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Regulation of metabolism in individual mitochondria during excitation-contraction coupling

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Abstract

The heart is an excitable organ that undergoes spontaneous force generation and relaxation cycles driven by excitation-contraction (EC) coupling. A fraction of the oscillating cytosolic Ca^{2+} during each heartbeat is taken up by mitochondria to stimulate mitochondrial metabolism, the major source of energy in the heart. Whether the mitochondrial metabolism is regulated individually during EC coupling and whether this heterogeneous regulation bears any physiological or pathological relevance have not been studied. Here, we developed a novel approach to determine the regulation of individual mitochondrial metabolism during cardiac EC coupling. Through monitoring superoxide flashes, which are stochastic and bursting superoxide production events arising from increased metabolism in individual mitochondria, we found that EC coupling stimulated the metabolism in individual mitochondria as indicated by significantly increased superoxide flash activity during electrical stimulation of the cultured intact myocytes or perfused heart. Mechanistically, cytosolic calcium transients promoted individual mitochondria to take up calcium via mitochondrial calcium uniporter, which subsequently triggered transient opening of the permeability transition pore and stimulated metabolism and bursting superoxide flash in that mitochondrion. The bursting superoxide, in turn, promoted local calcium release. In the early stages of heart failure, EC coupling regulation of superoxide flashes was compromised. This study highlights the heterogeneity in the regulation of cardiac mitochondrial metabolism, which may contribute to local redox signaling.

Keywords

Mitochondrial metabolism; Excitation-contraction coupling; Superoxide flash; Mitochondrial calcium uptake; Permeability transition pore

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1. Introduction

Excitation-contraction (EC) coupling underlies beat-to-beat pumping function of the heart [1]. A unique feature of EC coupling is the timely cycling of cytosolic calcium (Ca^{2+}) under control of a large group of proteins, including ryanodine receptor (RyR), phospholamban-regulated Ca^{2+} -ATPase (SERCA) and $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (NCX) [2]. In the beating heart, the normal function of these proteins and the contractile machinery depends on continuous energy supply. In humans, an estimated 6 kilograms of adenosine triphosphate (ATP) are produced and hydrolyzed in the heart daily [3, 4]. This vast amount of energy flow is primarily realized through energy metabolism in mitochondria [5], which occupy 20–30% of the total volume of a cardiac myocyte and are located in close proximity to sarcoplasmic reticulum (SR) and in between myofilaments [6]. Previous studies have shown that a number of factors can regulate mitochondrial metabolism, including ATP utilization [7], metabolic regulators such as Ca^{2+} [8], and respiration substrates [9]. In these studies, large populations of mitochondria are used, such as the measurement of oxygen consumption rate in isolated mitochondria or ATP levels in the heart tissue [10, 11], which detect the overall response rather than differentiating the response of individual mitochondrion. However, cardiac mitochondria are heterogeneous and exhibit less networking and dynamics as reflected by their unique morphology and discrete localization in between sarcomeres [6, 12, 13]. Nevertheless, very few studies focus on the functional heterogeneity of cardiac mitochondria and how the metabolism in individual mitochondria is regulated under physiological conditions.

Mitochondrial metabolism is also the major source of reactive oxygen species (ROS), which are short-lived signaling molecules and heavily buffered in the cell. It is suggested that endogenous ROS mainly modulate cell signaling locally. Mitochondrial Ca^{2+} uptake is a known stimulator of mitochondrial metabolism and ROS production. Whether and how the global Ca^{2+} oscillations during EC coupling affect local ROS signaling are questions remain to be addressed. It is conceivable that the heterogeneity in mitochondrial metabolism may underlie the generation of local ROS hot spots” or redox domains. However, due to the lack of appropriate approach to monitor ROS production in individual mitochondria, evidence showing the modulation of local ROS generation by mitochondria during EC coupling is missing.

The discovery of single mitochondrial superoxide production events, named superoxide flashes, provides a unique tool to disentangle mitochondrial function regulation at individual mitochondria level [14]. These bursting superoxide production events are detected by genetic superoxide indicators (cpYFP) and conventional reactive oxygen species (ROS) indicators (MitoSOX and DCF) in intact cells, in the beating heart and *in vivo* [14–16]. The pH sensitivity of cpYFP has raised concerns over the origin of signals it detected during flashes [17, 18]. Simultaneous monitor of flash and matrix pH revealed that majority of the cpYFP fluorescence increase during flash is superoxide with a mild contribution of pH [15, 19]. Importantly, despite disagreement over origin of the signal, consensus has been reached regarding the electron transport chain (ETC) dependence of flash events [14, 15, 18]. Flash event is accompanied by a loss of membrane potential, decline of matrix NADH level, and slight alkalization in the same mitochondrion [15, 20]. Further, depriving mitochondrial

DNA abolished flashes [14], while supplementing substrates for State 4 or 3 respiration or increasing workload stimulated flashes [15, 21, 22]. Collectively, these results suggest that flash is a composite phenomenon arising from mitochondrial metabolism, during which ETC electron flow consumes NADH, pumps out protons and generates bursting superoxide. Thus, monitoring flash frequency could be a novel approach for studying the metabolism in individual mitochondria in intact tissues and *in vivo* [21, 23], which is otherwise unable to be assessed by methods such as oxygen consumption measurements.

By using superoxide flash as an indicator for the metabolic status of individual mitochondria, we determined an intrinsic pathway that links EC coupling to the metabolic regulation in individual mitochondria in intact cardiac myocyte and the beating heart. This pathway is composed of mitochondrial Ca^{2+} uniporter (MCU) and subsequent transient openings of mitochondrial permeability transition pore (tMPT). The EC coupling regulation of individual mitochondrial metabolism bears important signaling roles as it modulates local Ca^{2+} release. In the failing heart, the EC coupling regulation of individual mitochondrial metabolism is compromised. These findings uncover a novel mechanism, through which a uniform and whole cell event specifically regulates the metabolism in individual mitochondria to provide bursting ROS for local redox signaling.

2. Methods

2.1 Recombinant adenovirus vectors and mt-cpYFP transgenic mice

Construction of recombinant adenovirus vectors containing mitochondrial targeted circularly permuted yellow fluorescent protein (Ad-mt-cpYFP) has been described previously [14]. Ad-mt-cpYFP was amplified in HEK 293 cells and purified by standard CsCl gradient centrifugation followed by overnight dialysis. Ad-SOD2 virus was a kind gift from Dr. John F. Engelhardt (University of Iowa) [24]. Concentration of the viruses were determined to be at $\sim 1 \times 10^{11}$ viral particles per ml. All the viruses were aliquoted and stored at -80°C .

Animal procedures used in this study were approved by the Internal Review Board of Institutional Animal Care and Use Committee (IACUC) at the University of Washington. Generation of pan-tissue mt-cpYFP transgenic mice used pUC-CAGGS-mt-cpYFP vector (a kind gift from Dr. Heping Cheng, Peking University) as previously reported [21]. 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 oocytes. Genotyping was performed by PCR using specific primers (upstream: 5'-GCGAGGAGCTGTTCACCG-3'; downstream: 5'-AGCCACCACTTCTGATAGGCA-3'). The mt-cpYFP transgenic mice were housed and bred in the animal facility on South Lake Union Campus of the University of Washington.

2.2 Adult cardiac myocyte culture and gene transfer

Adult cardiac myocytes were isolated from female Sprague Dawley (SD) rats (200~250 g, Harlan) following the protocol reported previously [14]. Briefly, rat was anesthetized by intraperitoneal (i.p.) injection of pentobarbital. The heart was quickly removed, cannulated via ascending aorta, and mounted on a modified Langendorff perfusion system. The heart was perfused with oxygenated Krebs-Henseleit Buffer (KHB) solution supplemented with

collagenase II (Worthington) and hyaluronidase (Sigma) at 37°C. Rod shaped adult cardiac myocytes were collected and plated on glass coverslips coated with laminin at a density of 2×10^4 cells per coverslip and cultured in serum-free M199 medium (Sigma) supplemented with 10 mM glutathione, 26.2 mM sodium bicarbonate, 0.02% bovine serum albumin and 50 U/ml penicillin-streptomycin for up to 3 days. Adenovirus-mediated gene transfer was done 2 hr after cell plating and at a multiplicity of infection (MOI) of 50–100. The experiments were done 2–3 days after gene transfer.

2.3 Confocal imaging of superoxide flashes, Ca^{2+} and NADH

Confocal imaging used a Zeiss LSM 510 Meta confocal microscope equipped with a 40x 1.3NA oil immersion objective and followed the procedure developed previously [14, 15, 25]. To detect superoxide flashes in intact myocytes, cells were incubated in modified KHB solutions (containing 138 mM NaCl, 3.7 mM KCl, 1.2 mM KH_2PO_4 , 5 mM Glucose, 20 mM HEPES and 1 mM CaCl_2 , pH 7.4) at room temperature and in a custom designed perfusion chamber mounted on confocal microscope stage as previously reported [15]. Dual excitation images of mt-cpYFP were taken by alternating excitation at 405 and 488 nm and collecting emissions at >505 nm. Time-lapse x,y images were acquired at 1024 resolution for 100 frames and at a sampling rate of 1 s per frame. During the scan, the spatiotemporal occurrence of flash is stochastic and no apparent laser or time dependent induction of flashes was observed. For experiments in Figure 4, myocytes were first incubated in normal KHB solution, and then changed to KHB without substrate for 20–30 min before adding pyruvate as the only substrate. For all other studies on intact cardiac myocytes, KHB solutions with glucose (5 mM) as substrate were used. For electrical stimulation, we used custom made electrodes to achieve local stimulation of a few selected myocytes at various frequencies for 2 min and the 100-frame serial scan was done on these cells before and right after the electrical stimulation. Each cell received no more than two serial scans. Flash frequency was calculated in each cell. For all other experiments, the myocytes were randomly chosen before and after the treatment and each cell was scanned only once (100 frames in ~100 seconds).

To simultaneously monitor flash and mitochondrial Ca^{2+} , myocytes were loaded with rhod-2 AM (5 μM , after quenching by NaBH_4) at 4°C following overnight washout at 37°C. This procedure has been shown to facilitate mitochondrial sequestration of the dye [26]. Since these myocytes also express mt-cpYFP, only the rhod-2 signal that colocalized with cpYFP fluorescence was used to generate the traces. This further suppressed the contamination of cytosolic rhod-2 signals. On the other hand, when cytosolic Ca^{2+} was monitored by rhod-2, rhod-2 AM (5 μM) was acutely loaded at room temperature for 20 min followed by 20 min washout. A uniform loading pattern with strong nuclear staining was observed, which suggested cytosolic localization of the dye. Tri-wavelength excitation images of mt-cpYFP and rhod-2 were taken by sequential excitation at 405, 488, and 543 nm, and emissions collected at 505–545, 505–545 and >560 nm, respectively. In a subset of experiments, cytosolic Ca^{2+} transients and sparks were measured by fluo-4 AM (10 μM). To simultaneously image flash and Ca^{2+} spark, we loaded myocytes with rhod-2 AM and line scan imaging was done at a sampling rate of 60 ms per line. Digital image processing used IDL software (Research Systems) and customer-devised programs.

To detect superoxide flashes in permeabilized cells, cardiac myocytes were first incubated in Ca^{2+} -free KHB solution for 3 min, changed to internal solution containing saponin (50 $\mu\text{g/ml}$) for 30 s and then maintained in saponin free internal solution for imaging [25]. The internal solution contains 120 mM potassium aspartate, 3 mM MgATP (free $[\text{Mg}^{2+}]$ 1 mM), 0.1 mM EGTA, 10 mM phosphocreatine, 5 U/ml creatine phosphokinase, 8% dextran (40,000) (pH 7.2). In a subset of experiments, permeabilization protocol was verified by incubating permeabilized cells with rhod-2 salt and imaging rhod-2 signal inside cells. Ca^{2+} was added to permeabilized myocytes following previously reported methods to ensure accurate concentrations of free Ca^{2+} in the solution [25].

To monitor intrinsic mitochondrial NADH autofluorescence, we used an Olympus FV1000 multi-photon microscope with a 25x objective. Myocytes were excited at 720 nm and emission collected at 390–465 nm.

2.4 Determination of gene expression

To detect SOD2 expression in cultured myocytes, three days after gene transfer, myocytes were harvested and protein samples were collected in lysis buffer (Cell signaling). To detect gene expression in tissues from mt-cpYFP transgenic mice or failing rat hearts, tissues were quickly frozen in liquid nitrogen, processed and homogenized in lysis buffer. Protein samples were quantified by BCA kit (Pierce) and equal amount of proteins were loaded onto SDS-PAGE gel. Separated proteins were transferred to nitrocellulose membrane and probed with specific antibodies. The antibodies used were: Anti-SOD2 (1:1000, Calbiochem), Anti-GFP (1:2000, Roch), Anti-cyclophilin D (1:2500, Abcam), Anti-MCU (1:100, Sigma), Anti-MICU (1:200, Santa Cruz), Anti-actin (1:2000, Sigma) and Anti-GAPDH (1:1000, Abcam). Secondary antibodies were conjugated to IRDye 800 (Rockland) or Alexa Fluor 680 (Invitrogen). Signals were visualized and quantified using Odyssey system (Licor).

2.5 Heart failure models in rat and mouse

Abdominal aorta constriction (AAC) surgery was done on female SD rats (220–250 g) following approved protocols by the IACUC. Animals were randomly assigned to surgery or sham operation groups. The rat was first anesthetized with pentobarbital (50 mg/kg, i.p.). An incision was made on the left side of abdomen along mid-axilla line below the rib cage. Abdominal cavity was entered and tissues around abdominal aorta at the level above renal artery branches were dissected to expose aorta. A 22-gauge needle was placed along the aorta, a 4–0 suture was tied around them, then the needle quickly removed, and incision closed by layers. Sham operated animals underwent the same procedure except for constriction of aorta. Echocardiography was performed using an ultrasound machine (Vevo 770, VisualSonics) to verify cardiac contractile performance at 2 and 4 weeks after the surgery. In a subset of experiments, hearts were fixed for histological studies on myocardium morphology and fibrosis.

Transverse aorta constriction (TAC) induced heart failure model in mouse used transgenic mice expressing mt-cpYFP (3–4 months old). TAC surgeries were performed as previously described [27]. Briefly, mice were anesthetized with sodium pentobarbital (75 mg/Kg, i.p.), the aorta was exposed via a left thoracotomy and a constriction was created using a 7–0

ligature around the vessel and tied against a 27-gauge blunt needle. Sham surgeries were performed as above without aorta constriction. Pressure gradient was measured by Vevo 770 High-Resolution Imaging System right after the surgery to evaluate success of aorta constriction. Mice were assessed at 2 and 4 weeks after surgery by echocardiography (Vevo 770 equipped with a 707B scan head). All measurements were averaged for six consecutive cardiac cycles and carried out in a blind fashion.

2.6 Confocal imaging of perfused heart

In situ imaging of single mitochondrial superoxide flashes in Langendorff perfused heart followed a procedure reported previously [23]. Briefly, mouse heart was perfused at Langendorff mode using a custom designed perfusion system that allows mounting of the heart into a chamber on confocal microscope stage. The heart was perfused with modified KHB buffer containing 0.5 mM pyruvate, 10 mM glucose, no insulin, and 10 μ M blebbistatin at 37°C and bubbled with 95% O₂ and 5% CO₂. This approach has been used to support adequate heart performance while slightly suppressing heartbeat [23]. Gentle pressure was applied when taking time lapse 2-D images of the myocardium. In some experiments, mitochondrial membrane potential indicator, TMRM (100–500 nM) was included in perfusion solution. Electrical stimulation was done by placing platinum electrodes on both sides of the heart.

2.7 Statistics

Data are presented as mean $\pm$ SEM. One way ANOVA and paired or unpaired Student's T test were used when appropriate to determine the statistical significance among groups. A p value of less than 0.05 was deemed significant.

3. Results

3.1 EC coupling stimulated superoxide flashes in single mitochondria

Since superoxide flash represents the composite mitochondrial metabolic function, we first tested whether the frequency of flash is under control of EC coupling. Cultured adult ventricular myocytes remain quiescent. Upon electrical stimulation, EC coupling is triggered. We monitored superoxide flashes before and after local electrical stimulation of the myocytes (0.5–2 Hz) and found a frequency-dependent increase in flash activity after electrical stimulation (flash frequency from 1.8 ± 0.2 to 3.5 ± 0.4 flashes/1000 μ m²/100 s after 2 Hz pacing, $p < 0.001$, Figure 1A–B and Movies S1 and S2). Despite the doubled flash frequency, electrical stimulation did not affect the amplitude and kinetics of each flash (Figure 1C–E). Thus, electrical stimulation triggered more mitochondria to produce bursting superoxide flashes. Next, we tested whether tMPT underlies EC coupling-induced flashes, since tMPT triggers flash in cardiac myocytes [14]. Indeed, EC coupling-induced flash activity was potentiated by tMPT activator, atractyloside (Atrac, 10 μ M), and abolished by tMPT inhibitor, CsA (2 μ M), or pre-incubation of an antioxidant, Tiron (1 mM) (Figure 1F–H). Moreover, specific inhibition of mitochondrial superoxide by overexpression of superoxide dismutase 2 (SOD2) suppressed flashes at baseline and abolished 2-Hz pacing-induced flashes (Figure 1H–J). Together, these results suggest that EC coupling promotes single mitochondrial superoxide flash production through tMPT opening.

3.2 Mitochondrial Ca^{2+} uptake underlay EC coupling-induced flash activity

To determine the mechanism of EC coupling modulation of tMPT opening and single mitochondrial superoxide flash activity, we focused on mitochondrial Ca^{2+} . Mitochondria is known to take up and accumulate Ca^{2+} during EC coupling [28], and matrix Ca^{2+} is a known activator of tMPT [29]. Therefore, we loaded a Ca^{2+} indicator, rhod-2, into mitochondria of intact myocytes to simultaneously monitor mitochondrial Ca^{2+} with superoxide flash during EC coupling (Figure 2A). Consistent with previous reports [28], EC coupling stimulation of Ca^{2+} uptake was observed in some mitochondria, in which subsequent induction of superoxide flash was accompanied by depletion of matrix rhod-2 signal (Figure 2B). The probability of detecting these sequential events in the same mitochondrion is tripled by 2 Hz pacing (Figure 2C). These results highly suggest that increased mitochondrial Ca^{2+} accumulation may trigger superoxide flash production during EC coupling. It should be noted that during pacing, whole cell mitochondrial Ca^{2+} level as determined by mitochondrial rhod-2 signal was largely stable likely due to the fast equilibrium of matrix and cytosolic Ca^{2+} or that only a small portion of the released Ca^{2+} during EC coupling enters mitochondria [30].

To test the causal role of mitochondrial Ca^{2+} in EC coupling-induced superoxide flash production, we manipulated mitochondrial Ca^{2+} uniporter (MCU), the major channel responsible for Ca^{2+} uptake [31]. A specific inhibitor of MCU, Ru360 (100 μM , pre-incubated for 30 min to facilitate membrane permeate), abolished EC coupling-induced flashes (Figure 2D). Furthermore, blockade of Ca^{2+} release from SR by ryanodine (100 μM) also attenuated EC coupling-induced flashes (Figure 2E). Taken together, these results indicate that cytosolic Ca^{2+} release followed by mitochondrial Ca^{2+} uptake via MCU is needed for EC coupling-stimulated superoxide flashes.

We also found that increased intracellular Ca^{2+} was sufficient to trigger superoxide flashes in cardiac myocytes. First, addition of a Ca^{2+} ionophore, A23187 (1 μM), dramatically elevated cytosolic Ca^{2+} levels followed by a gradual decay (Figure 3A). The increased cytosolic Ca^{2+} by A23187 was accompanied by increased superoxide flash frequency (Figure 3B, $p < 0.001$). Second, photo-bleaching by an ultraviolet laser (405 nm) “uncaged” Ca^{2+} from nitrophenyl-ethylene glycol tetraacetic acid (NP-EGTA), elevated cytosolic Ca^{2+} concentration locally, and stimulated superoxide flashes in the vicinity of the photo-bleached area (an area of $5 \mu\text{m} \times 10 \mu\text{m}$, Figure 3C–D and *S1*). This is evidence showing that cytosolic Ca^{2+} release directly promotes flash activity. Third, flashes were induced in permeabilized myocytes by increased intracellular Ca^{2+} concentrations with a significantly higher flash frequency observed at the Ca^{2+} concentration approaching systolic Ca^{2+} levels (Figure 3E–F, $p < 0.05$). EC coupling-associated ATP utilization or redox changes may also modulate single mitochondrial superoxide flashes. The data so far support that intracellular Ca^{2+} transients and mitochondrial Ca^{2+} uptake are both necessary and sufficient to stimulate superoxide flashes in single mitochondria.

3.3 Superoxide flash frequency reflected mitochondrial metabolic status

Since blockade of ETC activity suppressed superoxide flash in intact cells and energizing isolated mitochondria increased flash frequency [14, 15], we hypothesized that EC coupling

stimulation of superoxide flash bears physiological significance as an indication of the elevated metabolic status in individual mitochondria. To test this hypothesis, we first removed substrate from the incubating solution, then added back pyruvate as the sole substrate to trigger ETC activity. Pyruvate can be transported into cardiac myocyte through monocarboxylate transporter and has been used to stimulate mitochondrial respiration through Complex I. As shown in Figure 4A–B, pyruvate (0.1–20 mM) dose-dependently stimulated flash frequency in intact cardiac myocytes. Flash amplitude and duration were also increased by pyruvate (Figure 4C–E). Moreover, pyruvate-induced superoxide flashes were blocked by ETC inhibition (rotenone, Rot, 0.5 μ M), tMPT inhibition (cyclosporine A, CsA, 2 μ M), or antioxidants (Tiron, 1 mM or SOD2 overexpression) (Figure 4F). We also detected increased autofluorescence of NADH (10% over baseline) by pyruvate in intact myocytes (Figure 4G), suggesting the accumulation of reducing equivalents, which can fuel the ETC for flash generation. These results indicate that, in intact myocytes, superoxide flash is tightly coupled to mitochondrial metabolic status through ETC activity.

Excessive substrates may reduce the ETC and promote massive ROS generation in the absence of electron flow [32]. However, under physiological conditions, EC coupling-induced superoxide flash activity should be accompanied by an increased electron flow for ATP production. Therefore, we determined whether an increased electron flow is responsible for increased flash generation. We stimulated electron flow and increased oxygen consumption of the permeabilized cardiac myocytes by using pyruvate, malate and ADP to support State 3 respiration. We found that flash frequency was significantly increased under State 3 respiration, which was blocked by ETC or tMPT inhibitors (Figure 4H). Further we found that State 3 respiration increased MitoSOX red fluorescence (Figure 4I, $p < 0.001$), suggesting increased basal ROS production in the mitochondria during metabolic stimulation. Taken together, these results support that increased ETC activity mediates the substrate- or EC coupling-induced superoxide flashes. Since increased ETC activity would lead to increased ATP production and ROS production, the EC coupling modulation of single mitochondrial superoxide flashes could contribute to local energetic or signaling regulation in the beating heart.

3.4 Superoxide flash promoted local Ca^{2+} release

We further explored the physiological significance of EC coupling-induced superoxide flash activity. Many of the EC coupling proteins, including RyR, are known targets of ROS [33]. Therefore, we simultaneously monitored RyR function through Ca^{2+} sparks, the cytosolic Ca^{2+} release events, and superoxide flashes in intact adult cardiac myocytes by linescan confocal imaging (Figure 5A). Interestingly, clusters of Ca^{2+} sparks were found following and around each single mitochondrial superoxide flash (Figure 5A–D), as determined by spatiotemporal mapping. Given the fact that flashes and sparks are distinct events with very different frequencies and mechanisms, the concurrence of the two events highly indicates they are connected. Moreover, both baseline and electrical stimulation-induced Ca^{2+} sparks were attenuated by Tiron (1 mM, Figure 5E), supporting redox regulation of RyR function during EC coupling. Therefore, superoxide flashes may bear signaling function to promote local RyR Ca^{2+} release. Taken together, a positive feedback mechanism may exist, by which EC coupling stimulates individual mitochondrial metabolism and ROS production, which, in

turn, enhances local Ca^{2+} release. It should be noted that significant increase in SR Ca^{2+} release may cause arrhythmia. The low frequency and stochastic nature of superoxide flashes indicates that this is a well-controlled process that elevated local rather than global Ca^{2+} levels.

3.5 EC coupling modulation of flashes was compromised in the failing heart

If EC coupling-induced superoxide flash represents a mechanistic coupling between cardiac excitation and single mitochondrial metabolism, uncoupling of this regulatory pathway may contribute to the development of heart disease. To determine the EC coupling modulation superoxide flashes in heart failure, we generated pressure overload-induced heart failure model in rats by abdominal aorta constriction (AAC) surgery (Figure 6). Four weeks after AAC surgery, a decrease in cardiac contractility was detected by echocardiography (fractional shortening from $67 \pm 6\%$ in sham-operated rats to $39 \pm 6\%$ in heart failure (HF) rats, $p < 0.05$, Figure 6A and Figure S2A–B). Increases in heart size, heart weight/body weight ratio, heart weight/tibia length ratio, and myocyte size were observed (Figure 6A–B and S2C–D). At this stage, we didn't find myocardial fibrosis (Figure S2E–F). In addition, single myocyte Ca^{2+} transients and contractility were mildly compromised, as reflected by prolonged Ca^{2+} transient decay and lowered contraction amplitude (Figure 6C).

Next, we monitored superoxide flashes in myocytes from sham-operated and heart failure rats. As shown in Figure 6D, baseline flash frequency was significantly reduced in cultured failing rat myocytes ($p < 0.001$), suggesting a decrease in individual mitochondrial metabolism under basal conditions. Importantly, electrical stimulation did not increase flashes in failing myocytes compared to normal myocytes (Figure 6D). This indicates compromised response of individual mitochondria to EC coupling in the failing myocytes. However, addition of metabolic substrate (pyruvate) induced flashes in failing myocytes, suggesting that maximal ETC activity is largely unaffected at this early stage (Figure 6D). Because Ca^{2+} transient amplitude was not changed (Figure 6C), the decrease in electrical stimulation-induced flashes in failing myocytes was likely due to an impairment in mitochondrial Ca^{2+} uptake. Indeed, we detected a significant decrease in MCU protein levels in the failing myocardium (Figure 6E), this is consistent with previous report showing decreased mitochondrial membrane potential and compromised mitochondrial Ca^{2+} uptake in the failing heart [34]. Although mitochondrial Ca^{2+} overload can be found in the failing heart, this may happen at the later stages when cytosolic Ca^{2+} is severely disorganized.

To further explore the pathophysiological relevance of compromised individual mitochondrial response to EC coupling in the failing heart, we monitored superoxide flashes in Langendorff perfused heart in which myocardium remains intact and heart remains beating. We first generated transgenic mice bearing superoxide indicator, mt-cpYFP, for monitoring superoxide flashes in intact tissues or *in vivo* (Figure S3) [23]. Pressure overload-induced heart failure model was generated in these mice by transverse aortic constriction surgery [27]. The mice exhibited overt heart failure at 4 week post-surgery (Supplemental table 1). We then perfused the heart in Langendorff mode, and took serial 2D images using a confocal microscope (Figure 7A–B). Failing heart exhibited lower flash frequency under normal perfusion conditions and did not respond to 2 Hz pacing, in contrast

to the healthy heart (sham), which showed significantly increased flash frequency upon electrical stimulation (Figure 7C–D, $p < 0.05$). These findings not only confirm the results in cultured myocytes but also corroborate that EC coupling modulates individual mitochondrial metabolism in the intact beating heart. Taken together, compromised EC coupling modulation of individual mitochondrial metabolism is a prominent feature of heart failure.

4. Discussion

In this report, we discovered a novel mechanism by which EC coupling regulates the metabolism in individual mitochondria to provide local redox signaling in the heart. Cardiac energy homeostasis is under deliberate modulation in the beating heart. Impaired mitochondrial metabolism may compromise cardiac contractility and contribute to heart failure [35]. Mitochondrial metabolism also drives the production of ROS for cell signaling. In this study, we show that metabolism in individual mitochondria is modulated by cytosolic Ca^{2+} transients. During EC coupling, individual mitochondrion takes up Ca^{2+} via MCU, which triggers tMPT opening and leads to accelerated respiration and bursting superoxide production. Further, superoxide flash modulates local Ca^{2+} signaling and can be used as a biomarker for individual mitochondrial metabolism in the heart (Figure 7E).

The link between EC coupling and single mitochondrial superoxide flashes provides unique insight into cardiac metabolism regulation under physiologically relevant conditions. Until recently, evaluation of mitochondrial metabolism in physiologically relevant situations is hampered by the lack of appropriate approach [36, 37]. Oxygen consumption is the gold standard for determining the electron flow during respiration in isolated mitochondria and cells, but not in intact tissues or *in vivo*. Despite debate over the original signal of the flash event, agreement has been reached over its ETC dependence. Specifically, flash activity is modulated by respiration substrates, electron flow (during State 3 respiration) and energy utilization (during EC coupling). Single mitochondrial flash is also accompanied by decreased NADH and slightly increased pH in the matrix, consistent with an accelerated electron flow that drives superoxide generation during the flash. Taken together, superoxide flash is tightly coupled to the metabolic state of individual mitochondria, and its regulation by EC coupling suggests that metabolic control can happen at individual mitochondria level.

EC coupling modulation of superoxide flash activity suggests that there might be an “excitation-metabolism coupling” in individual mitochondria, which “switches on” the metabolism in that mitochondrion from quiescent (basal) to excited (accelerated) states. Such a switch is under control of whole cell Ca^{2+} signal and may in turn modulate local Ca^{2+} release. The current metabolic control theory suggests that matrix Ca^{2+} activates key metabolic enzymes [38]. Our findings provide a novel and additional mechanism, in which matrix Ca^{2+} acutely activates the metabolism in individual mitochondria through triggering tMPT opening (Figure 7E). Unlike sustained opening of the permeability transition pore, which is often detrimental, tMPT has been shown to bear essential physiological functions in the normal heart [39, 40]. Here, tMPT associated depletion of membrane potential and re-equilibrium of mitochondrial contents may drive the accelerated mitochondrial metabolism through ETC activity to rebuild proton motive force and also provide bursting ROS for local signaling.

These findings also highlight heterogeneity in mitochondrial metabolism. Previous studies investigate mitochondrial function almost exclusively at the population level, using a large group of mitochondria and excessive substrates and recording the overall outcome [12, 41]. The stochastic feature of EC coupling-induced superoxide flashes is strong evidence that individual mitochondria respond to a uniform cytosolic signal, such as oscillating Ca^{2+} , differentially. Therefore, it is likely that individual mitochondria can control their own metabolic status through an intrinsic mechanism. We speculate that different mitochondria may possess unique Ca^{2+} uptake/accumulation capacities or thresholds in Ca^{2+} -triggered tMPT opening. Under pathological conditions, when stimulations exceed the threshold of majority of the mitochondria, synchronized ROS production may be induced, which is likely detrimental. Such situation has been shown by laser-induced whole cell ROS and mitochondrial membrane potential oscillations in stressed myocytes [42].

Physiological significance of excitation-metabolism coupling in individual mitochondria is reflected by the finding that superoxide flashes modulate local Ca^{2+} release. It has been shown that exogenous superoxide or oxidants increase the open probability of RyR and promote Ca^{2+} release [43], while reducing reagents inhibit RyR activity [44]. Recent studies suggest a biphasic regulation of RyR function by ROS and propose that, within a certain threshold, oxidants stimulate Ca^{2+} release from RyR [45]. Due to the highly reactive nature of ROS and the high abundance of antioxidants in the cells, redox regulation of cellular signaling is likely compartmentalized. Here, the stimulation of RyR open probability by locally generated superoxide flashes provides the direct evidence that endogenous mitochondrial ROS modulates compartmentalized Ca^{2+} signaling. It should be noted that superoxide flash activity is low in cardiac myocytes under normal conditions and during EC coupling. Therefore, the accelerated metabolism in the “flashing” mitochondria during EC coupling is unlikely to directly impact whole cell energy/signaling homeostasis. Rather, they may play a more important role in a local environment. It is possible that, under certain pathological conditions, the local ROS and Ca^{2+} signals could be exaggerated or accumulated to eventually contribute to whole cell energy/signaling derangement.

Dissociation of excitation-metabolism coupling in the early stages of heart failure emphasizes a possible role of this regulatory mechanism in cardiac pathology. Most of the studies on heart failure therapy focus on boosting cardiac contractility and Ca^{2+} handling to improve heart performance [46]. However, without adequate energy supply, these approaches are unlikely effective. Our findings show that, at the early stage of heart failure, individual mitochondria fail to respond to stimulation, likely due to a lower Ca^{2+} uptake. It has been shown that enhancing cytosolic Ca^{2+} cycling by SERCA overexpression improves cardiac energetics [47]. Thus, restoring excitation-metabolism coupling by enhancing mitochondrial Ca^{2+} handling may benefit mitochondrial metabolism, cardiac Ca^{2+} cycling and heart performance. Furthermore, mitochondrial respiration-coupled ROS production is decreased at early stage, while oxidative stress is increased in overt heart failure [48]. Thus, a suppression of the physiological ROS production mechanism is accompanied by enhanced ROS production from other sources or impaired antioxidant systems at the end stage of heart failure.

In conclusion, we identify an intrinsic mechanism that modulates individual mitochondrial metabolism in cultured cardiac myocytes and the beating heart. The link between EC coupling and individual mitochondrial function is connected by mitochondrial Ca^{2+} uptake via MCU and subsequent opening of tMPT. Our findings demonstrate a chain reaction that integrates cytosolic Ca^{2+} , mitochondrial metabolism and local ROS signaling. Further, restoration of excitation-metabolism coupling could benefit the failing heart. Excitation-metabolism coupling may exist in individual mitochondria of other excitable cells, such as neurons and skeletal muscles, which share similar features in Ca^{2+} , metabolic and ROS regulation as the cardiac myocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Excitation contraction (EC) coupling stimulated single mitochondrial superoxide flashes. (A) Representative image of a cultured adult rat cardiac myocyte expressing mt-cpYFP showing the onset of superoxide flashes before and after electrical stimulation (2 Hz pacing for 2 min), and representative traces of a typical superoxide flash before and after electrical stimulation. Scale bar = 10 μm . (B) Summarized data showing frequency dependent increase in superoxide flashes by electrical stimulation in cultured myocytes. Data are mean $\pm$ SEM, $n = 16$ –38 cells from 4 rats. #, $\dagger$: $p < 0.01$ or 0.001 versus Basal, respectively. (C–E) Summarized data of superoxide flash amplitude ($\Delta F/F_0$, C), time to peak (Tp, D) and time to 50% decay (T50%, E) before and after 2 Hz pacing. Data are mean $\pm$ SEM, $n = 61$ –90 flashes from 10–21 myocytes. (F–H) Modulation of electrical stimulation-induced superoxide flashes by tMPT activator, atractyloside (Atrac, 10 μM , F), inhibitor, cyclosporine A (CsA, 2 μM , G), or antioxidant, Tiron (1 mM, H) in cultured rat myocytes. Data are mean $\pm$ SEM, $n = 7$ –16 cells from 2–3 rats. *: $p < 0.05$ versus Basal. #: $p < 0.05$ versus +Atrac. (I–J) Electrical stimulation-induced superoxide flashes were attenuated by

overexpression of SOD2 in cultured myocytes. Data are mean $\pm$ SEM, n = 21–28 cells from 3–5 rats. *: p < 0.05 versus Basal.

Figure 2.

Mitochondrial Ca^{2+} uptake underlay EC coupling-stimulated single mitochondrial superoxide flashes. (A) Representative images of a cultured rat myocyte (left) expressing mt-cpYFP and with mitochondrial loading of Ca^{2+} indicator, rhod-2. Scale bar = 20 μm . The enlarged images (right) are from a portion of the same myocyte showing the mitochondrial pattern of rhod-2 signal overlapping with mt-cpYFP signal. Scale bar = 5 μm . (B) Representative traces showing real time recording of mitochondrial Ca^{2+} and superoxide signal from a single mitochondrion during electrical stimulation of the rat myocyte (2 Hz). Arrow head indicates a superoxide flash. Note the gradual increase of rhod-2 signal during pacing and its abrupt decline upon the onset of a flash. (C) Summarized data showing the percentage of superoxide flashes with detectable mitochondrial Ca^{2+} accumulation before and after 2 Hz pacing. Data are mean $\pm$ SEM, $n = 7-9$ cells from 2 rats. †: $p < 0.001$ versus Basal. (D) Effect of mitochondrial Ca^{2+} uniporter inhibitor, Ru360 (100 μM) on electrical stimulation-induced superoxide flashes. Data are mean $\pm$ SEM, $n = 10$ cells from 2 rats. (E) Effect of blockade of SR Ca^{2+} release by ryanodine (100 μM) on basal and electrical stimulation-induced superoxide flashes. Data are mean $\pm$ SEM, $n = 12-15$ cells from 3 rats. †: $p < 0.001$ versus Basal.

Figure 3.

Local cytosolic Ca^{2+} release triggered single mitochondrial superoxide flashes. (A) Images of a cultured myocyte loaded with Ca^{2+} indicator, fluo-4 in the cytosol before (upper image) and after (lower image) addition of A23187 (1 μM , a Ca^{2+} ionophore), and representative trace of fluo-4 fluorescence change during the treatment. Similar results were obtained from 3 experiments. (B) Summarized data showing A23187 induced superoxide flashes. Data are mean $\pm$ SEM, $n = 9$ cells. #: $p < 0.01$ versus Control. (C) Local Ca^{2+} release stimulated superoxide flashes. *Left*: images showing a cultured myocyte loaded with rhod-2 in cytosol (upper, red) and expressing mt-cpYFP (lower, green). The myocyte was also loaded with NP-EGTA, a photolabile Ca^{2+} chelator that releases Ca^{2+} upon UV (405 nm) stimulation (photo-bleached area 5 $\mu\text{m} \times 10 \mu\text{m}$, highlighted with blue boxes). *Right*: fluorescent signal of rhod-2 from the corresponding areas highlighted in the image (upper, red traces) and signal of mt-cpYFP from areas highlighted by yellow boxes (lower, green traces). The dashed line indicates the onset of photo-bleaching. Arrow heads indicate the onset of flashes. (D) Summarized data showing superoxide flash frequency from whole cell or bleached area. Data are mean $\pm$ SEM, $n = 16$ –17 cells from 3 rats. †: $p < 0.001$ versus Whole cell. (E) Sample traces showing cytosolic Ca^{2+} (rhod-2) and superoxide (mt-cpYFP) fluorescence in permeabilized rat myocyte by increased intracellular Ca^{2+} levels. Arrow head indicates a randomly occurred flash during the treatment. Similar results were obtained in 3 experiments. (F) Summarized data showing superoxide flash frequency increased with

elevated intracellular Ca^{2+} in permeabilized rat myocytes. Data are mean $\pm$ SEM, n = 17–21 cells from 3 rats. *: p < 0.05 versus 0 Ca^{2+} .

Figure 4.

Superoxide flashes coupled to mitochondrial respiration. (A) Representative image of a cultured adult rat cardiac myocyte expressing mt-cpYFP showing onset of superoxide flashes before (yellow boxes) and after (red boxes) addition of pyruvate (20 mM), and representative traces of a typical superoxide flash before and after pyruvate treatment. Scale bar = 20 μm . (B) Summarized data on superoxide flash frequency in normal KHB solution (Control), in solutions without any substrate (No substrate) or with pyruvate (0.1–20 mM). Data are mean $\pm$ SEM, $n = 9$ –23 cells from 2–6 rats. #: $p < 0.01$ versus Control, †: $p < 0.001$ versus No substrate. (C–E) Summarized data of superoxide flash amplitude ($\Delta F/F_0$, C), time

to peak (Tpk, *D*) and time to 50% decay (T50%, *E*). Data are mean $\pm$ SEM, *n* = 79–351 flashes from 15–17 myocytes. †: *p* < 0.001 versus No substrate. (*F*) Pyruvate-induced superoxide flashes were blocked by cyclosporine A (CsA, 2 μ M), Tiron (1 mM), adenovirus-mediated SOD2 overexpression, and rotenone (Rot, 0.5 μ M). Data are mean $\pm$ SEM, *n* = 10–23 cells from 3–6 rats. †: *p* < 0.001 versus Control, #: *p* < 0.01 versus Pyruvate. (*G*) Representative trace of NADH autofluorescence during pyruvate treatment. Trace was averaged from 6 cells in one experiment. Similar results were obtained in 5 experiments. (*H*) Summarized data on superoxide flash frequency in permeabilized adult rat cardiac myocytes supplemented with Complex I substrates (Pyr/Mal/ADP: 10 mM pyruvate, 5 mM malate and 2 mM ADP) and with inhibitors. Data are mean $\pm$ SEM, *n* = 11–44 cells from 3–6 rats. †: *p* < 0.001 versus Control, #: *p* < 0.001 versus Pyr/Mal/ADP. (*I*) State 3 respiration (Pyr/Mal/ADP) in permeabilized myocytes increased MitoSOX signal (left) and the rate of signal change in MitoSOX fluorescence (right) at both 405 nm and 514 nm excitation. Data are mean $\pm$ SEM, *n* = 19 cells in 5 experiments. †: *p* < 0.001 versus Control.

Figure 5.

Superoxide flashes induced local Ca²⁺ release. (A) *Left*: Linescan confocal images of a myocyte loaded with rhod-2 (in cytosol) and expressing mt-cpYFP showing simultaneous recording of superoxide flashes and Ca²⁺ sparks in a cultured rat myocyte. *Right*: 3D surface plot of the areas labeled with yellow boxes showing the spatiotemporal change of mt-cpYFP (*upper*) and rhod-2 (*lower*) fluorescence during the linescan. Arrow head indicate the occurrence of a flash (*upper*), which is followed by a cluster of Ca²⁺ sparks (*lower*). (B) Time dependent changes of mt-cpYFP or rhod-2 fluorescence of the corresponding locations labeled in (A). Arrow head indicates the onset of a flash and * indicates the spark event. (C–D) Temporal (C) and spatial (D) mapping of Ca²⁺ spark events in linescan images showing the concurrence of Ca²⁺ sparks and superoxide flashes. n = 7 cells from 3 rats. (E) Regulation of Ca²⁺ sparks by antioxidant, Tiron (1 mM) at basal conditions and after 2 Hz

pacing. Data are mean $\pm$ SEM, n = 34–50 cells from 5–9 rats. *: p < 0.05 versus Basal in Control group, #: p < 0.05 versus Basal with the same treatment.

Figure 6.

EC coupling modulation of superoxide flashes was compromised in early stage of heart failure. (A) Characterization of pressure overload-induced heart failure model in rat. Four weeks after abdominal aortic constriction surgery (AAC), heart morphology and function were determined by direct visualization of heart size, echocardiography (M-mode), fractional shortening and heart weight/body weight ratio (HW/BW). Data are mean $\pm$ SEM, $n = 7$ rats from each group. *: $p < 0.05$ versus Sham. (B) HE staining of the myocardium. (C) Ca^{2+} transient amplitude and decay, and cell contraction amplitude measured in cultured intact myocytes. Data are mean $\pm$ SEM, $n = 72-74$ cells from 5 rats. *: $p < 0.05$ versus Sham. (D) Superoxide flashes in cultured rat myocytes from normal (Sham) or heart failure (HF) hearts at basal condition (*Left panel*), in response to electrical stimulation (*Middle*

panel), and in response to metabolic stimulation (*Right panel*). Data are mean $\pm$ SEM, n = 13–60 cells from 3 rats. *: p < 0.05 versus Sham, †: p < 0.001 versus Sham, #: p < 0.001 versus Control. (E) Protein levels of cyclophilin D (Cyp D), MCU and MICU in sham and failing rat hearts. Data are mean $\pm$ SEM, n = 3 for each group. *: p < 0.05 versus Sham.

Figure 7.

EC coupling modulation of superoxide flashes was compromised in perfused failing mouse heart. (A–B) Tri-wavelength confocal images showing expression of mt-cpYFP in intact myocardium from Sham (A) or heart failure (HF (TAC), B) mice 4 weeks after TAC surgery. The hearts were perfused in Langendorff mode and TMRM (100 nM) was used to evaluate mitochondrial membrane potential. (C–D) Representative images (C) and summarized data (D) showing decreased superoxide flash frequency in failing hearts under Langendorff perfusion. Heart failure was induced in mice by transverse aorta constriction (TAC) for 4 weeks. Data are mean $\pm$ SEM, $n = 8$ –20 cells from 2 mice. *: $p < 0.05$ versus Basal in Sham group, #: $p < 0.05$ versus Sham with the same treatment. (E) Schematic model of excitation-metabolism coupling. Intracellular Ca^{2+} transients during EC coupling not only drives cell contraction, but also promotes the metabolism in individual mitochondria through MCU-mediated Ca^{2+} uptake, mPTP opening, ETC electron flow and superoxide flash production. Superoxide flashes, in turn, modulate local Ca^{2+} signaling.