Superoxide Flashes in Mouse Skeletal Muscle Are Produced by Discrete Arrays of Active Mitochondria Operating Coherently

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Abstract

Reactive Oxygen Species (ROS) constitute important intracellular signaling molecules. Mitochondria are admitted sources of ROS, especially of superoxide anions through the electron transport chain. Here the mitochondria-targeted ratiometric pericam (RPmt) was used as a superoxide biosensor, by appropriate choice of the excitation wavelength. RPmt was transfected in vivo into mouse muscles. Confocal imaging of isolated muscle fibers reveals spontaneous flashes of RPmt fluorescence. Flashes correspond to increases in superoxide production, as shown by simultaneous recordings of the fluorescence from MitoSox, a mitochondrial superoxide probe. Flashes occur in all subcellular populations of mitochondria. Spatial analysis of the flashes pattern over time revealed that arrays of mitochondria work as well-defined superoxide-production-units. Increase of superoxide production at the muscle fiber level involves recruitment of supplemental units with no increase in per-unit production. Altogether, these results demonstrate that superoxide flashes in muscle fibers correspond to physiological signals linked to mitochondrial metabolism. They also suggest that superoxide, or one of its derivatives, modulates its own production at the mitochondrial level.

Introduction

Reactive oxygen species (ROS) refer to a group of oxygen containing molecules having the capability of reacting with reduced compounds. They were once viewed only as harmful molecules involved in disease and aging. Nowadays, this early perception is being replaced by a new concept whereby at low concentration ROS serve a physiological role as signaling molecules, while at high concentration they can damage critical cellular components, inducing cell death.

This dual role of ROS is fundamental in skeletal muscle physiology/pathology. On the one hand, ROS have been postulated to be involved in a panel of distinct muscle disorders, such as myotonic dystrophy, Duchenne dystrophy, central core disease, malignant hyperthermia and muscle fatigue [1,2]. On the other hand, numerous evidences support a role of ROS in several muscle functions, such as force production [3], initiation of adaptive changes in gene expression [4], and regulation of calcium channels, calcium transporters and calcium-sensing proteins [5–7].

Several sources of ROS are present in mammalian skeletal muscle [4,6,8] the main produced species being superoxide anions (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). Mitochondria are often cited as the major site of superoxide production in tissues. Superoxide is generated from molecular O$_2$ by the mitochondrial electron transport chain (ETC) and several other enzymes. ROS production by mitochondria is most likely of primary importance in skeletal muscle as these organelles are in close apposition with calcium stores [9] containing redox –sensitive calcium-signaling proteins. Indeed, close location between ROS source and target is mandatory since efficient cellular antioxidant defenses and ROS own reactivity restrain their spatial diffusion.

The recent identification of brief bursts of superoxide production –superoxide flashes– within the matrix of mitochondria in resting cells opened up a completely new area into ROS signaling studies [10]. Superoxide flashes have been described in a wide range of cells, including cardiac myocytes, skeletal myotubes, neurons, neuroendocrine cells and clonal cell lines.

In the present work, the mitochondria-targeted calcium biosensor ratiometric-pericam-mt (RPmt) [11] was transfected into mouse skeletal muscle fibers for use as a superoxide biosensor, by appropriate choice of the excitation wavelength. Results show that 1) Superoxide flashes in adult skeletal muscle fibers are physiological events linked to metabolism and involving the ETC.

2) Mitochondria operate as well defined units for the production of superoxide, active units being adjacent to quiescent ones. 3) Superoxide flashes cause depolarizations of mitochondria and releases of calcium. These phenomena involve channels distinct from the classical permeability transition pore (PpP) and inner membrane anion channel (IMAC). 4) Increase of superoxide production at the fiber level is mediated by a recruitment of mitochondrial units and not by an increase of the production per unit. Superoxide or one of its derivatives may be involved in the activation of quiescent mitochondria.
Results

Targeting of RPmt

RPmt is targeted to the mitochondrial matrix by the sequence encoding the N-terminal 12 amino acids presequence of subunit IV of cytochrome c oxidase. Confocal imaging revealed that RPmt was colocalised with TMRM, a mitochondrion-selective membrane potential indicator (fig. 1A, B, C). RPmt stained intermyofibrillar and subsarcolemmal mitochondria, with little if any cytosolic contamination (fig. 1B). 3D reconstruction (fig. 1D) of the intermyofibrillar mitochondrial network, as highlighted by RPmt, unveiled a very sharp pattern with paired slender mitochondria transversally aligned on both sides of the Z line, and thin and a few thick longitudinal mitochondrial columns, as previously described at electron microscopy resolution [12]. The results show that my experimental conditions allow a highly precise targeting of the protein.

Spontaneous bursts of fluorescence reflect an increase of superoxide

Repeated xy confocal imaging of RPmt revealed an exciting phenomenon: ~20 s-long bursts of fluorescence with an average ΔF/F₀ of 0.93±0.02 (n = 1328 flashes from 34 cells) (see fig. 2A and movie S1 in supplemental material).

Due to the nature of RPmt, a calcium sensor, these events were first interpreted as transient increases of mitochondrial matrix calcium concentration. However, spectral properties of these events presented discrepancies with the calcium-dependence of the excitation spectrum provided by Nagai et al. [11] and of the spectrum recorded here in transfected skeletal muscle fibers (fig. 2D). Increases in RPmt fluorescence were observed simultaneously at the excitation wavelengths of 488 and 458 nm (fig. 2B) whereas calcium-induced changes in fluorescence should have concomitantly at the excitation wavelengths of 405 nm (fig. 3A). This was not compatible with a calcium-induced change in fluorescence since, in this case, a strong decrease of fluorescence at 405 nm should accompany an increase of fluorescence at 491 nm (fig. 2D). Moreover, the simultaneous measurement of Rhod-2 fluorescence showed a decrease of mitochondrial [Ca²⁺] concomitant with the increase of RPmt fluorescence, strongly arguing against the possibility that the events reflect calcium movements.

During the course of this work, Wang et al. [10] showed that the fluorescent protein cpYFP of the ratiometric pericam can specifically sense superoxide. The authors created a new superoxide sensor, called mt-cpYFP by removing the calcium sensing part (calmodulin (nt 896–1323) and M13 (nt 49–126)) from the RPmt. Hence, in addition to sensing calcium, RPmt is also sensing superoxide, most likely with the same properties than mt-cpYFP. Interestingly, mt-cpYFP spectral properties are compatible with my records using different excitation wavelengths. Hence, the events could reflect transient increases of superoxide level, detected by the cpYFP part of the RPmt, rather than transient increases in calcium concentration. Several lines of evidences confirmed this hypothesis. First, the records showed simultaneous increases in the fluorescence intensity of MitoSox, a mitochondrial specific synthetic probe selective for superoxide, and RPmt fluorescence (fig. 3B). The continuous increase in the MitoSox fluorescence intensity during the time course of the experiment was undoubtedly due to non-reversibility of the fluorescence change in this dye. These results were reproduced on 6 other cells. Second, application of 15 mM tiron, a superoxide scavenger, to the fiber decreased the frequency of events from 12.4 ± 2.4 to 2.4 ± 1.1 μm²/1000 μm² cell.100 s (n = 7 cells), and the average events amplitude F/F₀ from 2.43 ± 0.37 to 2.06 ± 0.25 (fig. 3C).

For the sake of nomenclature uniformity, the spontaneous bursts of RPmt fluorescence were named “flashes”, following the terminology of Wang et al. [10]. In order to avoid calcium contamination in the superoxide signals, RPmt was then systematically excited at 477 nm. Superoxide flashes occurred randomly in time. Their size presented important variability. In order to encompass both their number and their size, flashes frequency is reported in this paper in total area of flashing mitochondria per area of cell per unit of time (μm²/1000 μm² cell.100 s). Average flash frequency was 18.2 ± 4.2 (n = 42 cells), but varied substantially between different fibers. Hence, to study the effect of a given condition on the properties of the events, records were performed in a same fiber under control condition and after treatment, and paired statistical tests were used. In order to discard any effect of laser exposure on events properties, which may interfere with the treatment, two 3 min time series were successively recorded on the same fiber. The frequency and properties of the flashes during the first and the second time series were similar (n = 7, see fig. 4A). This shows that, at least under the current experimental conditions, superoxide flashes properties are not modified by putative damaging effects induced by long lasting laser exposure.

Bursts of mitochondrial ROS have been shown in cardiac cells following artificial ROS production by photoactivation of the mitochondrion-selective membrane potential indicators TMRM and TMRE [13,14]. In the present work, superoxide flashes...
occurred also in cells transfected with RPmt but devoid of any synthetic dye (TMRM or other). In order to rule out the possibility that superoxide flashes result from artificial ROS production, for instance following excitation of RPmt, the following protocol was performed: muscle fibers transfected with RPmt were successively exposed to increasing intensities of a 477 nm laser line (laser transmission: 30 %, 50 %, 90 %). Neither the frequency nor the properties of the flashes were affected by the increase in laser exposure intensity (fig. 4B). This result demonstrates that the superoxide flashes recorded in this study are spontaneous cellular events and not artifacts induced by recording conditions.

Subcellular localization of superoxide flashes

Superoxide flashes were observed in subsarcolemmal mitochondria, as well as in intermyofibrillar mitochondria (fig. 5A). Their relative distribution between these two subcellular compartments was not random, as they were more frequent in subsarcolemmal mitochondria than in intermyofibrillar ones (25.9% ± 7 of flashing mitochondria among the subsarcolemmal ones vs 16% ± 5.6 among the intermyofibrillar ones, n = 6 cells, see fig. 5B).

Events recorded in intermyofibrillar mitochondria presented different patterns (fig. 6A). Some seemed to involve a single mitochondrion, point shaped or column-shaped, longitudinal or...
transversal. Point shaped events had an area of approximately 0.088–0.8 m\textsuperscript{2}, while column shaped events were roughly 0.8–1.6 m\textsuperscript{2}. Others (1.6–2.4 m\textsuperscript{2}) comprised a pair of longitudinal mitochondria. Finally, a third class of events encompassed large clusters of mitochondria and involved thick longitudinal columns.

The histogram of distribution of flashes size (fig. 6C) revealed an inverse correlation between size and frequency, the point-shaped events being the most frequent (n = 490 events on 7 cells). The large clusters, which areas could vary from 2.4 up to 18 m\textsuperscript{2}, were less common than the other patterns, and not found in every cell. It should however be kept in mind that, as shown in fig. 1D, mitochondrial network encompasses several confocal planes. Hence flashes size could be underestimated here, as measures are performed in a single confocal plan.

Flashes of any size occurred amidst a background of quiescent mitochondria, suggesting that flashes do not simply propagate to adjacent mitochondria under resting conditions. The spatial distribution of flashes was not random, but linked to the morphology of mitochondria. Hence, thick longitudinal columns were often involved in large clusters (fig. 6A). On the other hand, point shaped and column shaped events were found in longitudinal thin column, or transversal paired slender mitochondria.

Even the largest cluster-sized flashes presented a striking homogeneity in terms of increase of fluorescence intensity (fig. 6B), suggesting either a continuity of mitochondrial lumen in the entire cluster, or the existence of superoxide-permeant junctions between the mitochondria constituting a cluster. Furthermore, the same cluster could flash several times during a 5 min record (fig. 6B), the shape of the successive flashes being identical. It should be stressed that the possibility of changes in the flash shape due to mitochondrial movements should be discarded because mitochondria are essentially immobile in skeletal muscle at the records time scale, mostly due to their strong attachment to calcium release units [15].

Overall, results show the existence of intermyofibrillar mitochondrial units of variable size, characterized by their superoxide production. These units have well-defined boundaries and are stable in time. ‘Active’ units are surrounded by quiescent ones; this shows that superoxide does not diffuse randomly to adjacent mitochondria.

Flashes are metabolism-linked events

As flashes seem to be ubiquitous events in different cell types [10] and, in a same cell, among different types of mitochondria (fig. 5), they might be linked to the first role of the mitochondrion, the regulation of cellular metabolism. Indeed, flash frequency was low (7.2 ± 3.1 m\textsuperscript{2} 1000 m\textsuperscript{2} cell.100 s, n = 12) when fibers were incubated in a Tyrode solution devoid of metabolites and increased to 16.9 ± 4.2 (n = 12) upon application of 10 mM glucose

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and 5 mM pyruvate (fig. 7A). Surprisingly, neither the flashes amplitude nor the number of flashes per mitochondrial unit (fig. 7A) were affected. Furthermore, the distribution of inter-myoﬁbrillar unit size was identical in ﬁbers incubated with Tyrode devoid of or containing metabolites (fig. 7B), whereas the number of ﬂashing units was higher in ﬁbers incubated in Tyrode containing glucose and pyruvate. These results suggest that mitochondria work as restricted units of production of superoxide, and that increase in cell superoxide production is due to a recruitment of supplementary units rather than an increase of superoxide production per unit.

Inhibition of the electron transport chain with 2.5 μM antimycin A, an inhibitor of complex III, decreased ﬂashes frequency from 26.7±4.1 to 9.9±3.2 μm²/1000 μm² cell.100 s (n=5, fig. 7C), without affecting the ﬂashes amplitude or the number of ﬂashes per unit, demonstrating an involvement of the ETC in the ﬂashes production. Overall, these results show that ﬂashes are metabolic–linked events involving the ETC.

Superoxide ﬂashes are accompanied by mitochondrial depolarization

86% of superoxide ﬂashes were accompanied by a mitochondrial depolarization, as shown by the simultaneous records of RPmt ﬂuorescence and TMRM (fig. 8A, B). These depolarizations occurred exactly at the same spatial location than the ﬂashes, which reinforces the hypothesis of separated mitochondrial units...
for the production of superoxide. The depolarizations had a mean amplitude $\Delta F/F_0$ of 0.57 ± 0.01 (n = 391 flashes from 11 cells), the minimal amplitude being 0.11. Hence, the 14% of flashes that appeared to be devoid of depolarization were probably due to a lack of resolution of the technique rather than a lack of depolarization. These depolarizations did not have systematically the same kinetics as the flashes. They could be square-shaped or long lasting (with no recovery during the course of the record). In rare cases (2–3% of the flashes), the flash-linked depolarization was preceded by a transient mitochondrial hyperpolarization. Finally, some spontaneous depolarizations were devoid of flashes.

Wang et al. [10] suggested that mitochondrial depolarization causes the superoxide flash. In order to check this hypothesis in my model, I applied tiron to the cells, and measured flash and depolarization frequency. My results show that depolarization frequency is higher than flashes frequency (36.9 ± 11 mm$^2$/1000 mm$^2$ cell.100 s versus 26.4 ± 7.4 mm$^2$/1000 mm$^2$ cell.100 s, n = 5), which is expected since some depolarizations are independent of flashes. As described above, flash frequency decreased with tiron from 26.4 ± 7.4 mm$^2$/1000 mm$^2$ cell.100 s to 13 ± 6.8 (n = 5). Surprisingly, depolarization frequency also decreased, to the same extent (36.9 ± 11 mm$^2$/1000 mm$^2$ cell.100 s to 20.2 ± 8.5), arguing that the depolarization is caused by the superoxide flash, rather than the opposite (fig. 8C).

Tiron treatment affects the kinetics of the flashes

As discussed before, tiron treatment decreased the amplitude of the flashes, as well as the flashes frequency. Conversely, fig. 9 shows that tiron did not significantly affect the number of flashes per unit (fig. 9A) or the distribution of intermyofibrillar unit sizes (fig. 9B). However, time to peak was significantly increased upon tiron application (fig. 9C: 4.89 ± 0.47 s under control conditions vs 6.2 ± 0.54 s with tiron, n = 7). This strongly suggests that superoxide regulates its own production. Interestingly, neither the $\tau$ decay ($\tau = 8.23 ± 1.03$ s$^{-1}$ control vs $\tau = 10.23 ± 1.79$ s$^{-1}$ tiron, n = 5) nor the full width at half magnitude (8.06 ± 0.95 s control vs 6.98 ± 0.68 s tiron, n = 5) were affected.

Mechanisms underlying flashes genesis

Stochastic openings of the PtP have been shown to trigger superoxide flashes genesis in cardiac myocytes [10]. Hence, to test the hypothesis that PtP may be involved in flashes production in skeletal muscle, cyclosporin A (a PtP inhibitor) and atracyloside (a PtP activator) were applied. Neither inhibition of PtP with 5 μM cyclosporin A (fig. 10A) nor activation of PtP with 50 μM atracyloside (fig. 10B) affected flashes frequency or properties, suggesting that another mechanism is involved in superoxide flashes production in skeletal muscle fibers.

Another mitochondrial channel could be involved in the production of superoxide flashes: IMAC. IMAC mediated...
mitochondrial propagation of ROS events has been shown in cardiac myocytes following local perturbation of a few mitochondria by phototoxicity [14]. Therefore, the effects of 4’ chlorodiazepam (4-ChlDZP), a reported inhibitor of IMAC, were tested. Application of 40 μM 4-ChlDZP did not affect flashes frequency nor properties (fig. 11). Also, in 2 cells transfected with RPmt and loaded with TMRM, 4-ChlDZP application failed to inhibit mitochondrial depolarization induced by superoxide flashes (result not shown). These results show that IMAC is neither involved in physiological superoxide flashes production nor in flashes-induced mitochondrial depolarizations in skeletal muscle.

Discussion

In the present work, RPmt was used as a superoxide biosensor targeted to mitochondria of adult skeletal muscle fibers. Repeated xy confocal imaging of RPmt revealed superoxide flashes with an approximate duration of 20 s, and an average amplitude ΔF/F₀ of 0.93±0.02. Mitochondrial superoxide flashes appear as discrete events arising amidst a quiescent background. They involve clusters of active mitochondria, or active units, whose shape relies heavily on the organization of the mitochondrial network. Flashes are not a consequence of damaging processes, such as pathological phenomenon, laser exposure, oxidative stress or metabolic stress. Rather, they seem to be physiological events, linked to cellular metabolism. In their turn, superoxide flashes modulate mitochondrial function, as they cause mitochondrial depolarizations.

Superoxide flashes genesis is shown to involve ETC activity as ETC blockade by antimycin A dramatically decreases superoxide flashes frequency. Interestingly, no significant differences were found in basal mitochondrial level of superoxide (F₀) in cells under control and antimycin conditions (result not shown). Antimycin A is commonly seen as an enhancer of ROS production through accumulation of ubisemiquinone at complex III. However, discrepancies have been described in the literature. For instance, antimycin A has been reported to decrease superoxide flashes frequency in cardiac myocytes [10], or to reduce glucose deprivation-induced ROS production in cerebellar granule
neurons [18]. Discrepancies may arise from different experimental conditions or probes (DCF, Amplex Red indirectly measuring superoxide through its by-product hydrogen peroxide or hydroxyl radical vs hydroethidine, mt cpYFP which directly measure superoxide levels). Also, mitochondrial matrix levels of superoxide may be differently regulated from cytosolic ones. Indeed, Kudin et al. [19] showed that antimycin A induces an increase of superoxide levels in isolated mitochondria from rat brain and skeletal muscle, this increase taking place outside the mitochondrial matrix. Superoxide flashes are not randomly distributed among the subpopulations of mitochondria. They were found to be in higher frequency in subsarcolemmal mitochondria than in intermyofibrillar ones. Metabolic differences between these two populations of mitochondria were observed in permeabilized muscle fibers from mouse quadriceps, rat soleus and gastrocnemius [20,21]. By measuring FAD/NADH autofluorescence, which reflects the activity of the ETC, the authors showed that subsarcolemmal mitochondria are in a more oxidative state than intermyofibrillar ones. The higher flashes frequency observed in this work in subsarcolemmal mitochondria could be well explained by a higher ETC activity.

Superoxide flashes in intermyofibrillar mitochondria involve active mitochondrial clusters adjacent to quiescent ones. These active units have very well defined limits and are stable in time, as superoxide flashes can be detected several times at the same place during a 3 min record. The distribution and shape of active units in skeletal muscle relies on the mitochondrial organization, especially on the presence of thin and thick columns, as described in [12]. For instance, large units (clusters) systematically involve longitudinal thick columns. Unit size varies from 0.1 \( \mu \text{m}^2 \) up to 18 \( \mu \text{m}^2 \). The fact that mitochondrial depolarizations, as detected with TMRM, occur exactly at the same place as superoxide flashes shows that mitochondrial clusters are composed of electrically connected mitochondria. This connection could be due to a continuous lumen, or to conductive junctions between mitochondria. Junctions of high electric conductance have been described...
by Amchenkova et al. [22] in rat cardiomyocytes. These junctions define mitochondrial clusters by connecting together several mitochondria. Such networks are common and have been described in several cellular types [23]. These mitochondrial clusters may constitute subcellular microdomains for fine tune of energy production and delivery, and organisation of antioxidant defense, as discussed by [23].

Superoxide flashes are accompanied by transient depolarization of mitochondria. Spontaneous transient depolarizations of mitochondrial membrane potential, sometimes called mitochondrial flickers [24], are a widespread phenomenon. They have been for instance observed in neuroblastoma cells [25], cardiomyocytes [10,13,24,26], smooth muscle cells [17,27], vascular endothelial cell and isolated mitochondria [28]. Mitochondrial flickers have been linked to calcium movements [17,24], cytosolic calcium overload [16,17], and oxidative stress [13,17,29]. The present work, in accordance with Cheng’s group [10], propose a new model in which flickers are linked to bursts of superoxide in the mitochondrial matrix. However, the two studies disagree on the mechanism. In their pioneer paper, Wang et al. [10] showed that, in cardiac myocytes, inhibiting or silencing the PtP decreases the flash frequency. Their interpretation was that opening of the PtP, which causes mitochondrial depolarization, triggers the flash. Present results show that the mechanism is different in adult skeletal muscle fibers. Superoxide scavenging with tiron decreases the flashes frequency as well as the depolarization frequency, indicating that increase in superoxide level triggers the depolarization and not the opposite. The contradictory results obtained by the two groups could be reconciled by the model proposed in cardiac cells by Zorov et al. [13,30], according to which, in a single mitochondrion, increased ROS production triggers opening of the PtP, which in turn causes a burst of ROS deriving from the ETC. This phenomenon was called ROS induced ROS release. RIRR is accompanied by a depolarization of the mitochondrion, and in some case preceded by a hyperpolarisation of the same mitochondrion [13]. However, in my work, neither the activation of the PtP by atractyloside nor the inhibition of PtP by cyclosporin A affect the flashes frequency. In rare occasions a “pre increase” in RPmt fluorescence could be identified preceding the flash. Although consistent with the “trigger” ROS production described by Zorov et al [13, fig. 4C], it is far from being a systematic pattern. Still, this could be due to the difference in sampling rate (10 Hz line scan imaging in [13], vs 1 Hz xy imaging here). Indeed, in fig. 4D of [13], which has been realised with a sample rate of 2 Hz in line scan mode, this “Trigger”ROS is barely or not detected. Further work is obviously needed to unravel the mechanism involved in the genesis of flashes.

PtP is a well known redox-sensitive calcium release protein which opening triggers depolarization of mitochondria [31]. Several studies have shown an activation of the PtP by ROS, or more precisely by superoxide [13,16]. For this reason, despite the fact that PtP is not involved in flash genesis, I investigated the possible involvement of the PtP in the depolarization and calcium release accompanying the superoxide flash. However, inhibiting
PtP with cyclosporin A failed to prevent flash-induced depolarization. Cyclosporin A-resistant mitochondrial depolarization induced by ROS has been described in guinea pig cardiomyocytes by Aon et al. [14]. The authors showed that IMAC is involved in this phenomenon. However, inhibition of IMAC failed to affect superoxide production or mitochondrial depolarization in my work. Another possibility would be the uncoupling protein 3 (UCP3), which activation causes a depolarization of mitochondria. Mitochondrial depolarization can trigger a release of calcium from the mitochondrial matrix. Interestingly, UCP3 is activated by superoxide [28]. Obviously, further work will be needed to determine the protein(s) responsible for the depolarization accompanying the spontaneous mitochondrial superoxide flash.

The juxtaposition of active and quiescent units argues against a simple diffusion of superoxide within the cell that would be detected only in mitochondria, where the sensor is. Surprisingly, my results show that, upon metabolic stimulation, increase of superoxide production involves an increase in the number of active units rather than an increase of superoxide production per active unit. In the same way, scavenging superoxide by tiron only slightly decreases flashes amplitude, and most of all dramatically decreases flashes frequency. Neither the number of flashes per unit nor the distribution of intermyofibrillar unit sizes are significantly affected. It is rather the number of active units that decreases. These results raise the question of whether superoxide or one of its derivatives may act as a second messenger between mitochondrial clusters, further stimulating production of superoxide by supplementary units. Such a mechanism, “inter mitochondria” ROS induced ROS release, has already been described in cardiac myocytes [13,14,30]. In this model, ROS are released from mitochondria by a channel, either PtP or IMAC, and trigger RIRR in some neighboring mitochondria. Mitochondria behave as a percolation matrix: to be activated, mitochondria have to be near threshold, which could explain the fact that some

**Figure 10.** Superoxide flashes are not induced by openings of the permeability transition pore. A Total area of flashing mitochondria per 1000 μm² cell per 100 s (left), average amplitude of flashes (middle) and number of flashes per active unit (right) in 5 cells under control conditions and after a 20 min treatment with 5 μM cyclosporin A. Paired Wilcoxon Signed Rank tests have been performed. B Total area of flashing mitochondria per 1000 μm² cell per 100 s (left), average amplitude of flashes (middle) and number of flashes per active unit (right) in 8 cells under control conditions and after a 20 min treatment with 50 μM atractyloside. Paired Student’s t tests have been performed.

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neighboring mitochondria are devoid of flashes [32]. However, in the present work, altering the activity of PpP or IMAC neither affected the flashes frequency/properties, nor the depolarizations linked to superoxide flashes. Discrepancies with other studies may come from distinct cellular types (skeletal muscle fibers vs cardiac myocytes), or from differences in the recorded events (physiological spontaneous superoxide flashes vs photo-induced toxic propagating ROS increase). Although we can not rule out the hypothesis that mitochondria are activated by a derivative of superoxide which can freely diffuse across membranes (H$_2$O$_2$ for instance), or that superoxide diffuse to other mitochondria through a different channel, these results suggest that, under physiological conditions, superoxide production is restricted to the productive unit. IMAC or PpP may be involved in pathological propagation of ROS production and mitochondrial activation in skeletal muscle.

To conclude, this work demonstrates a physiological, basal production of superoxide in mitochondria from adult skeletal muscle fibers, that is linked to metabolism. Superoxide production is not homogenous in the fiber, but rather occurs in active mitochondrial units surrounded by quiescent ones. Increase in superoxide production involves activation of quiescent units rather than increase of production per unit. Subcellular localization of superoxide production is of primary importance, as mitochondria are adjacent to calcium release units, containing redox-sensitive calcium signaling proteins [5–7,9]. Numerous evidences support a role of ROS in several muscle functions, including regulation of calcium channels, calcium transporters and calcium-sensing proteins [5–7]. Could mitochondrial superoxide locally modulate calcium release unit activity at rest or/and during muscle contraction? Which consequences would have a change in the numbers of active mitochondrial units on calcium signaling? Could it, at some point, affect muscle homeostasis? The present work opens up a novel exciting framework regarding the role of mitochondrial superoxide production in skeletal muscle physiology and pathology.

Materials and Methods

Ethical approval

Experiments were performed on 5–8-week-old male OF1 mice (Charles River Laboratories, L’Arbresle, France). All experiments and procedures were in accordance with the guidelines of the French Ministry of Agriculture (37/348) and of the European Community (86/609/EEC). They were approved by the local animal ethic committee of Rhone-Alpes, approval numbers 692660602, 0292.

Transfection of ratiometric-pericam-mt in adult mice FDB muscles by electroporation

In vivo transfection of ratiometric-pericam-mt (RPmt), a kind gift of Dr Miyawaki (RIKEN, Saitama, Japan), was performed within the flexor digitorum brevis (FDB) of the animals, according to Di Franco et al. [33]. In brief, 20 µl of 2 mg/ml hyaluronidase dissolved in sterile saline were injected into the footpads of each hind paw of a mouse anaesthetized by isoflurane. 1 hour later, the mouse was anaesthetized by an intraperitoneal injection of a mixture of ketamine and xylazine dissolved in sterile saline (80 and 16 mg kg$^{-1}$, respectively). 15 µl of pcDNA3/RPmt solution (10 µg DNA in sterile saline) were then injected subcutaneously. 10 min later, two electrodes (gold plated stainless steel acupuncture needles) were placed subcutaneously at the starting lines of paw and toes, separated by ~1 cm. The standard protocol consisted of 20 pulses of 120 V cm$^{-1}$ amplitude and 20 ms duration delivered at a frequency of 1 Hz by a BTX ECM 830 square wave pulse generator (Harvard Apparatus, Holliston, MA, USA). Experimental observations and measurements were carried out 4 to 5 days later.

Isolation of single FDB fibers

Single fibers were isolated from the mouse FDB muscles using a previously described procedure [34]. In brief, mice were killed by cervical dislocation. Muscle were removed and placed in a Tyrode solution containing 0.2% collagenase (Sigma, type 1) for 50 min at 37°C. After this treatment, muscles were kept at 4°C in Tyrode and used within 10 hours. Single fibers were obtained by triturating the muscles within the experimental chamber. For standard observations, fibers were bathed in Tyrode.

Solution and dye

Tyrode solution contained (in mM): 136 NaCl, 5 KCl, 2.6 CaCl$_2$, 1 MgCl$_2$, 10 Hepes, 10 Glucose, 5 Pyruvate.

Loading of the dyes were carried out at room temperature in Tyrode. For 2 dyes-detection of mitochondrial superoxide, cells
were loaded with 5 μM MitoSox for 10 min. For measurement of mitochondrial membrane potential, cells were loaded with 10 nM Tetramethyl Rhodamine Methyl Ester (TMRM) for 10 min then washed with Tyrode containing 2.5 nM TMRM to avoid loss of the dye. For fluorescence imaging of mitochondrial [Ca²⁺], cells were loaded with 5 μM Rhod 2/AM for 10 min, following a protocol adapted from Hajnoczky et al. [35].

TMRM, Rhod-2/AM and MitoSox were from Molecular Probes (Eugene, OR, USA), antymycin A, 4’chlorodiazepam, cyclosporin A, tiron from Sigma (St Louis, MO, USA).

Confocal imaging and image processing

Experiments were performed at room temperature. Fibers were visualized using a Zeiss LSM 5 Exciter laser scanning confocal microscope (Zeiss, Jena, Germany) equipped with a ×63 oil immersion objective ×1.4 numerical aperture. For detection of RPmt the excitation was provided by an argon laser (477 nm unless otherwise specified) and fluorescence was collected between 505 and 545 nm. For the series of measurements performed with Rhod-2, MitoSox or TMRM, the excitation was from the 543 nm line of a HeNe laser and fluorescence was collected above 560 nm. Fibers were imaged in full frame (xy) mode at a rate of 1 frame/s, corresponding to 1.61 μm per pixel in case of simple staining and 0.8 μm in case of double staining. The zoom factor was set to 2.6, corresponding to a xy pixel size of 0.08 μm. Frame size was 512×512 pixels, with a pixel depth of 12 bit. Pinhole was set to 1 airy unit. For xyz images in fig. 1D, refractive index correction was performed.

For fig. 2B, fibers were visualized using a Leica TCS SP5 AOPS laser scanning confocal microscope equipped with a ×40 oil immersion objective×1.4 numerical aperture, available at the PLATIM of IFR 128 BioSciences Gerland - Lyon Sud. For detection of RPmt, the excitation was provided by a diode laser (405 nm) and an argon laser (491 nm). Fluorescence was collected between 500–550 nm. Rhod-2 was excited with a diode laser (405 nm) and an argon laser (491 nm). Fluorescence was collected between 505 and 545 nm. For the series of measurements performed with Rhod-2, MitoSox or TMRM, the excitation was from the 543 nm line of a HeNe laser and fluorescence was collected above 560 nm. A binary mask was created from the edge of the picture. Frequency count was performed on intermyofibrillar mitochondria, and excludes areas located on the edges of the picture.

Decay phase of the flash was fitted by a single exponential decay (Microcal Origin, Microcal Software Inc., Northampton, MA, USA). Goodness of fit was verified by a Pearson’s chi-square test.

Confocal imaging and image processing

Supporting Information

Movie S1 Superoxide flashes in a skeletal muscle fiber. Movie from which fig. 2a is taken. Images were acquired by time-lapse confocal microscopy using a laser-scanning confocal microscope (LSM 5 Exciter; Carl Zeiss, Inc). Frames were taken every second for 3 min and are shown here at 18 Hz. Images were filtered by a median filter of radius 2.

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Author Contributions

Conceived and designed the experiments: SP. Performed the experiments: SP. Analyzed the data: SP. Contributed reagents/materials/analysis tools: SP. Wrote the paper: SP.
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