Mitochondrial flash as a novel biomarker of mitochondrial respiration in the heart

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Gong G, Liu X, Zhang H, Sheu SS, Wang W. Mitochondrial flash as a novel biomarker of mitochondrial respiration in the heart. Am J Physiol Heart Circ Physiol 309: H1166–H1177, 2015. First published August 14, 2015; doi:10.1152/ajpheart.00462.2015.—Mitochondrial respiration through electron transport chain (ETC) activity generates ATP and reactive oxygen species in eukaryotic cells. The modulation of mitochondrial respiration in vivo or under physiological conditions remains elusive largely due to the lack of appropriate approach to monitor ETC activity in a real-time manner. Here, we show that ETC-coupled mitochondrial flash is a novel biomarker for monitoring mitochondrial respiration under pathophysiological conditions in cultured adult cardiac myocyte and perfused beating heart. Through real-time confocal imaging, we follow the frequency of a transient bursting fluorescent signal, named mitochondrial flash, from individual mitochondria within intact cells expressing a mitochondrial matrix-targeted probe, mt-cpYFP (mitochondrial-circularly permuted yellow fluorescent protein). This mt-cpYFP recorded mitochondrial flash has been shown to be composed of a major superoxide signal with a minor alkalization signal within the mitochondrial matrix. Through manipulating physiological substrates for mitochondrial respiration, we find a close coupling between flash frequency and the ETC electron flow, as measured by oxygen consumption rate in cardiac myocyte. Stimulating electron flow under physiological conditions increases flash frequency. On the other hand, partially block or cardiac myocyte. Stimulating electron flow under physiological conditions, we find a close coupling between flash frequency and the oxygen consumption rate highly suggests that flash frequency could be used as a novel biomarker for mitochondrial respiration in vivo.

mitochondrial respiration; mitochondrial flash; electron transport chain; real-time confocal imaging; biomarker

NEW & NOTEWORTHY

Mitochondrial flash activity is tightly coupled with the electron flow along mitochondrial electron transport chain in intact cardiac myocytes and intact heart. The positive correlation between flash frequency and oxygen consumption rate highly suggests that flash frequency could be used as a novel biomarker for mitochondrial respiration in vivo.

MITOCHONDRIAL RESPIRATION is at the center stage of bioenergetics in eukaryotic cells, especially the cells in energy craving tissues, such as the nervous system and the heart. It is estimated that the human heart generates and utilizes ~6 kg of ATP per day (54), and this high rate of ATP and energy flow mainly relies on mitochondrial metabolism (24). Central to mitochondrial metabolism is the oxidative phosphorylation, also known as mitochondrial respiration, which is the sequential process of electron flow along the electron transport chain (ETC) and ATP generation by F0F1 ATPase (42). Mitochondrial respiration is also the driving force for establishing the negative inner membrane potential, ion transportation (4), and generation of reactive oxygen species (ROS), which are physiologically indispensable signaling molecules (17, 38), as well as culprit of a variety of diseases and aging (6, 24, 46). Meanwhile, mitochondrial dysfunction, which is originated from impaired mitochondrial respiration, has been linked to numerous human diseases and is the target for therapy (12, 49). However, evaluating mitochondrial respiration mainly relies on in vitro measurement of oxygen consumption rate (OCR), which may not reflect mitochondrial respiration status in vivo or under physiologically relevant conditions (9, 32). The lack of a real-time, in vivo, and tissue/cell-specific approach for monitoring mitochondrial respiration is a major challenge for the study of mitochondrial function and dysfunction in health and disease.

We have discovered that individual mitochondrion under physiological condition and in vivo display a transient and bursting signal, named superoxide flash, which is detected by a mitochondrial targeted superoxide indicator, mt-cpYFP (mitochondrial-circularly permuted yellow fluorescent protein) (51). Since then, other groups have also used this probe and other probes and detected similar bursting single mitochondrial events, which are associated with mitochondrial ROS production and redox signaling and play critical roles in cell physiology and pathology (10, 40, 45, 55, 57). However, concerns over the pH and superoxide sensitivity of the cpYFP probe have also been raised, which suggested that the bursting flash events detected by cpYFP could be interpreted as transient alkalization signals in mitochondrial matrix, named pH flashes (41, 43, 44). This controversy could arise from the differences in in vitro calibration of cpYFP and will need to be resolved most likely with the elucidation of crystal structure of cpYFP (14, 44). Nevertheless, we simultaneously monitored the cpYFP signal and a pH indicator loaded in mitochondrial matrix and found a major contribution of superoxide with a minor component of alkalization in each of the flash events (52). Considering the mixed signal in the flash event and the ongoing debate, superoxide flash has been renamed to mitochondrial flash (45) or mitochondrial flash. Meanwhile, regardless of the interpretation of its nature, accumulated evidence has sug-
gested that flash event is closely associated with mitochondrial energy metabolism and depends on mitochondrial ETC activity (10, 20, 41, 51). Indeed, both superoxide production and proton pumping are originated from and can be integrated by ETC electron flow. Moreover, a simultaneous loss of mitochondrial NADH signal was observed during each of the single mitochondrial flashes (52). Taken together, it is highly probable that mitochondrial flash is a composite event arising from mitochondrial respiration in vivo or in intact cells. Although mitochondrial flash has been monitored under several disease conditions, no study has systematically investigated the electron flow dependence of flash and whether flash is coupled with mitochondrial respiration (e.g., oxygen consumption). The aim of this study is to answer these important questions and provide new evidence to justify the use of flash as a novel biomarker for mitochondrial respiration in vivo or under pathophysiological conditions.

To achieve this aim, we manipulated mitochondrial substrate availability in Langendorff perfused heart and in intact adult cardiac myocytes. We found that respiration substrates initiated electron flow to stimulate oxygen consumption and induce mitochondrial flashes. In permeabilized myocytes, initiation of forward or reverse electron flows increased flash frequency. Interestingly, inhibition of F1F0 ATP synthase, which slows down electron flow, augmented electron accumulation and transiently increased mitochondrial flash frequency. Cardiac-specific deletion of an assembling subunit of complex I, transiently increased mitochondrial flash frequency. Cardiac-specific deletion of an assembling subunit of complex I, transiently increased mitochondrial flash frequency. Cardiac-specific deletion of an assembling subunit of complex I, transiently increased mitochondrial flash frequency. Cardiac-specific deletion of an assembling subunit of complex I, transiently increased mitochondrial flash frequency.

**METHODS**

**Recombinant adenovirus vectors and transgenic mice.** All of the animal procedures used in this study were approved by Internal Review Board of the Institutional Animal Care and Use Committee at the University of Washington. Animals were maintained on rodent diet with water available ad libitum and in a vivarium with a 12:12 h light-dark cycle at 22°C. Generation of pan-tissue mt-cpYFP transgenic (TG) mice used pUC-CAGGS-mt-cpYFP vector and pronuclear microinjection was conducted by transgenic core facility at the University of Washington. Linearized expression vector was injected into pronucleus of fertilized C57BL/6 mouse (Charles River) oocytes. Genotyping used primers for cpYFP (upstream: 5′-AGGCCACCACTTCTGATAGGCA-3′; downstream: 5′-AGGCCACCACTTCTGATAGGCA-3′) for mt-cpYFP TG mice at 3–4 mo old. GADPH was used as internal control (upstream: 5′-AGCCACCACTTCTGATAGGCA-3′; downstream: 5′-AGCCACCACTTCTGATAGGCA-3′). cKO mice were further bred with mt-cpYFP TG mice to obtain cKO mt-cpYFP mice. cKO mt-cpYFP mice at 3–4 mo old were used. At this age the Ndufs4 protein level is significantly decreased, while cardiac function is normal.

Construction of recombinant adenovirus vectors containing mt-cpYFP (Ad-mt-cpYFP) has been described previously (51). Ad-mt-cpYFP was amplified in human embryonic kidney-293 cells and purified by standard CsCl gradient centrifugation followed by overnight dialysis. The titer of virus was determined to be ~1×1011 viral particles/ml. Ad-SOD1 and Ad-SOD2 viruses were kind gifts from Dr. John F. Engelhardt (University of Iowa). All adenoviruses were divided into aliquots and stored at ~80°C.

**Confocal imaging of perfused heart.** To imaging mitochondrial flashes in the perfused heart, mt-cpYFP TG mouse was heparinized (intraperitoneal heparin, 50 U/mouse) and euthanized (intraperitoneal pentobarbital, 150 mg/kg). The heart was quickly removed and perfused in Langendorff mode using a custom-designed perfusion system that allows mounting of the heart onto a chamber on the confocal microscope stage (23). The heart was perfused with physiological solutions containing 118 mM NaCl, 25 mM NaHCO3, 5.3 mM KCl, 2 mM CaCl2, 1.2 mM MgSO4, 0.5 mM EDTA, and equilibrated with 95% O2 and 5% CO2 (pH 7.4) at 37°C. In addition, substrates were added to generate glucose-only solution (10 mM glucose and 0.5 mM pyruvate) or mixed substrate solution (5.5 mM glucose, 0.4 mM M199 long-chain fatty acids (palmitic acid 56.7%, palmitoleic acid 11.7%, stearic acid 1.9%, oleic acid 17.1%, linoleic acid 10.8%, and linolenic acid 1.7%) bound with 1.2% albumin, 1.2 mM lactate, 1.3 mM ketone, and 50 µM (methyl insulin) (30, 36). Mitochondrial membrane potential indicator, tetramethylrhodamine, methyl ester (TMRM) (100 nM, Invitrogen) was included in the perfusion solution. For substrate removal and restoration, the heart was perfused with oxygenated and substrate-free solution for 30–40 min before changing back to substrate-containing solution. Blebbistatin (10 µM) was added in the perfusion solution to suppress (but not completely stop) the heartbeat, and gentle pressure was applied to further prevent motion artifact when taking the images. Confocal imaging followed the procedure developed in our laboratory’s previous report (23).

**Adult cardiac myocyte culture and gene transfer.** Adult rat cardiac myocytes were isolated from female Sprague-Dawley rats (200–250 g, Harlan) following the protocol reported previously (50). Briefly, rats were anesthetized by intraperitoneal injection of 100 mg/kg pentobarbital. The heart was quickly removed and cannulated via ascending aorta and mounted on a modified Langendorff perfusion system. The heart was perfused with oxygenated Krebs-Henseleit buffer (10.8 mM glucose and 0.5 mM pyruvate) or mixed substrate solution (5.5 mM glucose, 0.4 mM M199 long-chain fatty acids (palmitic acid 56.7%, palmitoleic acid 11.7%, stearic acid 1.9%, oleic acid 17.1%, linoleic acid 10.8%, and linolenic acid 1.7%) bound with 1.2% albumin) and hyaluronidase (0.15 mg/ml, Sigma) at 37°C for 30 min. The heart was cut into small pieces for further digestion under gentle agitation in enzyme solution. Rod-shaped adult rat cardiac myocytes were collected by brief centrifugation.

Adult mouse cardiac myocytes were isolated from mt-cpYFP TG mice (20–25 g) following a protocol reported previously (29). Briefly, mice were anesthetized by intraperitoneal injection of 150 mg/kg pentobarbital. The heart was quickly removed and cannulated via ascending aorta and mounted on a perfusion system. The heart was perfused with oxygenated myocyte isolation solution (113 mM NaCl, 4.7 mM KCl, 0.6 mM KH2PO4, 0.6 mM NaH2PO4, 1.2 mM MgSO4, 11 mM HEPES and 20 mM glucose) supplemented with collagenase II (80 U/ml, Worthington) and hyaluronidase (0.15 mg/ml, Sigma), 50 µM CaCl2, and 10 µM blebbistatin at 37°C for 10 min. The heart was taken down, disaggregated with forceps, and gently triturated five to six times and incubated in enzyme solution for 5 min at room temperature to allow further digestion. Rod-shaped adult mouse cardiac myocytes were collected by brief centrifugation.

The isolated myocytes from rat or mouse were plated on coverslips (20 mm, round) precoated with laminin (40 µg/ml for 1 h, Invitrogen) at a density of 2×104 cells per coverslip and in M199 medium (Sigma) supplemented with 10 mM glutathione, 26.2 mM sodium bicarbonate, 5 mM creatine, 2 mM t-carnitine, 5 mM taurine, 0.1% insulin-transferrin-selenium-X, 0.02% bovine serum albumin, 50 U/ml penicillin-streptomycin, and 5% fetal bovine serum. Two hours after the plating, the medium was changed to serum-free M199. Freshly isolated mouse myocytes right after 2-h plating were used for confocal imaging.
confocal imaging. Adenovirus-mediated gene transfer was done at a multiplicity of infection of 50–100 on rat cardiac myocytes. The rat myocytes were kept in culture for 48–72 h to allow adequate gene expression before imaging.

**Determination of gene expression.** Protein levels of SOD1 and SOD2 after adenovirus-mediated gene expression in cultured myocytes were determined by Western blot. Rat cardiac myocytes were harvested 48–72 h after gene transfer. Protein samples were collected in 1× lysis buffer (Cell Signaling), quantified by a BCA kit (Pierce), and loaded (20 μg) onto SDS-PAGE gel. Separated proteins were transferred to nitrocellulose membrane and probed with antibodies specific for SOD1 (1:1,000, Calbiochem), SOD2 (1:1,000, Calbiochem), or actin (1:2,000, Sigma). Secondary antibodies were conjugated to IRDye 800 (Rockland) or Alexa Fluor 680 (Invitrogen), and signals were visualized and quantified using Odyssey system (Licor).

**Confocal imaging of cultured adult cardiac myocytes.** Confocal imaging used a Zeiss LSM 510 Meta confocal microscope equipped with a 40× 1.3 numerical aperture oil immersion objective and followed a procedure developed previously (51). Intact myocytes were incubated in physiological solutions containing 138 mM NaCl, 3.7 mM KCl, 1.2 mM KH2PO4, 5 mM glucose, 20 mM HEPES, and 110 mM mannitol, 0.3 mM dithiothreitol, and 3.7 mM KCl, 1.2 mM KH2PO4, 5 mM glucose, 20 mM HEPES, and 110 mM mannitol, 0.3 mM dithiothreitol, and 1 mM CaCl2; at room temperature and in a custom-designed perfusion chamber mounted on the confocal microscope stage. Dual-excitation images of mt-cpYPFP were taken on randomly selected cells by alternating excitation at 405 and 488 nm and collecting emissions at >505 nm. Time-lapse x-y images were acquired at 1,024 resolution for 100 frames and at a sampling rate of 1 s/frame. For substrate stimulation, myocytes were first incubated in glucose-free solution for 30–40 min before changing to the solution with various substrates.

To detect mitochondrial flashes in permeabilized rat myocytes, the cells were first incubated in Ca2+-free solution for 3 min, changed to internal solution containing 120 mM potassium aspartate, 3 mM MgATP (free [Mg2+]i ~ 1 mM), 0.1 mM EGTA, 10 mM phospho-creatine, 5 U/ml creatine phosphokinase, 8% dextran, (40,000), and 50 μg/ml saponin (pH 7.2) for 30 s and then maintained in saponin-free internal solution (35). In a subset of experiments, permeabilization protocol was verified by adding rhod-2 salt (5 μM, Invitrogen), a membrane-impermeable indicator, right after permeabilization, and visualizing the intracellular rhod-2 signals. To test substrate-induced respiratory activity, permeabilized cells were incubated in mitochondrial respiration solution containing 0.5 mM EGTA, 3 mM MgCl2, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 10 mM potassium-lactobionate, 110 mM mannitol, 0.3 mM dithiothreitol, and 1 g/l BSA (32). After the baseline recording was obtained, various substrates were added [10 mM pyruvate, 5 mM malate, and 1 mM ADP; 10 mM succinate and 1 mM ADP; or 0.5 mM N,N,N',N'-tetramethyl p-phenylenediamine (TMPD), 2 mM ascorbate, and 1 mM ADP], and inhibitors were added subsequently (0.5 μM rotenone, 5 μM antimycin A, 1 mM NaN3, or 1 μM FCCP).

To monitor mitochondrial superoxide, intact cardiac myocytes were loaded with MitoSOX Red (5 μM, Invitrogen) at 37°C for 10 min, followed by washing two times. Dual-wavelength imaging was done by tandem excitation at 405 and 514 nm, and emission collected at >560 nm (52). Since MitoSOX signal increases irreversibly and reaches saturation quickly, the experiments were completed within 30 min after MitoSOX loading.

To monitor mitochondrial membrane potential, TMRM (20 nM, Invitrogen) was loaded into intact myocytes at room temperature for 20 min. Tri-wavelength excitation imaging of mt-cpYPFP and TMRM was done by tandem excitation at 405, 488 and 543 nm, and emission collected at 505–545, 505–545, and 560–657 nm, respectively (52).

**Polarography assays for OCR.** Measurement of OCR in permeabilized rat cardiac myocytes used a Clark electrode and the same solution and substrates as for the confocal imaging of permeabilized cells and followed the protocol reported previously (30, 32).

**Statistics.** Data are presented as means ± SE. One-way ANOVA was used to compare the differences among three or more groups, followed by unpaired Student’s t-test to compare between any two groups. In some experiments (see Fig. 3, B and C), when the two groups were from the same set of samples (before and after a treatment), paired Student’s t-test was used. Nonparametric correlation was used to determine the relationship between OCR and mitochondrial flash frequency or OCR and MitoSOX signal. A P value < 0.05 was deemed significant.

**RESULTS**

**Respiration substrates modulated mitochondrial flash in the perfused heart.** To determine mitochondrial flash activity under physiologically relevant condition, we manipulated the substrate availability in Langendorff perfused beating hearts from mt-cpYPFP TG mice (Fig. 1). The respiration manipulation protocol was carried out by removing substrate (perfuse with oxygenated and no substrate solution for 30–40 min) and then restoring substrate (Fig. 1A). TMRM (100 nM) was used to evaluate mitochondrial membrane potential simultaneously. We tested mixed substrate (glucose plus mixed fatty acids) and glucose-only substrate, both of which have been used for functional and metabolic evaluation of the perfused heart (30, 56). We found that, in the intact myocardium, mitochondrial flash frequency decreased after substrate removal and rebounded after substrate restoration (Fig. 1, B–D). Each flash is accompanied with loss of membrane potential (Fig. 1, B and C). These results suggest that flash frequency is modulated by respiration substrates in the perfused beating heart.

**ETC dependence of substrate-induced mitochondrial flash in intact myocytes.** To further determine whether the substrate-induced mitochondrial flash depends on mitochondrial respiration via ETC activity, we used adult rat cardiac myocytes, which can be cultured for 3–4 days to facilitate adenovirus-mediated gene manipulation. The physiological substrates, glucose, palmitate, or a mixture of both, acutely increased the frequency of mitochondrial flash in cultured rat cardiac myocytes (Fig. 2, A and B). These effects were abolished by rotenone, an inhibitor of complex I of the ETC (Fig. 2B).

Importantly, galactose, which is not a respiratory or glycolytic substrate for the heart (39), was unable to induce flashes in the cultured rat myocytes, while pyruvate, a substrate that mainly generates NADH and provides electrons entering ETC at complex I, significantly increased flash activity (Fig. 2C). The glucose-induced flash activity was sensitive to antioxidants (Tiron or SOD2 overexpression) and transient mitochondrial permeability transition (tMPT) inhibition by cyclosporine A (Fig. 2D), consistent with previous reports (51). Unitary properties of flashes, including amplitude (ΔF/Δt), time to peak, and time to 50% decay were mildly altered by these physiological substrates (Fig. 2E). These results suggest that substrate-induced mitochondrial flash is dependent on ETC activity.

**ETC electron flow underlay mitochondrial flash generation.** To further determine whether substrate-induced mitochondrial flash generation requires the electron flow along ETC, we monitored flash in permeabilized adult rat cardiac myocytes, which largely preserved the intracellular environment/structure, while allowing the assessment of electron flow associated with specific substrates of the ETC complexes (Fig. 3A) (32). Successful permeabilization of plasma membrane was confirmed by visualization of the fluorescence of rhod-2 salt, a membrane-impermeable dye, in the cytosol (data not shown).
We then supplemented the permeabilized rat myocytes with ETC substrates to start electron flow from complex I [pyruvate, malate, and ADP (Pyr/Mal/ADP)], complex II (succinate and ADP), or complex IV (TMPD, ascorbate, and ADP) (Fig. 3, B–D). We first determined the OCR, which confirmed the stimulation of electron flow by these substrates and subsequent blockade by ETC inhibition (8, 31, 32) (Fig. 3B). Next, we monitored the fluorescence of MitoSOX, a pH-insensitive and mitochondrial targeted superoxide indicator, in permeabilized rat myocytes. We found increased MitoSOX signal when complex I (22), complex II, or complex IV substrate was added (Fig. 3C), consistent with increased superoxide production upon the initiation of ETC electron flow. There is a positive correlation between OCR and MitoSOX fluorescence during the above substrate manipulations (Fig. 3D). These results suggest that, in permeabilized myocytes, the specific substrates for ETC complexes can stimulate electron flow along the ETC.

Next, we monitored mitochondrial flash in permeabilized rat myocytes (Fig. 4). Addition of Pyr/Mal/ADP significantly induced flash activity (Fig. 4A). This effect was abolished by blockers of ETC electron flow or uncoupler (Fig. 4B). Although uncoupler maximizes ETC electron flow and OCR, it completely dissipates membrane potential and diminishes mitochondrial flashes (51). A similar increase in flash frequency was found when using complex II or IV substrate to initiate electron flow in permeabilized rat myocytes (Fig. 4B). In addition, flash amplitude was increased and kinetics was largely unchanged by these substrates (Fig. 4C). Interestingly,
the OCR in permeabilized rat myocytes was positively correlated with mitochondrial flash frequency during respiration stimulation by these substrates (Fig. 4D). Taken together, ETC electron flow underlies mitochondrial flash generation. Regarding the direction of electron flow, complex I substrates mainly induce forward electron flow, complex II substrates can induce both forward and reverse flows (8, 31, 47), and complex IV substrates has been shown to induce reverse flow to quinone pool or NAD\(^+\) (13, 37). However, we speculate that forward electron flow may be the major direction that supports mitochondrial flash in intact cells, in the perfused hearts, and in vivo (20, 23, 51).

**Slowdown ETC electron flow transiently increased mitochondrial flashes.** ETC electron flow is controlled by downstream ATP generation and utilization (4, 5). Our laboratory has shown that long-term blockade of F0F1 ATP synthase...
(complex V) by oligomycin A, which decreases ETC electron flow, abolished flashes (51). This is in line with the above results showing electron flow along ETC underlies flash activity. To further explore how ETC electron flow supports mitochondrial flashes, we followed the time-dependent effect of oligomycin A on flash frequency in intact myocytes supplemented with glucose as substrate. Surprisingly, we found a biphasic change of flash frequency: an initial increase peaked at 20 min, followed by a decline and eventually a decrease to a level lower than control at 40 min (Fig. 5A). The transient increase in flash frequency was abolished by addition of uncoupler or ETC inhibitors, likely due to the fact that they either dissipate membrane potential or totally block electron flow (Fig. 5B). In intact myocytes without substrate, oligomycin A only moderately induced flashes. Subsequent addition of pyruvate further increased flash frequency (Fig. 5C). The additive effect suggests that providing more electrons to a slowed ETC further augments mitochondrial flash generation. When oligomycin A was added after pyruvate in intact (Fig. 5C) or permeabilized myocytes (Fig. 5D), no further increase in flash frequency was observed, probably due to the already saturated electron flow by pyruvate. Oligomycin A-stimulated mitochondrial flashes were attenuated by tMPT inhibitor, cyclosporine A (Fig. 5E), or mitochondrial overexpression of the superoxide scavenger, SOD2, but not overexpression of the cytosolic SOD1 (Fig. 5F). Finally, oligomycin A induced a significant increase in MitoSOX signal (Fig. 5G), supporting that oligomycin A increased ROS production. Oligomycin A has been shown to slow down electron flow (1), block transition from state 2 to state 3 respiration, and induce mitochondrial ROS production (48). Previously, our laboratory has shown that excessive electrons in ETC, such as during state 4 respiration in isolated mitochondria, supported the maximal mitochondrial flash activity (52). Therefore, in the presence of substrates, slowdown electron flow transiently induces mitochondrial flash, probably through promoting electron accumulation and/or reverse electron flow to enhance proton motive force and ROS production. Taken together, these results further support that flash generation relies on electron flow.

**Complex I deficiency suppressed mitochondrial flash.** The above results suggest that electron flow through ETC is responsible for mitochondrial flash generation in the heart and adult cardiac myocytes. Next, we sought to determine the molecular mechanism of ETC complexes in controlling mitochondrial flash. Complexes I and III have been shown repeatedly as the major complexes for electron transport, proton pumping, and ROS production within the ETC, and complex I likely plays a more important role in vivo, since it is the major acceptor of electrons transported from reducing equivalents (8, 31, 33, 38, 47). Therefore, we focused on complex I in mitochondrial flash generation. We crossed mt-cpYFP TG mice with cardiac-specific complex I-deficient mice (Ndufs4 knockout, cKO), which had lost 75% of complex I content and activity in the heart with unchanged activity of other com-
Fig. 4. ETC electron flow supported mitochondrial flash generation in permeabilized adult rat cardiac myocyte. A, left: representative images of a permeabilized rat cardiac myocyte showing flash events (highlighted in white boxes) during the 100-s scan in the absence (No sub) or presence of complex I substrates (Pyr/Mal/ADP). Scale bar = 10 μm. Right: representative traces showing a typical flash before or after the complex I substrate. B: flash frequency supported by Pyr/Mal/ADP, Succ/ADP, or TMPD/Asc/ADP and subsequent inhibition by ETC inhibitors: Rot (0.5 μM), AA (5 μM), FCCP (1 μM) or NaCN (1 mM). N = 11–58 cells from 3–8 rats. C: unitary properties of mitochondrial flash in permeabilized rat myocytes with or without substrates. N = 194–524 flashes in 16–57 cells from 6–8 rats. D: correlation between OCR and mitochondrial flash frequency in permeabilized rat myocytes. The data points are from Figs. 3B and 4B. Values are means ± SE. ††P < 0.001 vs. No sub.
plexes (30). Freshly isolated adult cardiac myocytes from wild-type or cKO mice exhibited mitochondrial flash activity accompanied by loss of membrane potential (Fig. 6, A and B). Flash frequency was significantly decreased in cKO myocytes under basal conditions, while unitary properties of flash remained unchanged (Fig. 6, C and E). Furthermore, pyruvate-induced flash activity was diminished in cKO myocytes compared with wild-type myocytes (Fig. 6D). In addition, we have shown that basal mitochondrial superoxide and H2O2 levels are decreased in the cKO hearts (30). Taken together, these results suggest that complex I, which accepts electrons mainly from pyruvate-generated NADH, plays a critical role in mitochondrial flash generation in the heart.

**DISCUSSION**

In this study, we sought to explore the mechanistic coupling between mitochondrial respiration and mitochondrial flash generation in intact cardiac myocyte and the beating heart under physiological relevant conditions. The major findings are that metabolic substrates initiated electron flow along ETC to support mitochondrial flash generation. Slowdown of the electron flow transiently enhanced mitochondrial flash activity, while restricting electron entering complex I attenuated flashes. These results provide new evidence to show, for the first time, the tight coupling between ETC electron flow and mitochondrial flash generation (Fig. 7). These findings also...
justify the use of mitochondrial flash as a novel biomarker for monitoring mitochondrial respiration in situ or in vivo, at single mitochondrion resolution, and under physiologically relevant conditions.

The gold standard for studying mitochondrial respiration is the OCR, which is measured by using a Clark electrode on isolated mitochondria or permeabilized cells (9). The Seahorse XF Analyzer and Oroboros Oxygraph system are newly developed techniques that can be used for intact cell OCR measurement. However, these methods require sophisticated systems, have limited ability for real-time manipulation of respiration status, and are not suitable for in vivo measurement. Here, we show that the frequency of a recently discovered transient and single mitochondrial event, mitochondrial flash, is tightly coupled with the electron flow along ETC and has the potential to be used as a novel biomarker for mitochondrial respiration evaluation. Previously, we and others have shown that mitochondrial flash requires intact ETC activity (51), and its frequency can be modulated by metabolic perturbations in intact cells and in vivo (20, 22, 52). In this study, we systematically evaluated the coupling between mitochondrial flash and ETC electron flow and found a positive correlation between OCR of the population of mitochondria and the flash frequency of single mitochondria. It should be noted that flash frequency is usually low, and the flash events are confined within single mitochondria. However, the positive correlation between flash frequency and OCR and the fact that under various conditions the electron flow along ETC determines flash generation highly suggest that flash frequency is an appropriate readout for real-time evaluation of mitochondrial respiration status in intact cells, intact heart, and in vivo.

Fig. 7. Model of the coupling between ETC electron flow and mitochondrial flash. Respiration substrate initiates electron flow to support mitochondrial respiration and flash generation. The amount of electrons transported along ETC is a determining factor for the respiration-flash coupling. The electron flow leads to superoxide production and matrix alkalization, which are components of mitochondrial flash. IMM, inner mitochondrial membrane; Qo, quinol-oxidizing center; Qi, quinone-reducing center; CytoC, cytochrome C.
We and others have shown that mitochondrial flashes are triggered by tMPT pore openings (28, 51), which are mainly regulated by ROS and Ca^{2+} (11). The tMPT opening under physiological condition has been shown to bear important roles in mitochondrial Ca^{2+} regulation and preventing matrix Ca^{2+} overload (11, 18, 19, 26). Results from this study further suggest that TMPT may play a role in mitochondrial respiration regulation under physiological conditions, as reflected by the triggering of single mitochondrial flashes. Besides ROS and Ca^{2+}, a number of factors can also modulate tMPT activity, including proton motive force, phosphate, Mg^{2+}, and ADP/ATP (11, 16, 25). Importantly, all of these factors are integrated by mitochondrial respiration and ETC electron flow. In this regard, a positive feedback mechanism may exist, in which mitochondrial respiration through ETC electron flow may provide the triggering signal, such as ROS and Ca^{2+} (22, 27), to stimulate the stochastic opening of TMPT in individual mitochondrion and accelerate respiration in single mitochondrion. The accelerated electron flow in the flashing mitochondria further support the ROS and pH changes for local signaling. Recent studies have shed light on a close association between TMPT and ETC. For instance, the TMPT regulator, cyclophilin D, can bind ATP synthase, modulate local phosphate level, and regulate permeability transition (15). Furthermore, it has been proposed that ATP synthase may be part of the permeability transition pore or its subunit, or dimerization may form the pore (2, 7, 21). These data indicate that a physical coupling between ETC and TMPT may exist. Finally, the ETC-coupled TMPT and flash activity is compatible but not identical to ROS-induced ROS release phenomenon (58) or whole cell ROS oscillations (3), which are laser-induced phenomena in stressed cells. Moreover, the ROS-induced ROS release or whole cell ROS oscillations are propagating processes initiated by ROS released from individual mitochondrion that subsequently trigger ROS release from the adjacent mitochondria. Here, the TMPT regulation of respiration and ROS production is stochastic and locally confined within the same mitochondrion, with no signs of propagation.

The application of mitochondrial flash in monitoring mitochondrial respiration in intact cells, tissues, and in vivo has clear advantage over the current in vitro methods. However, interpretation of the changes in flash frequency under various situations should take into consideration the context and other regulatory factors. In most cases, when ETC electron flow is not blocked, mitochondrial flash frequency reliably reflects substrate availability (this study), electron flow rate (measured by OCR in this study), intracellular energy utilization/demand (22), and mitochondrial metabolic status (20, 52, 53). In other cases, however, slowdown electron flow, such as by oligomycin A (this study) or under state 4 respiration (52), will further stimulate flashes. This could be explained by the increased electron accumulation in ETC, which may facilitate reverse electron flow and lead to increased flash activity. Moreover, it should be noted that uncoupling ATP production with ETC electron flow (e.g., by FCCP) abolishes flashes, largely due to the dissipation of mitochondrial membrane potential. In addition and as we discussed above, since flash is a composite event arising from serial processes, including tMPT and ETC activity within single mitochondria, flash frequency can be modulated by factors/regulators within ETC or targeting tMPT, such as Ca^{2+} and basal levels of ROS. Therefore, simultaneous or parallel monitoring of other key parameters, including OCR, membrane potential, ATP, pH, and ROS, may be needed to provide a comprehensive picture of mitochondrial respiration status under certain conditions. Although this study focuses on the flash-respiration coupling under physiological condition, the same mechanism may apply to pathological conditions, such as heart failure, ischemia reperfusion, neurotoxicity, and muscle disorders (22, 34, 45, 51, 53). We speculate that the establishment of flash-respiration coupling will stimulate its application in diseases and, together with other indicators, advance our understanding of the role of mitochondria in human disease.

In summary, through monitoring mitochondrial flashes and OCR in parallel, we found a close coupling between mitochondrial respiration and flash frequency in permeabilized cells, intact cells, and the perfused beating heart. The frequency of mitochondrial flash is positively correlated with increased ETC electron flow under physiological conditions. Under pathological conditions, when electron flow is blocked or slowed down, the accumulated electrons in ETC can also transiently support increased flash activity. Complex I is a critical site in ETC for the respiration-coupled mitochondrial flash activity. Therefore, we propose that flash frequency could be a useful biomarker for mitochondrial respiration evaluation in vivo and under pathophysiological conditions.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


