Cardiac Subsarcolemmal and Interfibrillar Mitochondria Display Distinct Responsiveness to Protection by Diazoxide

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Abstract

Objective: Cardiac subsarcolemmal (SSM) and interfibrillar (IFM) mitochondrial subpopulations possess distinct biochemical properties and differ with respect to their protein and lipid compositions, capacities for respiration and protein synthesis, and sensitivity to metabolic challenge, yet their responsiveness to mitochondrially active cardioprotective therapeutics has not been characterized. This study assessed the differential responsiveness of the two mitochondrial subpopulations to diazoxide, a cardioprotective agent targeting mitochondria.

Methods: Mitochondrial subpopulations were freshly isolated from rat ventricles and their morphologies assessed by electron microscopy and enzymatic activities determined using standard biochemical protocols with a plate reader. Oxidative phosphorylation was assessed from State 3 respiration using succinate as a substrate. Calcium dynamics and the status of Ca2+-dependent mitochondrial permeability transition (MPT) pore and mitochondrial membrane potential were assessed using standard Ca2+ and TPP+ ion-selective electrodes.

Results: Compared to IFM, isolated SSM exhibited a higher sensitivity to Ca2+ overload-mediated inhibition of adenosine triphosphate (ATP) synthesis with decreased ATP production (from 375 ± 25 to 83 ± 15 nmol ATP/min/mg protein in SSM, and from 875 ± 39 to 583 ± 45 nmol ATP/min/mg protein in IFM). In addition, SSM exhibited reduced Ca2+-accumulating capacity as compared to IFM (230 ± 13 vs. 450 ± 46 nmol Ca2+/mg protein in SSM and IFM, respectively), suggestive of increased Ca2+ sensitivity of MPT pore opening. Despite enhanced susceptibility to stress, SSM were more responsive to the protective effect of diazoxide (100 μM) against Ca2+ overload-mediated inhibition of ATP synthesis (67% vs. 2% in SSM and IFM, respectively).

Conclusion: These results provide evidence for the distinct sensitivity of cardiac SSM and IFM toward Ca2+-dependent metabolic stress and the protective effect of diazoxide on mitochondrial energetics.

Introduction

Two distinct mitochondrial subpopulations – subsarcolemmal (SSM), situated underneath the sarcolemmal membrane, and interfibrillar (IFM), distributed between myofibrils – have been previously identified in myocardium [1–5]. These mitochondrial subpopulations differ in respect to their protein and lipid compositions, capacities for respiration and protein synthesis, and in their sensitivity to metabolic challenge [4–13]. Differences in the responsiveness of mitochondrial subpopulations to metabolic stress with enhanced susceptibility of SSM have been demonstrated in the heart [14,15]. SSM appears to be more vulnerable to ischemic injury and mitochondrial Ca2+ overload when compared to IFM [3,8–11,16–20]. Despite distinct biochemical properties and sensitivity to stress, the differences between SSM and IFM in responsiveness to mitochondrially active therapeutics have not been completely characterized. Here, we demonstrate that diazoxide, a cardioprotective mitochondria-targeting agent [21–24], effectively protects mitochondria against Ca2+ loading and restores Ca2+-inhibited oxidative phosphorylation to a greater extent in SSM than in IFM. These results thus provide evidence of distinct sensitivity of cardiac mitochondrial subpopulations toward the protective effect of diazoxide, indicating that SSM could be the preferred target for drug treatment.

Materials and Methods

Ethic statement

The study was approved by the Mayo Clinic Institutional Animal Care and use Committee (Protocol # A28201), and all
procedures were in accordance with recommendations published in Guide for the Care and Use of Laboratory Animals, National Academic Press, Washington, D.C., 1996.

Mitochondrial isolation

Mitochondria were isolated from the hearts of pentobarbital (100 mg/kg intraperitoneal injection)-anesthetized male adult rats (Sprague-Dawley; Harlan Laboratories, Indianapolis, IN). Following thoracotomy, the heart was rapidly removed from the chest and ventricles were trimmed of atria and connective tissue. The ventricles were placed in ice-cold media containing (in mmol/L): sucrose 50, mannitol 200, KH2PO4 5, EGTA 1, 0.2% BSA, MOPS 5 (pH = 7.3) as described by Holmuhamedov et al. [22]. SSM and IFM were isolated from Polytron®-homogenized (Brinkmann Instruments, Westbury, NY) ventricles using differential centrifugation as previously described [9,21,22]. Briefly, isolation of SSM was achieved by mechanical rupture of ventricular tissue with Polytron followed by differential centrifugation, whereas IFM isolation was performed in tissue depleted of SSM by an additional enzymatic digestion with Nagarse and mechanical disruption of residual ventricular tissue to release IFM. A subset of experiments was repeated to rule out nonspecific effect of enzymatic treatment with Nagarse and mechanical disruption of residual ventricular tissue to release IFM. No significant differences were observed in mitochondrial respiration and Ca2+ functions in isolated SSM. No significant differences were observed in mitochondrial respiration and Ca2+ handling of isolated SSM in the presence or absence of Nagarse treatment. Protein concentration was determined using DC® Protein Determination Kit (Bio-Rad Laboratories, Hercules CA).

Electron microscopy

SSM and IFM were fixed using Trump’s buffer (1% glutaraldehyde, 4% formaldehyde, 0.1-M phosphate buffer, pH 7.2), rinsed and post-fixed in phosphate-buffered 1% osmium tetroxide [25–27]. Samples were stained en bloc with 2% uranyl acetate for 30 min at 60°C, rinsed, dehydrated, and embedded in Spurr’s resin. Thin sections were cut on an Ultracut E ultramicrotome (Reichert-Jung, Vienna, Austria), placed on copper grids and stained with lead citrate. Mitochondria were micrographed with a 1200 EX II electron microscope (Jeol, Tokyo, Japan).

Citrate synthase activity

The activity of citrate synthase (CS) in SSM and IFM was determined as described by Short et al. [28] with minor modifications. Aliquots of mitochondria were transferred into the incubation buffer, which contained (in mmol/L): 5,5′-dithiobis-(2-nitrobenzoic acid) = 0.1; acetyl-CoA = 0.12; oxaloacetate = 0.5; TRIZMA = 100; Triton X-100 = 0.1%; pH = 8.1. The activity of CS was monitored as absorbance change of 412 nm and expressed as μmoles of thionitrobenzoic acid (TNB)/min/mg protein.

Western blot

Aliquots of mitochondria solubilized in Laemmle sample buffer were separated on polyacrylamide gels (Criterion™, Bio-Rad Laboratories) and then transferred to polyvinylidene fluoride membranes as described by Short et al. [28]. Brieﬂy, membranes were blocked in 5% milk in Tris-buffered saline with 0.1% Tween-20 for 1 hour and then incubated overnight with primary antibody. Dilutions for the primary antibodies were: citrate synthase 1:1000 (a kind gift from J.O. Holloszy, MD, Washington University, St. Louis, MO), adenine nucleotide transporter 1 (ANT1) 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were subsequently exposed to secondary horseradish peroxidase-labeled antibodies at 1:10,000 (Amersham Biosciences, Piscataway, NJ) and then chemiluminescent substrate (ECL Plus™, Amersham) was used for detection. Images captured on Kodak Onfilm (Kodak Scientiﬁc, Rochester, NY) were then used for densitometry of bands using Kodak Image Station 1000.

Respiration, membrane potential and calcium transport

Respiration, membrane potential and Ca2+ transport of isolated mitochondria were determined using a multichannel system (ABMT-USA, Durham, NC) equipped with oxygen-, tetraphenylphosphonium (TPP+) and Ca2+-selective minielectrodes, as previously described [21–23]. Brieﬂy, mitochondria (1 mg/ml) were added into the incubation buffer containing (in mM): KCl 110, KH2PO4 5, succinate 5, pyruvate 5, and MOPS 10 (pH = 7.35) and respiration was measured using calibrated Clark-type O2 minielectrode. Mitochondrial membrane potential was measured simultaneously with respiration using TPP+-selective minielectrode, manufactured and calibrated as described by Kami et al. [29]. Concentration of TPP+ was 200 nM, and mitochondrial membrane potential was calculated as previously described [21,22,29]. Mitochondrial Ca2+ uptake was measured from changes in the free Ca2+ concentration within the suspension using calibrated Ca2+-selective minielectrodes (Microelectrodes Inc., Bedford, NH) as described [22,25]. Mitochondrial Ca2+-accumulating capacity was determined as the total amount of Ca2+ accumulated into the matrix from a train of 50-μM Ca2+ pulses added at 1-min intervals until the load reached a threshold pulse after which mitochondria underwent irreversible and rapid Ca2+ release [30,31].

Adenosine triphosphate synthesis

Adenosine triphosphate (ATP) production in mitochondria was determined using K2CO3/MOPS-neutralized HClO4-soluble mitochondrial extracts by high-pressure liquid chromatography (Hewlett-Packard, Waldbronn, Germany) as described by Holmuhamedov et al. [23]. Brieﬂy, 200 μl of mitochondrial suspension were treated with 20 μl of 3.3-M HClO4, and precipitated proteins were removed by centrifugation (60 s, 14,000 rpm, 4°C). After neutralization of the supernatant with 80 μl of a mixture containing 2.5-M K2CO3 in 1-M HEPES, the precipitate was separated by centrifugation (60 s, 14,000 rpm, 4°C), and the concentration of ATP within the extract was determined in coupled enzymatic reactions [32]. The time course of adenosine diphosphate (ADP)-to-ATP conversion within mitochondrial suspension was monitored from changes in NADPH fluorescence (Ascent FL, Scientific Resources, Saint Paul, MN) in a coupled hexokinase/glucose-6-phosphate dehydrogenase assay [21–23,32].

Drugs

Diazoxide (Research Biochemical International, Natick, MA) was dissolved as a concentrated stock solution in dimethylsulfoxide (DMSO), and the maximal concentration of DMSO in the incubation medium was kept under 0.5%. All other chemicals were from Sigma Chemicals (St. Louis, MO).

Statistical analysis

Data are expressed as mean ± standard error of mean, and “n” represents the number of mitochondrial isolations. Comparison between groups was made using analysis of variance (ANOVA) with post-hoc test. ANOVA was performed for multiple comparisons between groups using two-way comparison of means by Tukey-Kramer HSD test, and p<0.05 was considered to be statistically significant.
Results

Biochemical similarity and differences in isolated cardiac SSM and IFM

Electron micrographs of the heart muscle demonstrate intracellular localization of mitochondrial subpopulations and morphological appearance of SSM and IFM in situ and after isolation (Fig. 1A). Intracellular SSM have a round shape and a less electron-dense “light” matrix, while IFM are elongated and rod-shaped with the matrix containing a greater electron-dense material (Fig. 1A). The content of CS and ANT1, specific mitochondrial matrix and membrane proteins were all determined by Western blot (Fig. 1B, top panels). Both subpopulations of isolated mitochondria demonstrated similar levels of expression of CS and ANT1 (Fig. 1B, top panels). In addition, the activity of CS in mitochondrial subpopulations was similar (2.04±0.03 vs. 1.98±0.04 μmoles TNB/min/mg protein in SSM and IFM, respectively; Fig. 1B, lower panel, n = 6, p = NS).

Ca²⁺ handling and oxidative phosphorylation capacity of SSM and IFM

The sensitivity of mitochondria toward Ca²⁺-induced mitochondrial permeability transition (MPT) pore opening was determined from the number of Ca²⁺ pulses required to reach the threshold for rapid and spontaneous Ca²⁺ release [31]. There was no difference in the baseline content of endogenous Ca²⁺ in isolated SSM and IFM measured immediately after isolation (2.1±0.9 vs. 2.2±1.1 nmol Ca²⁺/mg protein, respectively, n = 6, data not shown). However, the maximal Ca²⁺-accumulating capacity of mitochondrial subpopulations (determined from experiments with multiple Ca²⁺ pulses, described in Materials and Methods) was significantly decreased in SSM and was 230±13 nmol Ca²⁺/mg protein as compared with 450±24 nmol Ca²⁺/mg protein in IFM (Fig. 2A and 2B, n = 6, p<0.05).

The capacity for oxidative phosphorylation assessed from ADP-mediated increase in mitochondrial respiration and membrane depolarization was also different in SSM and IFM (Fig. 2C and 2D). On average, ADP-stimulated respiration (State 3) in SSM was 49% lower than in IFM (267±15 vs. 518±37 ng-atoms O₂/min/mg protein, respectively; Fig. 2D, n = 6, p<0.05). Decreased State 3 respiration in SSM correlated with a longer period of ADP-to-ATP conversion, as monitored from reversible and transient ADP-stimulated respiration (State 3) in SSM and IFM, respectively; Fig. 2D, n = 6, p<0.05). Thus, compared to IFM, SSM demonstrated lesser Ca²⁺ handling capacity, enhanced susceptibility to MPT pore opening, decreased rate of oxidative phosphorylation and enhanced sensitivity to inhibition of mitochondrial energetics by excessive Ca²⁺ loading.

Cardiac Mitochondria Responsiveness to Diazoxide

Diazoxide decreases Ca²⁺ loading preferentially in SSM

Diazoxide, an opener of sarcolemmal ATP-sensitive K⁺ channels with cardioprotective properties also known to target and depolarize isolated mitochondria [21,22,35–36], differentially affected mitochondrial membrane potential and Ca²⁺ handling in these two mitochondrial subpopulations. The SSM-oxidizing succinate, demonstrated a higher sensitivity to diazoxide (100 μM)-induced depolarization of the inner membrane (19±2 mV) compared with IFM (10±3 mV; Fig. 3A, n = 6, p<0.05). Similarly, when added to mitochondria prior to Ca²⁺ loading, diazoxide (100 μM) suppressed the rate of Ca²⁺ uptake preferentially in SSM (from 347±9 to 137±7 nmol Ca²⁺/min/mg protein) than in IFM (from 503±13 to 326±10 nmol Ca²⁺/min/mg protein), demonstrating a 61% vs. 35% inhibition of Ca²⁺ uptake in SSM and IFM, respectively (Fig. 3B and 3C, n = 6, p<0.05). In the absence of diazoxide, neither SSM nor IFM demonstrated release of accumulated Ca²⁺ during 20 min of observation (data not shown). However, diazoxide had a differential effect on Ca²⁺ release from Ca²⁺-loaded mitochondria. In mitochondria preloaded with 100 nmol Ca²⁺/mg protein, the same concentration of diazoxide (100 μM) induced faster release of accumulated Ca²⁺ from SSM than from IFM (Fig. 3D). On average, the rate of diazoxide-induced Ca²⁺ release was fivefold higher (10±2 vs. 2±1 nmol Ca²⁺/min/mg protein) in SSM and
and after (stimulated (State 3) respiration of SSM and IFM (n = 6, p
induced changes in oxygen consumption (mg protein, diazoxide restored the rate of ATP production by
53% in SSM compared with 5
3% in IFM. Thus, diazoxide
restores Ca2+
release was dose-
dependent and the concentration of diazoxide causing 50%
release was 114±21 and 377±44 μM in SSM and IFM, respectively (Fig. 3G, n = 6, p<0.05). The
SSM and IFM exhibit differential responsiveness toward diazoxide-mediated membrane
depolarization, Ca2+
uptake and release of accumulated Ca2+
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Diazoxide restores Ca2+-inhibited ATP production
Excessive mitochondrial Ca2+
loading inhibited oxidative
phosphorylation (Fig. 2) and diazoxide in a dose-dependent
manner restored State 3 respiration in both SSM and IFM (Fig. 4A). The rescuing effect of diazoxide on Ca2+-inhibited State 3 respiration was more prominent in SSM compared to IFM (Fig. 4) and was dependent upon the level of mitochondrial Ca2+
overload (Fig. 4B and 4C). In mitochondria loaded with 150 nmol Ca2+/mg protein, diazoxide restored Ca2+-inhibited ADP-stimulated respiration by 35±4% in SSM compared with 5±3% in IFM (Fig. 4C, p<0.05, n = 6, open bars), whereas at 30 nmol Ca2+/mg protein, the protective effect of diazoxide was only by 12±3% and 4±0.9% in SSM and IFM, respectively (Fig. 4C, n = 6, hatched bars). ATP synthesis in mitochondria, monitored using coupled enzymatic reactions [22,23], confirmed that diazoxide was more efficient in recovering the rate of Ca2+-inhibited ATP production in SSM than IFM. In mitochondria loaded with 150 nmol Ca2+/mg protein, diazoxide restored the rate of ATP production by 48±5% in SSM compared with 5±3% in IFM. Thus, diazoxide restores Ca2+-inhibited mitochondrial ATP synthesis in both SSM and IFM populations, but the magnitude of this protective effect is markedly higher in SSM compared to IFM.

Discussion
This study demonstrates that SSM and IFM, two subpopulations of cardiac mitochondria, exhibit differential susceptibility to Ca2+-dependent inhibition of oxidative phosphorylation, opening of MPT pore and sensitivity to the protective effect of diazoxide, a mitochondrially active cardioprotective agent. These results provide additional insights into the functional and pharmacological differences between the mitochondrial subpopulations previously shown to differ in their biochemical characteristics, protein and lipid composition, and susceptibility toward metabolic challenge [6,8–12,17,37]. Here we demonstrate that SSM were more vulnerable to the damaging effects of Ca2+
overload and inhibition of oxidative phosphorylation when compared to IFM, in line with previous observations [7,10,11,16–18]. While Ca2+-mediated inhibition of oxidative phosphorylation was more prominent in SSM compared to IFM [8,10], diazoxide was more effective in restoring Ca2+-inhibited oxidative phosphorylation in SSM than IFM (Figs. 3 and 4). This finding could be of great clinical relevance, as SSM has been shown to be more susceptible to injury than the IFM [6,10,11,17,38]. Mitochondrial energy production is determined by the activity of key enzymes of tricarboxylic cycle, which are regulated by Ca2+ ions in the physiological range of Ca2+ concentrations [39,40]. However, excessive Ca2+
loading under pathological conditions has a detrimental effect on mitochondrial ATP synthesis [39–44]. Diazoxide has been demonstrated to protect mitochondrial energetic function and preserve cellular ATP level under metabolic stress [22,45–48]. Here, we demonstrate that diazoxide-mediated decrease in mitochondrial Ca2+
loading is accompanied by partial restoration of Ca2+-inhibited ATP production in both mitochondrial subsets, but the responsiveness of SSM to diazoxide was much greater. The levels of expression of the mitochondrial-specific matrix enzyme
(citrate synthase) and membrane protein (adenine nucleotide transporter) were not different in the two subpopulations, suggesting that distinct properties were not introduced due to differences in the isolation protocol or the number of mitochondria but are intrinsic features of mitochondrial subpopulations. The salvaging effect of diazoxide on ATP production in Ca\(^{2+}\)-loaded mitochondria was greater in mitochondria with a higher level of Ca\(^{2+}\) load, indicating that the effect of diazoxide is condition-
selective, and it is rather the release of inhibited oxidative phosphorylation than the activation of mitochondrial ATP synthesis. In accordance with this notion is the fact that in the absence of Ca\(^{2+}\) loading, diazoxide had little effect on State 3 respiration in both preparations, and may even slow the rate of ATP production as reported previously [22,23,33,49,50].

The precise mechanism of diazoxide action on mitochondria in vivo remains unknown, [26,33,34,46,47,51] and likely involves multiple effects, including mitochondrial uncoupling by a protophoric effect [24], potassium transport, and substrate metabolism reported in isolated rat hearts [24,33,34,36,50,52] and mitochondria [23,26]. Additional factors may influence the overall effect of diazoxide on cardiac energetics and protection, including the intracellular locale of biochemically and functionally different mitochondrial subpopulations exposed to a different degree of Ca\(^{2+}\) load and metabolic stress. This is of significance in view of the different responsiveness of subsarcolemmal and interfibrillar mitochondria in intact cardiomyocytes [8–11,16,17,53] and the heterogeneity in mitochondrial Ca\(^{2+}\) loading demonstrated in various cellular microdomains [54–58]. Our observation that SSM are more sensitive toward diazoxide-mediated protection from Ca\(^{2+}\) and reduced tolerance of this mitochondrial population toward Ca\(^{2+}\)-mediated functional and structural damages. Therefore, our observation indicates that by preferentially targeting SSM (the more vulnerable subpopulation of cardiac mitochondria) diazoxide will be protective against ischemia/reperfusion-mediated injury.

Under normal conditions, both SSM and IFM are efficient in meeting demands of the cellular ATP-dependent processes and maintaining ionic homeostasis of cells (Fig. 5A). During ischemic insult and decreased delivery of oxygen, mitochondrial ATP production drops and ion pumps fail to maintain required gradients of Na\(^{+}\) and K\(^{+}\) ions across the sarcotubular membrane, resulting in increased cytosolic Ca\(^{2+}\) (Fig. 5B). At reperfusion, oxygen availability quickly restores mitochondrial membrane potential and leads to excessive uptake of Ca\(^{2+}\) from the cytosol. By promoting MPT pore opening or inhibition of oxidative phosphorylation, this oxygen availability causes greater injury to the more vulnerable SSM than IFM, resulting in additional structural and functional derangements that limit the capacity of SSM, which are located in the close vicinity of plasma membrane ion pumps [4,5,9–11,13] in order to synthesize ATP, a critical function for maintaining homeostasis at the time of reperfusion (Fig. 5B). From our findings, we speculate that during and/or following metabolic stress, cardioprotective diazoxide moderately depolarizes mitochondria and prevents SSM against excessive Ca\(^{2+}\) overload by decreasing the rate of Ca\(^{2+}\) uptake or releasing accumulated Ca\(^{2+}\) or both, resulting in preservation of ATP production in the more vulnerable and strategically distributed SSM (Fig. 5), thus rescuing the energy source for ATP-dependent cellular processes, such as the maintenance of transsarcolemmal ionic homeostasis.

In summary, SSM are a preferential target for the cardioprotective drug diazoxide in the setting of ischemia and heart reperfusion. Our data suggest that the mechanism of protective action of diazoxide could be through decreased Ca\(^{2+}\) uptake, reduction of mitochondrial Ca\(^{2+}\) loading and through release of excessively loaded Ca\(^{2+}\) and restoration of Ca\(^{2+}\)-inhibited ATP production in postischemic heart muscle.

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Author Contributions

Conceived and designed the experiments: EH AJ. Performed the experiments: EH AO KS. Analyzed the data: EH AJ KS. Contributed reagents/materials/analysis tools: AJ KS AT. Wrote the paper: EH AJ AT.

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