for RG and WG muscle were similar (Fig. 5B, right). Activity of glutathione peroxidase, the primary enzyme responsible for H$_2$O$_2$ detoxification in mitochondria (2, 54), was approximately twofold higher ($P < 0.05$) in soleus and RG (7.02 ± 0.44 and 6.85 ± 0.68 μU·ml$^{-1}$·mg dry wt$^{-1}$, respectively) than in WG (3.39 ± 0.37 μU·ml$^{-1}$·mg dry wt$^{-1}$) muscle when expressed in absolute terms; however, once normalized for mitochondrial content, glutathione peroxidase activity was similar among the three muscle types (not shown).

**DISCUSSION**

The major finding of the present study is that regulation of mitochondrial ROS production varies substantially in the three major fiber types of adult skeletal muscle tissue. Under basal conditions (e.g., non-ADP-stimulated state 4 respiration), free radical leak is significantly higher in WG than in RG or soleus muscle (Fig. 4), whether basal respiration is supported by electrons feeding complex I (low rate of H$_2$O$_2$ production; Fig. 2) or complex II (high rate of H$_2$O$_2$ production; Fig. 3). Remarkably, under conditions that favor high rates of O$_2^-$ generation (e.g., in the presence of the complex III inhibitor antimycin A), the absolute rates of H$_2$O$_2$ production in permeabilized WG fibers are nearly as high as those observed in RG muscle and rates in both RG and WG muscle are markedly higher than rates obtained from soleus muscle. This is somewhat counterintuitive, given that the respiratory capacity (Table 1) and mitochondrial content in type IIB (WG) muscle fibers are approximately half of those in type IIA (RG) or type I (soleus) fibers (9, 32, 49). If the regulation of H$_2$O$_2$ production were solely a function of mitochondrial content, then soleus and RG muscle would be expected to generate the highest rates of H$_2$O$_2$ formation—clearly not the case. Thus intrinsic differences appear to exist in type II fibers, particularly type IIB, that potentiate mitochondrial O$_2^-$ generation and/or H$_2$O$_2$ production/emission.

It is generally thought that basal respiration (state 4) supported by substrates feeding exclusively to complex I in the absence of respiratory chain inhibitors elicits little to no H$_2$O$_2$ production (41). However, the recent development of a highly sensitive fluorescent H$_2$O$_2$ indicator (Amplex Red) has enabled characterization of ROS production in isolated mitochondria respiring on NADH-linked substrates at levels previously con-
considered insignificant (40). In the present study, low but consistent rates of H$_2$O$_2$ production were observed in all three types of permeabilized fiber bundles during state 4 respiration supported by the complex I substrate combination of pyruvate plus malate (Fig. 2). This basal level of H$_2$O$_2$ production occurred only in the presence of substrate (i.e., no H$_2$O$_2$ production observed in degenerated fiber bundles; Fig. 2B, inset) and was completely eliminated by addition of ADP (lowers Δψ by increasing H$^+$ flow through ATP synthase; Fig. 2B) or FCCP (protonophore that lowers Δψ by increasing H$^+$ conductance; data not shown), confirming mitochondria as the source of H$_2$O$_2$ in respiring permeabilized fiber bundles. Surprisingly, basal rates of H$_2$O$_2$ production were considerably different among the three types of muscles (WG > RG ≥ soleus; Fig. 2B). H$_2$O$_2$ production in isolated mitochondria from skeletal muscle, heart, and brain is particularly sensitive to Δψ, increasing exponentially at the upper range of values (i.e., ~170–185 mV; Refs. 17, 22, 40, 50). Whether differences in basal rates of H$_2$O$_2$ production among different skeletal muscle fiber types reflect corresponding differences in Δψ requires further study. However, the percentage of O$_2^-$ giving rise to H$_2$O$_2$ release during complex I-supported state 4 respiration was ~3.5-fold higher in WG than in either RG or soleus muscle (Fig. 4), consistent with the notion that mitochondrial function (i.e., inherent proton leak, respiratory control, electron transport chain stoichiometry) differs among mitochondria found within the three major types of skeletal muscle (20, 23).

In the absence of inhibitors, succinate-supported state 4 respiration generated extremely high basal rates of mitochondrial H$_2$O$_2$ production in permeabilized fiber bundles from all three types of skeletal muscle (Fig. 3). Succinate donates its electrons directly via complex II (succinate dehydrogenase) of the respiratory chain. The high rate of H$_2$O$_2$ production from this source was virtually eliminated by addition of the complex I inhibitor rotenone in permeabilized soleus and RG muscle and partially inhibited in permeabilized WG muscle. These findings largely confirm those of previous studies on isolated mitochondria from rat brain, heart, and skeletal muscle (14, 16, 17, 26, 43, 48, 50), demonstrating that complex I is the major site of O$_2^-$ generation during succinate-supported respiration due to reverse electron flow from complex II to complex I. The remaining H$_2$O$_2$ production evident in WG fibers likely stems from O$_2^-$ generation at complex III (see below).

Addition of antimycin A, a complex III inhibitor, to permeabilized fibers supported by either pyruvate-malate or succinate-rotenone (blocking reverse electron flow) also generated extremely high rates of H$_2$O$_2$ production (Figs. 2 and 3). Complex III catalyzes the oxidation of reduced coenzyme Q (QH$_2$, quinol) by using a bifurcated series of single electron transfers, with cytochrome c serving as the final electron acceptor (31). Antimycin A, which blocks electron transfer from the cytochrome b proteins to the Q$_1$ site (“inside” or matrix side of inner membrane) of complex III, dramatically increases O$_2^-$ production during respiration supported by pyruvate-malate or succinate-rotenone (Figs. 2 and 3), presumably due to accumulation of an unstable semiquinone at the Q$_0$ site (“outside” or cytosolic side of inner membrane) (47). Subsequent addition of stigmatellin, which prevents the transfer of the first electron from QH$_2$ to the Rieske protein, completely abrogated (succinate/rotenone) or significantly reduced (pyruvate-malate) H$_2$O$_2$ production induced by antimycin A, confirming complex III as the site of O$_2^-$ generation. O$_2^-$ production at complex III has recently been implicated in the cellular response to hypoxia, providing some hint of the potential physiological relevance of O$_2^-$ production at this site (13).

Of considerable relevance to the potential mechanisms and consequences of ROS production is the topology or sidedness of O$_2^-$ production, a matter that has been somewhat controversial with respect to complex III. Initial studies on submitochondrial particles indicated that O$_2^-$ is released exclusively to the matrix (4, 48), whereas more recent studies using isolated mitochondria (±SOD) have provided evidence that O$_2^-$ is released almost exclusively to the intermembrane space (15, 29, 41). Using both indirect (H$_2$O$_2$) and direct measures of O$_2^-$ production in mitochondria isolated from mouse whole hindlimb muscles (containing a mixed population of myofiber types), Muller et al. (30) found that the Q$_0$ site of complex III releases O$_2^-$ in approximately equal portions to both the cytoplasmic and matrix sides of the inner membrane, offering two potential mechanisms by which this may occur. The data presented in Figs. 2 and 3 of the present study, however, provide evidence that the topology of O$_2^-$ release from complex III in skeletal muscle may be fiber type specific. In permeabilized fiber bundles from soleus and RG muscle, antimycin A-stimulated H$_2$O$_2$ production was similar in the presence or absence of SOD, whereas in WG permeabilized fibers, H$_2$O$_2$ detection was reduced >65% when SOD was not included in the reaction buffer. The simplest interpretation of these data is that virtually all of the O$_2^-$ produced at complex III in types I and IIA fibers is released to the mitochondrial matrix, whereas in type IIB fibers, the majority is released to the intermembrane space. An alternative possibility is that mitochondria present in oxidative fibers may possess the means of rapidly dismutating O$_2^-$ released directly into the intermembrane space, which would explain the apparent insensitivity to exogenous SOD in RG and soleus fibers observed in the present study. Indeed, the recent confirmation of Cu,Zn-SOD in the intermembrane space of mitochondria from rat liver and yeast (33, 42) lends credence to this possibility. However, complex III-mediated H$_2$O$_2$ production is similar in skeletal muscle mitochondria isolated from mice lacking Cu,Zn-SOD and wild-type mice (30), implying that Cu,Zn-SOD may not be localized to the intermembrane space of mitochondria in skeletal muscle or that activity of the enzyme is lost during the isolation of mitochondria but retained in permeabilized fibers. Further work is required to determine the fiber type-specific topology of O$_2^-$ release from complex III in skeletal muscle.

Surprisingly, ROS production at complex I also appeared to be partially sensitive to SOD, because rotenone-stimulated H$_2$O$_2$ production decreased by ~25% in RG muscle and ~50% in WG muscle during pyruvate-malate-supported respiration when SOD was not included in the reaction buffer (Fig. 2). These findings suggest that at least a fraction of complex I-generated O$_2^-$ may be released to the intermembrane space, an interpretation that is at odds with the widely held notion that complex I releases O$_2^-$ exclusively to the matrix (30, 41). Complex I consists of 46 distinct subunits, the majority of which have been identified but remain uncharacterized in terms of function (8, 18). Whether mitochondria preserved in their native reticulum configuration in permeabilized fibers retain a O$_2^-$-producing site in complex I that is
otherwise lost or masked in preparations of isolated mitochondria requires further study.

The fact that mitochondrial H$_2$O$_2$ production rates observed among the three types of muscles did not mirror differences in respiratory capacity/mitochondrial content suggests that mitochondria housed within each myofiber type possess intrinsic features that affect ROS production and/or ROS removal. Measurements of H$_2$O$_2$ scavenging rate in permeabilized fibers indicated that ROS removal capacity is significantly greater in soleus > RG > WG muscle when expressed relative to milligrams of dry weight (Fig. 5B, left). When normalized to citrate synthase activity (index of mitochondrial content), H$_2$O$_2$ scavenging rate was still highest in soleus muscle, whereas rates were similar in RG and WG muscle (Fig. 5B, right). H$_2$O$_2$ detoxification in mitochondria is largely a function of glutathione peroxidase activity (2, 54). Although higher in soleus and RG muscle when expressed in absolute terms, glutathione peroxidase activity was similar in all three types of muscle when normalized for mitochondrial content. Thus it appears that soleus myofibers may possess a slightly greater capacity to scavenge mitochondrial H$_2$O$_2$, limiting overall ROS emission. By contrast, the similar mitochondrial-specific scavenging capacity evident in RG and WG fibers suggests that the higher state 4 rate of free radical leak observed in WG muscle (Fig. 4) may be due to an elevated sensitivity to conditions that favor mitochondrial ROS production/emission. Potentially, the much thinner ultrastructure of the mitochondrial reticulum in type IIB fibers (32) may limit the efficiency of intramitochondrial scavenging and thus contribute to an overall greater rate of ROS emission.

In summary, with a novel in situ approach to examine the control of mitochondrial ROS production in skeletal muscle, the findings of the present study suggest that both basal and maximal rates of mitochondrial H$_2$O$_2$ production, as well as the topology of O$_2$$^•$− release/dismutation, differ markedly among soleus > RG > WG muscle when expressed relative to absolute terms. Differences in the stoichiometry-activity ratios of the respiratory complexes (25), susceptibility to proton pump “slip” at complex IV (21), or other mechanisms. Alternatively, the unique mitochondrial-specific scavenging capacity evident in RG and WG fibers suggests that the higher state 4 rate of free radical leak observed in WG muscle (Fig. 4) may be due to an elevated sensitivity to conditions that favor mitochondrial ROS production/emission. Potentially, the much thinner ultrastructure of the mitochondrial reticulum in type IIB fibers (32) may limit the efficiency of intramitochondrial scavenging and thus contribute to an overall greater rate of ROS emission.

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