

The Mitochondrial Phenotype of Peripheral Muscle in Chronic Obstructive Pulmonary Disease

Disuse or Dysfunction?

Martin Picard^{1,2*}, Richard Godin^{1,2*}, Michael Sinnreich³, Jacinthe Baril², Jean Bourbeau², H el ene Perrault^{1,2}, Tanja Taivassalo¹⁻³, and Yan Burelle⁴

¹Department of Kinesiology and Physical Education, McGill University, ²Respiratory Epidemiology and Clinical Research Unit, Montreal Chest Institute, ³Neuromuscular Research Group, Montreal Neurological Institute, and ⁴D epartement de Kin esiologie, Universit e de Montr eal, Montreal, Quebec, Canada

Rationale: Peripheral muscle alterations have been recognized to contribute to disability in chronic obstructive pulmonary disease (COPD).

Objectives: To describe the mitochondrial phenotype in a moderate to severe COPD population and age-matched controls.

Methods: Three primary aspects of mitochondrial function were assessed in permeabilized locomotor muscle fibers.

Measurements and Main Results: Respiration rates per milligram of fiber weight were significantly lower in COPD muscle compared with healthy age-matched control muscle under various respiratory states. However, when variations in mitochondrial volume were taken into account by normalizing respiration per unit of citrate synthase activity, differences between the two groups were abolished, suggesting the absence of specific mitochondrial respiratory impairment in COPD. H₂O₂ production per mitochondrion was higher both under basal and ADP-stimulated states, suggesting that mitochondria from COPD muscle have properties that potentiate H₂O₂ release. Direct assessment of mitochondrial sensitivity to Ca²⁺-induced opening of the permeability transition pore (PTP) indicated that mitochondria from patients with COPD were more resistant to PTP opening than their counterparts in control subjects.

Conclusions: Comparison of these results with those of studies comparing healthy glycolytic with oxidative muscle suggests that these differences may be attributable to greater type II fiber expression in COPD muscle, as mitochondria within this fiber type have respiratory function similar to that of mitochondria from type I fibers, and yet are intrinsically prone to greater release of H₂O₂ and more resistant to PTP opening. These results thus argue against the presence of pathological mitochondrial alterations in this category of patients with COPD.

Keywords: chronic obstructive pulmonary disease; skeletal muscle; mitochondrial function; oxidative stress; permeability transition pore

Peripheral muscle alterations are now recognized as an important contributor to exercise intolerance, reduced quality of life, and ultimately to disability and poor outcome in patients with chronic obstructive pulmonary disease (COPD) (1). These alter-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

It is presently unclear whether the abnormal locomotor muscle condition in patients with chronic obstructive pulmonary disease (COPD) is a result of specific muscle dysfunction or the result of prolonged disuse.

What This Study Adds to the Field

This study presents evidence demonstrating that the mitochondrial phenotype of patients with moderate to severe COPD is normal, thereby disproving a specific mitochondrial dysfunction hypothesis in this population.

ations, which include muscle fiber atrophy, lower proportion of type I fibers, reduced muscle capillarization, and reduced oxidative capacity (2, 3), have been largely attributed to muscle inactivity associated with disuse in the context of consequences of a sedentary lifestyle. However, given the severity of these changes relative to those observed in sedentary but otherwise healthy individuals, it has been suggested that reduced muscle activity may not be the only underlying factor (4, 5). In support of this notion, studies have shown that locomotor skeletal muscle from patients with COPD can display signs of oxidative stress (6), activation of proteolytic pathways (7), and enhanced apoptosis (8). Together, these observations have led to the proposal that an intrinsic muscle disease (i.e., a myopathic state) could play an additional role in atrophy, weakness (4), and overall exercise intolerance. However, evidence of muscle dysfunction, or the period of disease progression at which this myopathic state could become a significant contributor to dysfunction, is still lacking; moreover, the putative mechanisms involved in its development remain unknown.

Maintenance of mitochondrial integrity is crucial to the preservation of cellular energy homeostasis. The role of mitochondria also extends far beyond energy production, as they constitute important generators of reactive oxygen species (ROS) that can act either as second messengers or as a source of cellular damage depending on the amount being produced (9). In addition, it is now well established that in response to specific signals or after internal damage to mitochondria, these organelles have the capacity to activate necrosis and apoptosis by increasing their membrane permeability, through opening of the permeability transition pore (PTP) (10). Therefore, it is increasingly being proposed that, in addition to the potential contribution of steroid-induced toxicity from corticosteroid treatments (11), chronic low-grade systemic inflammation (12), and/or hypoxemia (13), the development of specific mitochondrial abnor-

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*These authors contributed equally to this study.

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Correspondence and requests for reprints should be addressed to Tanja Taivassalo, Ph.D., Assistant Professor and FRSQ Research Scholar, Department of Kinesiology and Physical Education, 475 Pine Avenue West, Room 222, McGill University, Montreal, PQ H2W 1S4, Canada. E-mail: tanja.taivassalo@mcgill.ca

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malities could be an integral part of muscle disease in patients with COPD (4). However, direct experimental evidence in support of this notion is currently lacking.

In the present study, we used permeabilized muscle fibers taken from the quadriceps of healthy control individuals and patients with COPD to perform an integrative analysis of mitochondrial function. Respiratory function was determined in different states of activity in the presence of substrates and inhibitors for various respiratory chain complexes. In addition, we used novel approaches developed in our laboratory (14) to provide the first direct assessment of mitochondrial ROS production and sensitivity to PTP opening in human muscles fibers. We hypothesized that in addition to a reduced amount of mitochondria (15, 16) caused by adoption of a sedentary lifestyle, muscle from patients would display signs of specific mitochondrial abnormalities such as impairment of respiratory responses, increased production of ROS, and vulnerability to opening of the PTP in response to a Ca^{2+} challenge, a typical trigger of permeability transition. Some results of this study have been previously reported in abstract form (17).

METHODS

Study Participants

Seven male patients with COPD based on the Global Initiative for Chronic Obstructive Lung Disease classification of disease severity (18) ($\text{FEV}_1 = 1.32 \pm 0.2$ L; $47 \pm 6\%$ predicted) and eight healthy male sedentary control subjects matched for age (± 5 yr) were investigated. See the online supplement for inclusion criteria. The study was approved by the ethics review board of the Montreal Chest Institute (Montréal, PQ, Canada), and all participants provided written informed consent. All study participants underwent, on two separate days, a clinical evaluation followed by a needle biopsy of the vastus lateralis muscle.

Clinical and Physiological Evaluation

Anthropometric measurements were obtained followed by pulmonary function testing to assess FEV_1 , residual volume, and diffusing capacity of the lung for carbon monoxide. Patients with COPD and control subjects completed a standard incremental peak exercise test for determination of peak work capacity and aerobic power (peak rate of oxygen consumption) with continuous monitoring of ECG, ventilatory parameters, and arterial oxygen saturation through finger oximetry. The partial pressure of oxygen in arterial blood (Pa_{O_2}) at rest and during submaximal exercise was determined from blood samples drawn from the radial artery catheter and analyzed with a Rapidlab 840 (Bayer Healthcare, Tarrytown, NY) blood gas analyzer.

Muscle Needle Biopsy

Percutaneous biopsy samples of the right vastus lateralis muscle were obtained by standard Bergstrom needle technique as previously described (19). Each sample was immediately put on ice in precooled stabilizing buffer, and transported to the laboratory for immediate dissection and permeabilization.

Preparation of Permeabilized Myofiber and Ghost Fiber Bundles

Dissection and permeabilization of fiber bundles with saponin were performed as described previously (20, 21). Ghost fibers were prepared as per Saks and coworkers (20) by incubating saponin-permeabilized bundles in a high-KCl medium, which allows extraction of myosin. Permeabilized myofibers and ghost fiber bundles were then kept on ice until use. All mitochondrial parameters were determined at least in duplicate for every subject. See the online supplement for further details on muscle biopsy, preparation of myofibers, and methods to assess the various mitochondrial parameters described in subsequent sections.

Mitochondrial Respiration

Mitochondrial respiratory function of permeabilized myofibers was determined as per Picard and coworkers (14). Rates of oxygen consumption were expressed as nanomoles of oxygen per minute per milligram dry weight, or per unit of the marker enzyme citrate synthase (CS) to normalize for differences in mitochondrial content between individuals.

Mitochondrial Production of Reactive Oxygen Species

Mitochondrial H_2O_2 production was measured in permeabilized fiber bundles with the fluorescent probe Amplex red (Invitrogen, Carlsbad, CA) as described previously (14, 22). Rates of H_2O_2 production were calculated from a standard curve established under the same experimental conditions except that fibers were absent. All H_2O_2 measurements were expressed per milligram of dry weight or per unit of CS.

Sensitivity to Ca^{2+} -induced PTP Opening

Accumulation of Ca^{2+} in the mitochondrial matrix is the most important and obligatory trigger for PTP opening (23). Sensitivity to permeability transition is therefore commonly assessed in isolated mitochondria by determining mitochondrial calcium retention capacity (CRC) in response to a Ca^{2+} challenge (24). In the present study, a novel method developed in our laboratory was used to determine CRC in ghost fibers (14). Briefly, mitochondrial Ca^{2+} uptake after the addition of a single Ca^{2+} pulse (20 nM) was followed by monitoring the decrease in extramitochondrial calcium concentration, using the fluorescent probe Calcium green-5N (Invitrogen). Progressive uptake of Ca^{2+} by mitochondria was monitored until mitochondrial Ca^{2+} release caused by opening of the PTP was observed. The CRC, which is a reliable index of PTP sensitivity (25), was calculated as total amount of Ca^{2+} taken up by mitochondria before Ca^{2+} release. CRC values were expressed per milligram of dry fiber weight and per unit of CS.

Citrate Synthase Assay

The activity of CS in fiber bundles extracts was measured as per Picard and coworkers (14) and reported as milliunits per milligram of dry fiber weight. We and others have previously shown the validity of CS as an indirect marker of mitochondrial volume density in skeletal muscle (14, 26).

Statistical Analyses

Results are expressed as means \pm SEM. Mean comparisons of baseline physiological characteristics and peak exercise performance between patients and control subjects were achieved through a two-tailed Student *t* test. Similarly, planned comparisons of CS activity, the rate of oxygen consumption at each stage of respiration, H_2O_2 production, and CRC were performed by two-tailed Student *t* test. Significance was assumed at $P \leq 0.05$.

RESULTS

Study Participant Characteristics

Table 1 shows the characteristics of the patients with COPD and control subjects. Subjects were of similar age and sex. As per the inclusion criteria, patients showed moderate to severe disease severity ($\text{FEV}_1 = 1.32 \pm 0.5$ L; $47 \pm 17\%$ predicted). Patients showed a lower body weight that was related to a lower lean mass and a lower body mass index (BMI) than the control group, although it fell within the normal range (27). The fat-free mass index was above the threshold of cachexia (less than 17 kg/m^2) (28) for all subjects. As expected, patients showed a reduced peak exercise capacity compared with healthy control subjects. None of the patients displayed resting hypoxemia, although exercise-induced hypoxemia was observed (Pa_{O_2} at 65% peak power: 100 ± 9 and $72 \pm 12\%$ in control and COPD, respectively; $P < 0.05$).

Oxidative Capacity and Respiratory Function

As shown in Figure 1B, respiration per unit of fiber weight was significantly lower in fibers of patients with COPD than in fibers

TABLE 1. CHARACTERISTICS OF STUDY PARTICIPANTS

	Control (n = 8)	COPD (n = 7)
Age, yr	68 ± 2	68 ± 2
Sex (male), n	8	7
Height, cm	175 ± 2	169 ± 3
Weight, kg	91 ± 6	72 ± 2*
Body mass index, kg/m ²	29 ± 3	25 ± 1*
Body fat, %	26 ± 2	26 ± 2
Fat-free mass, kg	68 ± 5	54 ± 2*
FFMI, kg/m ²	21.7 ± 1.2	18.9 ± 0.6
FEV ₁ , L	3.19 ± 0.3	1.26 ± 0.4*
FEV ₁ , % predicted	102 ± 9	45 ± 15*
FEV ₁ /FVC, %	73 ± 1	44 ± 6*
RV, % predicted	101 ± 11	189 ± 9*
RV/TLC, %	35 ± 4	61 ± 2*
Dl _{CO} , % predicted	103 ± 9	48 ± 20*
Peak \dot{V}_{O_2} , L · min ⁻¹	2.09 ± 0.38	0.90 ± 0.27*
Peak work capacity, W	159 ± 8	61 ± 15*
Pa _{O₂} , rest	85.0 ± 3	77.5 ± 4
Sa _{O₂} , rest	96.5 ± 0.3	95.3 ± 1

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; Dl_{CO} = carbon monoxide diffusion capacity; FFMI = fat-free mass index; RV = residual volume; TLC = total lung capacity; \dot{V}_{O_2} = rate of oxygen consumption. Values represent means ± SEM.

* $P < 0.05$.

from healthy control subjects under all respiratory states with the exception of the rate of glutamate-malate-driven respiration (\dot{V}_{GM}) and the rate of TMPD-ascorbate-driven respiration (\dot{V}_{TMPD}), for which differences did not reach statistical significance. The activity of the mitochondrial marker enzyme CS was 37% lower in fibers from patients with COPD compared with healthy control subjects (52 ± 4 vs. $84 \pm 4 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ dry weight; $P \leq 0.0001$). As shown in Figure 1C, normalization of respiration rates per unit of CS abolished all differences observed between the two experimental groups. There was no significant difference in the acceptor control ratio, representing the coupling between oxidation and phosphorylation in COPD compared with the control subjects ($\dot{V}_{ADP}/\dot{V}_{GM}$, 3.44 ± 0.45 vs. 3.38 ± 0.64 in control and COPD; $P = 0.67$). Similarly, no significant differences were observed between the two experimental groups for the V_{succ}/V_{ADP} ratio (1.20 ± 0.23 vs. 1.04 ± 0.08 ; $P = 0.18$) and the maximal capacity of cytochrome *c* oxidase (COX) to drive oxidative respiration when stimulated directly (COX excess capacity, 5.72 ± 0.76 vs. 6.74 ± 0.62 ; $P = 0.37$).

Production of Reactive Oxygen Species

Figure 2A shows a representative tracing of H₂O₂ production obtained in permeabilized fibers in response to sequential addition of substrates and inhibitors. In general, net H₂O₂ release by fibers was low in the absence of respiratory substrates in both groups but increased by approximately twofold after addition of the complex I donors glutamate and malate. However, in line with previous observations (14, 22, 29), the highest rates of H₂O₂ release under basal conditions were observed in the presence of the complex II donor succinate. Subsequent addition of ADP at increasing concentrations led to a progressive reduction in H₂O₂ release, which is expected when electron flow through the respiratory chain is stimulated (29). Finally, interruption of normal electron flow with addition of the complex III inhibitor antimycin A induced a substantial increase in H₂O₂ release, as expected.

In comparing patients with COPD with healthy control subjects, the net release of mitochondrial H₂O₂ per milligram of fiber weight was similar under all states of respiration (data not shown) in spite of a lower mitochondrial volume density in

COPD muscle. Therefore, when normalized per unit of CS activity, net H₂O₂ release was significantly higher in COPD than in control subjects during baseline succinate-driven respiration, and at all rates of ADP-stimulated respiration. H₂O₂ release also tended ($P = 0.09$) to be higher in COPD fibers in the presence of antimycin A, which was used to promote H₂O₂ release by blocking electron flow at complex III.

Sensitivity to Ca²⁺-induced PTP Opening

Figure 3A shows typical Ca²⁺ kinetics measured in ghost fibers energized with glutamate-malate. In all experiments the addition of exogenous Ca²⁺ to the incubation medium caused a large increase in Calcium green-5N fluorescence, which was immediately followed by a progressive reduction due to the electrophoretic uptake of Ca²⁺ by mitochondria. Ca²⁺ uptake then reached a plateau followed by the release of accumulated Ca²⁺. When experiments were repeated in the presence of the PTP inhibitor cyclosporin A (CsA) the amount of Ca²⁺ required to trigger Ca²⁺ release (i.e., CRC) was approximately doubled, confirming that opening of the PTP caused this phenomenon. In previous studies we have also shown that the Ca²⁺ uptake was specific to mitochondria and entirely dependent on the presence of respiratory substrates (14). As shown in Figure 3B, CRC values per unit of mitochondrial CS were 56% higher in fibers from patients with COPD compared with healthy control subjects (214 ± 40 vs. $137 \pm 31 \mu\text{mol Ca}^{2+}/\text{mU CS}$; $P = 0.019$), indicating a lower sensitivity to Ca²⁺-induced opening of the PTP in COPD muscle.

DISCUSSION

To our knowledge, this study provides the first integrative assessment of mitochondrial function in skeletal muscle from patients with moderate to severe COPD. We investigated three primary aspects, namely, mitochondrial respiration, production of reactive oxygen species, and susceptibility to permeability transition pore opening in COPD and a group of healthy, age-matched control subjects. Our results indicate that although whole muscle oxidative capacity was reduced in patients with COPD, respiratory properties of individual mitochondria did not differ from those of healthy control subjects. On the other hand, it was found that in COPD, production of reactive oxygen species per mitochondrion was increased and sensitivity to Ca²⁺-induced opening of the permeability transition pore was diminished compared with control subjects.

Respiratory Function

Tissue oxidative capacity is determined by the number and size of mitochondria (i.e., mitochondrial volume density), and by the respiratory function of individual mitochondria. In peripheral muscle from patients with COPD, there is clear evidence that oxidative capacity is impaired as a result of a reduction in mitochondrial volume density (assessed by marker enzyme activities or directly with electron microscopy) (16, 30) and expression of key transcription factors for mitochondrial biogenesis (31). In the present study, the systematic reduction in respiratory rates per milligram of fiber weight in the muscle of patients with COPD is in line with these previous results. Moreover, the activity of the mitochondrial marker enzyme CS was reduced by approximately 40% in COPD muscle, which is comparable to values reported by others (16, 30).

However, our results indicate that mitochondrial respiration expressed per unit of CS was not significantly different between patients and control subjects irrespective of the respiratory state (basal, ADP stimulated, and uncoupled) and type of substrate

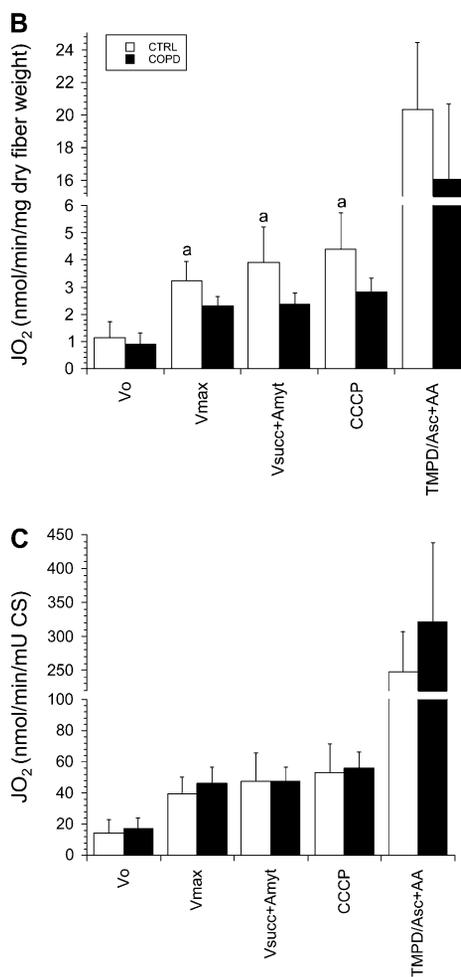
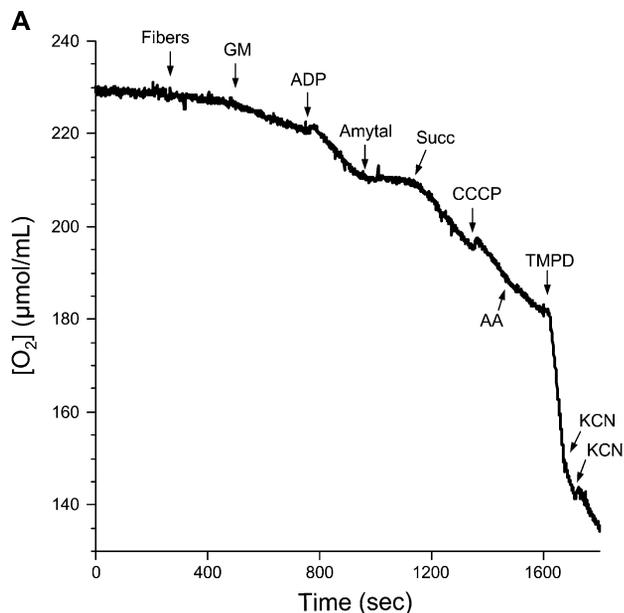


Figure 1. Respiratory function in permeabilized muscle fiber bundles from patients with chronic obstructive pulmonary disease (COPD) and healthy control subjects (CTRL). (A) Representative tracing of changes in oxygen concentration as determined by oximeter in response to the sequential addition of glutamate-malate (GM, 5:2.5 mM), ADP (2 mM), amytal (Amyt, 2 mM), succinate (Succ, 5 mM), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 1 μ M), antimycin A (AA, 8 μ M), tetramethylphenylenediamine plus ascorbate (TMPD/Asc, 0.9:9 mM), and potassium cyanide (KCN, 0.6 mM). (B) Respiration rates (J_{O_2}) expressed per unit of dry fiber weight under various states in fibers from patients with COPD and healthy control subjects. (C) Respiratory rates (J_{O_2}) expressed per unit of the marker enzyme citrate synthase (CS). Values represent means \pm SEM. ^aSignificantly different from control ($P < 0.05$).

oxidized (complex I, II, and III). Reporting mitochondrial respiratory rates per unit of CS constitutes an important feature of this study, by providing the distinct advantage of assessing respiratory function per mitochondrion. To date, this study is the first to report it as such for a group of patients with COPD. The normalization of mitochondrial respiratory rates relative to citrate synthase provides a more valuable index of mitochondrial function than measurements of respiratory chain enzyme activities or respiratory rates relative to muscle mass because it is not confounded by differences in mitochondrial content, which are known to vary significantly according to physical activity levels. The absence of a difference in respiration rates per unit of CS reported in the present study therefore rules out any specific mitochondrial respiratory impairments. This is further supported by the fact that several important respiratory parameters (acceptor control ratio, V_{succ}/V_{ADP} ratio, and COX excess capacity) were not different in fibers from control subjects and COPD, which would not have been the case if specific impairments had been present.

In general, these data are in agreement with results by Rabinovich and coworkers (32), who reported no difference in the acceptor control ratio in succinate-energized mitochondria from patients with COPD when BMI was normal (i.e., above 21 kg/m²), although a reduced acceptor control ratio was observed in a subgroup of patients with low BMI (<19 kg/m²). Similarly, increased expression of COX I subunit and COX activity has been reported in patients with COPD with chronic

arterial hypoxemia ($Pa_{O_2} < 60$ mm Hg) (33) but not in patients with higher Pa_{O_2} , suggesting that hypoxia induces specific changes at the level of COX to improve its efficiency at low arterial oxygen tensions (34). In the present study, although patients exhibited exercise-induced arterial oxyhemoglobin desaturation, resting levels of Pa_{O_2} remained between 62 and 88 mm Hg, with an average value (77.5 mm Hg) considered within the normal range. It may therefore be suggested that transient hypoxemic spells, as may result from brief periods of exercise-induced arterial oxygen desaturation, may not be sufficient to trigger COX1-related adaptation. Taken together, these results indicate that in patients with COPD who are not hypoxemic at rest, with moderate to severe disease involvement but with normal BMI, mitochondrial function appears to remain intact.

Mitochondrial ROS Production

Oxidative stress in peripheral muscle is increasingly being positioned as a potentially important contributor to muscle dysfunction in COPD (4). Studies have reported increased levels of lipid peroxidation by-products (35, 36) and accumulation of both nitrosylated (35, 37) and carbonylated proteins (38) in the muscle of patients with COPD at rest. Moreover, an abnormal decrease in reduced glutathione content has been reported in response to exercise training in patients with COPD, suggesting that muscle from patients with COPD can produce excessive amounts of ROS when confronted with repetitive metabolic stress (39). However, several sources of

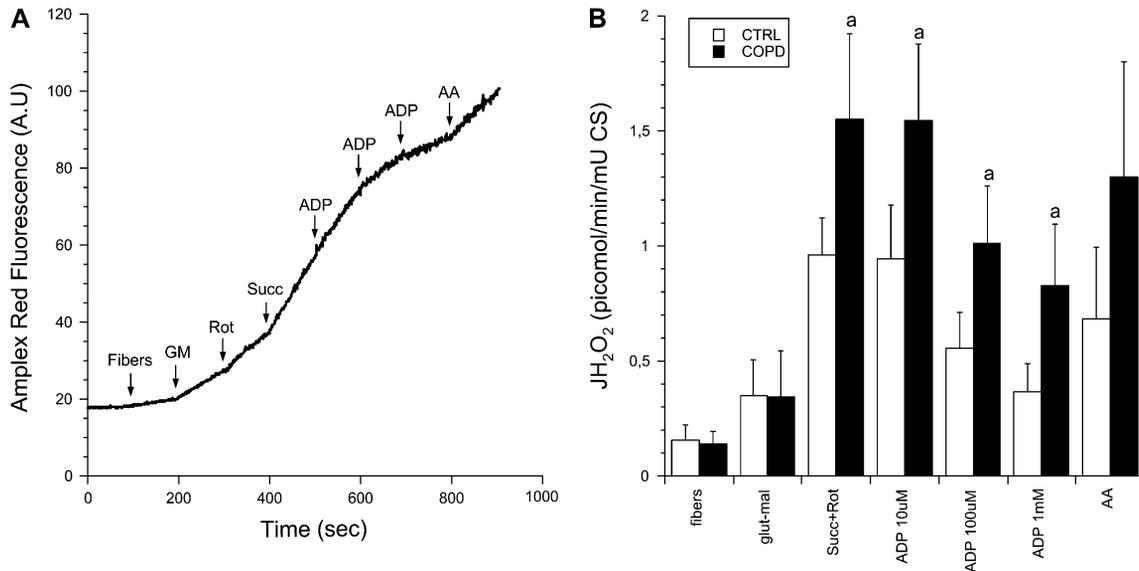


Figure 2. Rate of mitochondrial H_2O_2 (JH_2O_2) release in fibers from patients with chronic obstructive pulmonary disease (COPD) and healthy control subjects. (A) Representative tracing of change in Amplex red fluorescence in response to the sequential addition of fibers, glutamate–malate (GM, 5:2.5 mM), rotenone (Rot, 1 μM), succinate (Succ, 5 mM), ADP (10, 100, and 1,000 μM), and antimycin A (AA, 8 μM). (B) Rate of H_2O_2 production calculated from the slope of change in fluorescence in fibers from patients with COPD and healthy control subjects. All values are expressed per unit of the marker enzyme citrate synthase (CS). ^aSignificantly different from control ($P < 0.05$).

free radicals may be responsible for this phenomenon and the extent to which mitochondria contribute, if at all, is not established.

To our knowledge, the present study is the first to provide direct measurements of mitochondrial H_2O_2 production in mus-

cle of patients with COPD. We observed that total H_2O_2 production per unit of fiber weight was similar in the two experimental groups (data not shown). However, when expressed relative to CS activity, net release of H_2O_2 per mitochondrion was significantly higher in fibers of patients with COPD

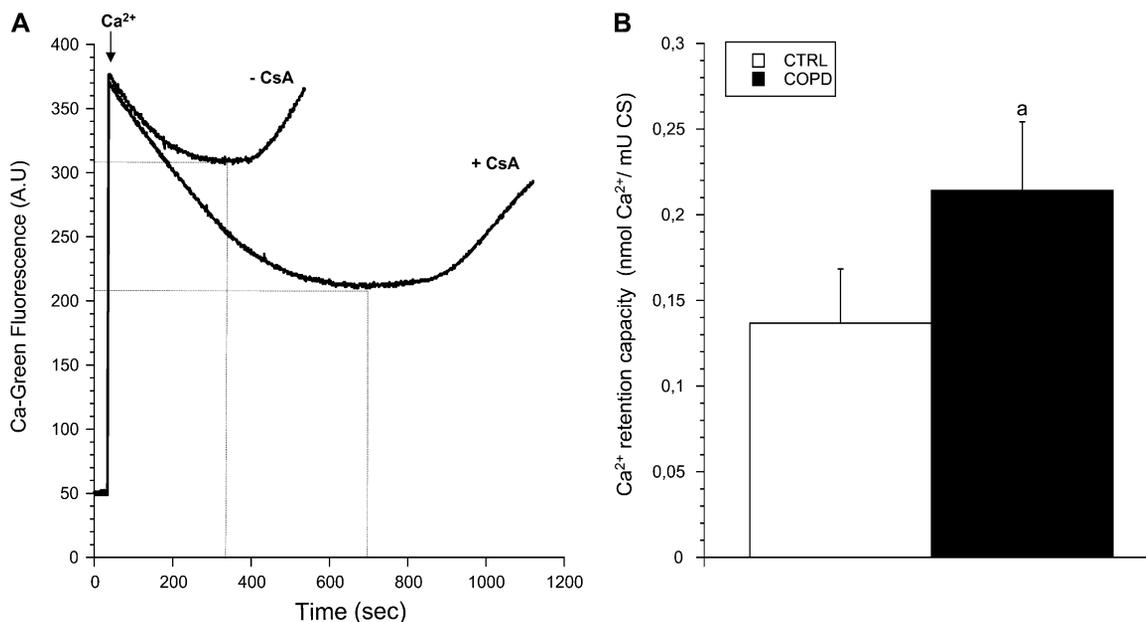


Figure 3. Sensitivity to Ca^{2+} -induced permeability transition pore (PTP) opening in ghost fibers from patients with chronic obstructive pulmonary disease (COPD) and healthy control subjects. (A) Typical changes in $[\text{Ca}^{2+}]$ in the incubation medium as a result of mitochondrial Ca^{2+} uptake and release. Experiments were initiated by the addition of ghost fibers (data not shown) followed by a single pulse of 20 nmol of Ca^{2+} . A progressive reduction in fluorescence was observed as a result of mitochondrial Ca^{2+} uptake followed by the release of accumulated Ca^{2+} due to PTP opening. Calcium retention capacity (CRC) represents the amount of Ca^{2+} taken up by mitochondria before the initiation of Ca^{2+} release (denoted by thin gray lines). As expected, the PTP inhibitor cyclosporin A (CsA, 1 μM) significantly increases the CRC. Although not shown, the response to CsA was similar in fibers from both experimental groups. (B) Mitochondrial calcium retention capacity in fibers from patients with COPD and healthy control subjects. Results are normalized per unit of the marker enzyme citrate synthase (CS). ^aSignificantly different from control ($P < 0.05$).

compared with those of healthy control subjects. This phenomenon was observed during baseline complex II-driven respiration, which is associated with high rates of superoxide production, and persisted under conditions of active phosphorylation, which is known to reduce superoxide generation (29). These results thus provide clear evidence that mitochondria from patients with COPD possess properties that potentiate H_2O_2 production. Of note, this phenomenon, which was observed *in vitro* with standardized levels of oxygenation, could be expected to be further enhanced *in vivo* when muscles of patients with COPD experience hypoxia, a condition associated with the promotion of superoxide generation by the respiratory chain (40, 41).

Factors responsible for the enhanced mitochondrial H_2O_2 release in COPD muscle currently remain unclear. Defects in respiratory chain complexes are a common cause of oxidative stress in pathologies involving mitochondria, such as ischemia-reperfusion (42). This, however, cannot account for the enhanced ROS release seen in the present study as no specific mitochondrial respiratory dysfunction could be observed. Interestingly, we (14) and others (22) have reported that mitochondria within fast glycolytic type II fibers release significantly greater amounts of H_2O_2 than their counterparts in slow oxidative type I fibers in rats. This phenomenon is attributed to the fact that mitochondria from type II fibers display a lower H_2O_2 -scavenging capacity and possibly other intrinsic differences in factors that modulate superoxide production by the respiratory chain (22). Although it was not possible to quantify fiber type composition in the present study, there is strong agreement that the proportion of type II fibers is increased in COPD (3, 43–47). As an example, one study of a COPD population similar to ours reported a 24% decrease in type I fiber proportion (34 vs. 58%) with a concomitant increase in type II fibers in patients with COPD compared with control subjects (45). Moreover, in our previous animal study (14) we reported that the difference in ROS production between type I and type II fibers was 250%. On the basis of this value, the 63% difference in ROS production observed between patients with COPD and healthy control subjects in the present study would represent a 25% difference in fiber type proportion, which is well within the range of change in fiber type reported for this patient population (3, 43–47). Consequently, the higher mitochondrial H_2O_2 release observed in muscle from patients with COPD in the present study could be reflective of a normal healthy muscle fiber type shift in response to reduced muscle activity and associated sedentary lifestyle, rather than the result of pathological alterations in mitochondrial function.

Susceptibility to Opening of the Permeability Transition Pore

Another key property of mitochondria that is receiving much attention is their capacity to trigger cell death. A key event in this process is the opening of the PTP, a nonspecific proteinaceous channel of the inner mitochondrial membrane (23, 48–54). Prolonged opening of the PTP results in mitochondrial swelling, loss of membrane potential ($\Delta\Psi$), uncoupling of oxidative phosphorylation, ATP hydrolysis, and release of several proapoptotic proteins normally confined to mitochondria including cytochrome *c*, AIF, Endo-G, Smac/Diablo, and Omi-HTrA2.

In view of this central role of the PTP, we determined whether sensitivity to Ca^{2+} -induced opening of the PTP differed in mitochondria from patients with COPD compared with age-matched healthy control subjects. To our knowledge, PTP regulation has not previously been studied in human skeletal muscle tissue samples, be it from healthy or diseased individuals. Our results show that mitochondria from COPD muscle

are significantly more resistant to Ca^{2+} -induced PTP opening than are those of healthy control subjects. Results from animal studies clearly suggest that mitochondria generally become less, not more, resistant to PTP opening in the presence of myopathic conditions such as denervation (25), congenital dystrophies such as Duchenne (55) and Bethlem (56, 57), bupivacaine-induced myotoxicity (58), or aging-induced sarcopenia (59), all of which have been associated with activation of cell death either by apoptosis or necrosis. Evidence of enhanced apoptosis has been reported in patients with COPD with low BMI (8) but not in patients with a less severe condition (8, 47), such as the patients in the present study. Our findings are therefore in direct contradiction to the expected myopathic patterns, which may be taken to suggest that at least in moderate to severe COPD, mitochondria are not more prone to permeability transition. It will therefore be of interest in future studies to determine whether vulnerability to PTP opening develops as the disease evolves to a more severe state.

The factors accounting for the greater resistance to Ca^{2+} -induced PTP opening observed in patients with COPD currently remain unclear. An intriguing possibility may be that, as discussed above for H_2O_2 release, the observed resistance to PTP opening may also be related to the greater proportion of type II fibers within COPD muscle (3, 43–47).

In support of this suggestion is our observation that mitochondria from type II rat fibers are intrinsically more resistant to Ca^{2+} -induced PTP opening than are mitochondria from type I fibers (14), as demonstrated by a 300% greater calcium retention capacity per unit of CS. On the basis of this value, the 56% difference in CRC observed between healthy control subjects and patients with COPD in the present study would represent an 18% difference in fiber type proportions, again consistent with previous reports (3, 43–47). In the animal study, we argued that a higher Ca^{2+} threshold for PTP opening in fast fibers could represent a basic cellular mechanism to prevent accidental activation of cell death signaling because fast fibers typically display higher and more frequent Ca^{2+} surges than slow fibers during contraction.

Perspective

The present study indicates that in patients with moderate to severe COPD, but who do not display aggravating conditions that worsen disease prognosis (such as cachexia or hypoxemia), the reduction in peripheral locomotor muscle oxidative capacity is due to a lower mitochondrial volume density but not to any specific mitochondrial respiratory abnormalities. We propose that the enhanced ROS production and resistance to PTP opening observed in mitochondria from COPD muscle could be related to a greater proportion of type II fibers compared with healthy control subjects. This is supported by observations from animal models showing that mitochondria from fast glycolytic muscle produce more ROS (14, 22) and are more resistant to PTP opening (14), but yet display similar respiratory function as their counterparts in slow oxidative muscle (14). Therefore, from the perspective of mitochondrial function integrity, our results do not support the presence of a myopathic state at this stage of disease but rather point to disuse as the main factor underlying mitochondrial alterations within moderate to severe COPD muscle. Future studies are required to determine whether mitochondrial dysfunction develops in patients with more severe disease as a result of aggravating conditions such as hypoxemia, chronic inflammation, and cachexia.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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**THE MITOCHONDRIAL PHENOTYPE OF PERIPHERAL MUSCLE IN COPD: DISUSE
OR DYSFUNCTION?**

Martin Picard, Richard Godin, Michael Sinnreich MD, PhD, Jacinthe Baril, Jean Bourbeau MD, PhD, H el ene Perrault PhD, Tanja Taivassalo PhD, and Yan Burelle PhD

ONLINE DATA SUPPLEMENT

METHODS

Subjects

Seven male COPD subjects and 8 male healthy sedentary controls (CTRL) matched for age (± 5 years) were investigated. To be eligible, COPD subjects had to meet the following criteria: (1) aged 40 years or older; (2) physician-diagnosed COPD; (3) post-bronchodilator FEV₁/FVC ratio less than 0.7 and FEV₁ less than 80% of predicted value. To be eligible CTRL subjects had to meet the following criteria: (1) aged 40 years or older; (2) non smokers and not known to have respiratory disease; (3) pre- and post-BD FEV₁/FVC \geq 0.70. Participants taking anti-coagulant drugs, or with a history of serious cardiovascular problems were excluded from the study. Participants were told to take their medications as usual during study participation with the exception of aspirin, which was stopped at least 3 days before the second visit to avoid bleeding during the muscle biopsy procedure.

Clinical and physiological evaluation

Anthropometric measurements (weight, height, % body fat, lean mass) were obtained followed by pulmonary function testing (Medisoft body box 5500 ®) to assess forced vital capacity (FEV₁), residual volume (RV) and diffusing capacity of the lung for carbon monoxide (DLCO). Patients completed an incremental peak exercise test utilizing 10W per minute increments until exhaustion on a cycle ergometer (Ergoline 800) for determination of peak work capacity for aerobic power (peak VO₂) with continuous monitoring of ECG, ventilator parameters and arterial oxygen saturation (SaO₂) through finger oxymetry. For control individuals, the increments were 15 or 20W every minute.

Muscle needle biopsy

Percutaneous biopsy samples of the right *vastus vateralis* muscle were obtained from mid-thigh level (about 15 cm above patella) using the standard Bergstrom needle technique as previously described (19). Briefly, after application of local anesthetic (lidocaine 5%), a 1 cm incision was made through which the needle was inserted. Suction was applied and approximately 100 mg wet weight of tissue was obtained. The sample was immediately put on ice in pre-cooled stabilizing buffer, Solution A (in mM: (2.77 CaK₂EGTA, 7.23 K₂EGTA, 6.56 MgCl₂, 0.5 Dithiothreitol (DTT), 50 K-MES, 20 imidazol, 20 taurine, 5.3 Na₂ATP, 15 phosphocreatine, pH 7.3 @ 4°C) and transported to the laboratory for immediate dissection and permeabilization. All bundles were dissected within two hours post-biopsy. The time at which each type of assay (*i.e.* respirometry, H₂O₂ production and Ca²⁺ challenge) was performed after the biopsy was standardized in order to minimize potential effect of *in vitro* aging on mitochondrial function. Respirometry and ROS measurements were followed by Ca²⁺ challenge experiments, initiated three and six hours post-biopsy, respectively.

Preparation of permeabilized myofiber bundles

Manual dissection of the muscle sample to yield approximately 8-10mg dry weight of fiber bundles was performed in buffer A. Muscle bundles were dissected by gently teasing apart fibers, while preserving areas or contact at the extremities of fibers, to a point where within a bundle of tens to hundreds of fibers, only aggregates of 2-5 individual fibers remained (14). Once dissection was completed, fibers were placed in Solution A supplemented with 0.05 mg/ml saponin to allow selective permeabilization of the sarcolemma. Following 30 minutes of incubation at low rocking speed, fiber bundles were subjected to three 10 minutes rinses in buffer B (in mM: 2.77 CaK₂ EGTA, 7.23 K₂EGTA, 1.38 MgCl₂, 3.0 K₂HPO₄, 0.5 dithiothreitol, 20 imidazole, 100 K-MES, 20

taurine, pH 7.3, at 4°C) supplemented with fatty acid free Bovine Serum Albumin (BSA: 2 mg/ml). Fiber bundles were kept on ice until use.

Preparation on ghost fiber bundles

Ghost fibers without myosin were prepared as previously described (20) with minor modifications. Fiber bundles were first permeabilized with saponin and washed 3 times in buffer B as described above, then washed three times for 10 min in buffer C (in mM: K-MES 80, HEPES 50, taurine 20, DTT 0.5, MgCl₂ 10, ATP 10, pH 7.3 at 4°C). Fibers were then incubated for 30 min with intermittent manual agitation at 4°C in buffer D (in mM: KCl 800, HEPES 50, taurine 20, DTT 0.5, MgCl₂ 10, ATP 10, pH 7.3 at 4°C) to extract myosin, washed three times in low-EGTA sucrose buffer (in mM: 250 sucrose, 10 Tris, 0.005 EGTA, 10 MOPS, pH 7.3 at 4°C) and kept on ice until use for Ca²⁺-induced PTP opening assays. All CRC measurements were performed at least in triplicates for every subject.

Mitochondrial respiration protocol

Mitochondrial respiratory function was determined in an oxymeter equipped with a Clark type electrode (Oxygraph, Hansatech Instruments). The chamber was filled with 1ml of buffer B with supplemented BSA. After recording baseline oxygen content in the chamber, one bundle of 1-2mg dry weight of permeabilized myofibers was placed into the chamber which was then sealed shut. All measurements were performed at 23°C. Baseline readings were taken in the absence of any exogenous respiratory substrates (V_{fibers}). As depicted in Figure 1A, the following additions were sequentially made: the complex I substrates glutamate-malate (5:2.5 mM, V_{GM}), the complex I blocker amytal (2 mM), ADP (2 mM, V_{ADP}), the complex II substrate succinate (10 mM, V_{succ}), the

uncoupler CCCP (1 μM , V_{CCCP}), the complex III blocker antimycin-A (8 μM), the complex IV substrates TMPD-Ascorbate (0.9:9 mM, V_{TMPD}), and the complex IV blocker cyanide (KCN: 0,6 mM) (Figure 1A). At the end of each test, fibers were carefully removed from the oxygraphic cell, blotted, wet weighted and frozen at -80°C until the determination of citrate synthase (CS) activity. Dry weight was calculated from fiber wet weight using a dry/wet weight ratio of 0.23 (60). This ratio has been used previously for permeabilized fibers studies (61) and we have verified its appropriateness in a previous study (14).

Report of mitochondrial respiration

Rates of O_2 consumption (JO_2) were expressed in nanomoles of O_2 per minute per milligram dry weight, or per unit of the marker enzyme citrate synthase (CS) to normalize for differences in mitochondrial content between subjects. The sequential addition of substrates, inhibitors and uncoupler allowed to determine on each bundle *i*) basal respiration with complex I donors (V_{GM}), *ii*) maximal ADP-stimulated respiration when the respiratory chain is energized with complex I (V_{ADP}) or complex II substrates (V_{succ}), *iv*) uncoupled respiration (V_{CCCP}) and maximal complex IV activity (V_{TMPD}). Three ratios were also calculated from these respiratory rates: 1) the acceptor control ratio (ACR) or $V_{\text{ADP}}/V_{\text{GM}}$, which represents the degree of coupling between oxidation and phosphorylation with complex I substrates; 2) $V_{\text{succ}}/V_{\text{ADP}}$, which represents the ability of complex II substrates to stimulate phosphorylation; and 3) $V_{\text{TMPD}}/V_{\text{succ}}$ which represents the excess capacity of complex IV relative to the maximal rate of oxidative phosphorylation. All respirometry experiments were performed at least in duplicates for every subject. TMPD-ascorbate driven respiration was systematically corrected for auto-oxidation of TMPD as previously described (14).

Mitochondrial production of ROS

Mitochondrial H₂O₂ production was measured in permeabilized fiber bundles with the fluorescent probe Amplex red (20 μM: excitation-emission: 563-587) as described previously (14, 22). Following preparation of permeabilized fibers, samples destined to ROS measurements were rinsed 3 times in buffer Z (in mM: 110 K-MES, 35 KCl, 1 EGTA, 5 K₂HPO₄, 3 MgCl₂·6H₂O, and 0.5mg/ml BSA, pH 7.3 at 4°C) before measurements could be performed. Fiber bundles (0.3-1.0 mg dry weight) were incubated at 37 °C in a quartz micro-cuvette with continuous magnetic stirring in 600 μL of buffer Z (pH 7.3 at 37°C) supplemented with 1.2 U/mL horseradish peroxidase.. Baseline fluorescence readings were taken in the absence of any exogenous respiratory substrates. The following additions were then made sequentially: glutamate-malate (5:2.5 mM), rotenone (1 μM), succinate (5 mM), ADP (10, 100 and 1000 μM) and antimycin-A (8 μM). Rates of H₂O₂ production were calculated from a standard curve established in the same experimental conditions except that fibers were absent. This protocol allowed quantification of mitochondrial H₂O₂ release under basal ADP-restricted respiration supported either by complex I or complex II donors and at various levels of ADP. This latter condition was used because of its relevance to the *in vivo* situation where mitochondria are subject to constant but variable levels of stimulation by cellular ADP. All H₂O₂ measurements were performed at least in triplicates for every subject.

Sensitivity to Ca²⁺-induced PTP opening

Accumulation of Ca²⁺ in the mitochondrial matrix is the most important and obligatory trigger for PTP opening (23). A Ca²⁺ challenge was used because of the well-established fact that accumulation of Ca²⁺ in the mitochondrial matrix is the obligatory and most important factor to trigger of PTP opening. In addition, variations in several other regulators of PTP opening (such as ROS, membrane and redox potentials,

[adenylates], [Pi], pH) often translate into changes in the Ca^{2+} threshold required for pore opening. The measure of calcium retention capacity therefore provides an integrated assessment of PTP vulnerability. Sensitivity to permeability transition is therefore commonly assessed in isolated mitochondria by determining mitochondrial calcium retention capacity (CRC) in response to a Ca^{2+} challenge (62, 63). Similar measurements have also been previously reported in permeabilized hepatocytes and various cell lines (64-66). However, in contrast to these non-contractile cells, muscle fibers possess contractile filaments and a well developed sarcoplasmic reticulum (SR). Therefore, binding of exogenous Ca^{2+} to contractile filaments as well as Ca^{2+} uptake by the SR could interfere with the measurement of mitochondrial CRC. For these reasons we have recently described a novel approach that allows to quantify CRC without these pitfalls (14). Briefly, this method makes use of ghost fibers, which are devoid of contractile filaments. In addition, SR Ca^{2+} uptake is effectively abolished during these measurements by omitting adenylates from the incubation medium and by adding oligomycin in order to prevent oxidative phosphorylation from residual adenylates, which may provide energy to support to SR Ca^{2+} ATPases.

For this assay, ghost fibers (0.3 – 1.0 mg dry fiber weight) incubated at 23 °C in a quartz micro-cuvette with low-to-medium continuous magnetic stirring in 600 μL of CRC buffer supplemented with glutamate-malate (5:2.5 mM) and 0.5 nM oligomycin. Mitochondrial Ca^{2+} uptake was followed by monitoring the decrease in extra-mitochondrial calcium concentration using the fluorescent probe Calcium-green 5N (1 μM , excitation-emission: 505-535 nm). At the beginning of each test, residual calcium concentration present in the buffer was adjusted to the same level using a small amount of EGTA prior to adding fiber bundles. Following addition of ghost fibers, a single pulse of Ca^{2+} (20 nmoles) was added (see Figure 3A). Progressive uptake of Ca^{2+} by

mitochondria was monitored until mitochondrial Ca^{2+} release caused by opening of the PTP was observed. Calcium retention capacity (CRC) was calculated as total amount of Ca^{2+} taken by mitochondria prior to Ca^{2+} release. It has been previously established that this value represents a reliable index of the Ca^{2+} threshold for PTP opening in the whole mitochondrial population studied (25). CRC values were expressed per mg of dry fiber weight and by unit of CS. Ca^{2+} concentration in the cuvette was calculated from a standard curve relating $[\text{Ca}^{2+}]$ to the fluorescence of Ca-Green 5N. Of note, since variations in several other regulators of PTP opening (such as ROS, membrane and redox potentials, [adenylates], [Pi], pH) often translate into changes in the Ca^{2+} threshold required for pore opening, the CRC assay provides an overall measure of PTP susceptibility, although it does not allow identification of the precise regulatory mechanisms contributing to it.

Citrate synthase assay:

For the measurement of CS activity, frozen fibers bundles (1.0 – 2.5 mg dry wt) that were previously subjected to respiration, were homogenized with a vibrating micro-bead homogenizer in 200 μl of homogenization buffer (in mM: 250 sucrose, 40 KCl, 2 EGTA, 20 Tris-HCl, pH 7.3 at 4°C). Homogenate was then supplemented with 0.1 % Triton X-100 and incubated on ice for 60 min. Following centrifugation for 8 min at 10 000 g, the activity of CS was determined spectrophotometrically at 37°C as previously described (25) and reported in mU/mg dry fiber weight. We and others have previously shown the validity of CS as an indirect marker of mitochondrial volume density in skeletal muscle (14, 26).