

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

Ekhsan L. Holmuhamedov<sup>1</sup>, Andrew Oberlin<sup>2</sup>, Kevin Short<sup>3</sup>, Andre Terzic<sup>2</sup>, Arshad Jahangir<sup>1,2\*</sup>

**1** Center for Integrative Research on Cardiovascular Aging, Aurora University of Wisconsin Medical Group, Aurora Health Care, Milwaukee, Wisconsin, United States of America, **2** Division of Cardiovascular Diseases, Mayo Clinic, Rochester, Minnesota, United States of America, **3** Division of Endocrinology, Mayo Clinic, Rochester, Minnesota, United States of America

## 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 Ca<sup>2+</sup>-dependent mitochondrial permeability transition (MPT) pore and mitochondrial membrane potential were assessed using standard Ca<sup>2+</sup> and TPP<sup>+</sup> ion-selective electrodes.

**Results:** Compared to IFM, isolated SSM exhibited a higher sensitivity to Ca<sup>2+</sup> 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 Ca<sup>2+</sup>-accumulating capacity as compared to IFM (230±13 vs. 450±46 nmol Ca<sup>2+</sup>/mg protein in SSM and IFM, respectively), suggestive of increased Ca<sup>2+</sup> sensitivity of MPT pore opening. Despite enhanced susceptibility to stress, SSM were more responsive to the protective effect of diazoxide (100 μM) against Ca<sup>2+</sup> 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 Ca<sup>2+</sup>-dependent metabolic stress and the protective effect of diazoxide on mitochondrial energetics.

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\* E-mail: arshad.jahangir@aurora.org

## 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 Ca<sup>2+</sup> overload when compared to IFM [5,8–11,16–20]. 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 Ca<sup>2+</sup> loading and restores Ca<sup>2+</sup>-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,  $\text{KH}_2\text{PO}_4$  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<sup>®</sup>-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 on basic mitochondrial functions in isolated SSM. No significant differences were observed in mitochondrial respiration and  $\text{Ca}^{2+}$  handling of isolated SSM in the presence or absence of Nagarse treatment. Protein concentration was determined using DC<sup>™</sup> 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-Co-A = 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  $\mu\text{moles}$  of thionitrobenzoic acid (TNB)/min/mg protein.

### Western blot

Aliquots of mitochondria solubilized in Laemmle sample buffer were separated on polyacrylamide gels (Criterion<sup>™</sup>, 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<sup>™</sup>, Amersham) was used for detection. Images captured on Kodak Omat film (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  $\text{Ca}^{2+}$  transport of isolated mitochondria were determined using a multichannel system (ABMT-USA, Durham, NC) equipped with oxygen-, tetraphenylphosphonium ( $\text{TPP}^+$ )- and  $\text{Ca}^{2+}$ -selective minielectrodes, as previously described [21–23]. Briefly, mitochondria (1 mg/ml) were added into the incubation buffer containing (in mM): KCl 110,  $\text{K}_2\text{HPO}_4$  5, succinate 5, pyruvate 5, and MOPS 10 (pH = 7.35) and respiration was measured using calibrated Clark-type  $\text{O}_2$  minielectrode. Mitochondrial membrane potential was measured simultaneously with respiration using  $\text{TPP}^+$ -sensitive minielectrode, manufactured and calibrated as described by Kamo et al. [29]. Concentration of  $\text{TPP}^+$  was 200 nM, and mitochondrial membrane potential was calculated as previously described [21,22,29]. Mitochondrial  $\text{Ca}^{2+}$  uptake was measured from changes in the free  $\text{Ca}^{2+}$  concentration within the suspension using calibrated  $\text{Ca}^{2+}$ -selective minielectrodes (Microelectrodes Inc., Bedford, NH) as described [22,25]. Mitochondrial  $\text{Ca}^{2+}$ -accumulating capacity was determined as the total amount of  $\text{Ca}^{2+}$  accumulated into the matrix from a train of 50- $\mu\text{M}$   $\text{Ca}^{2+}$  pulses added at 1-min intervals until the load reached a threshold pulse after which mitochondria underwent irreversible and rapid  $\text{Ca}^{2+}$  release [30,31].

### Adenosine triphosphate synthesis

Adenosine triphosphate (ATP) production in mitochondria was determined using  $\text{K}_2\text{CO}_3$ /MOPS-neutralized  $\text{HClO}_4$ -soluble mitochondrial extracts by high-pressure liquid chromatography (Hewlett-Packard, Waldbronn, Germany) as described by Holmuhamedov et al. [23]. Briefly, 200  $\mu\text{l}$  of mitochondrial suspension were treated with 20  $\mu\text{l}$  of 3.3-M  $\text{HClO}_4$ , and precipitated proteins were removed by centrifugation (60 s, 14,000 rpm, 4°C). After neutralization of the supernatant with 80  $\mu\text{l}$  of a mixture containing 2.5-M  $\text{K}_2\text{CO}_3$  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  $\pm$  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 ( $2.04 \pm 0.03$  vs.  $1.98 \pm 0.04$   $\mu$ moles TNB/min/mg protein in SSM and IFM, respectively; Fig. 1B, *lower panel*,  $n = 6$ ,  $p = \text{NS}$ ).

### Ca<sup>2+</sup> handling and oxidative phosphorylation capacity of SSM and IFM

The sensitivity of mitochondria toward Ca<sup>2+</sup>-induced mitochondrial permeability transition (MPT) pore opening was determined from the number of Ca<sup>2+</sup> pulses required to reach the threshold for rapid and spontaneous Ca<sup>2+</sup> release [31]. There was no difference in the baseline content of endogenous Ca<sup>2+</sup> in isolated SSM and IFM measured immediately after isolation ( $2.1 \pm 0.9$  vs.  $2.2 \pm 1.1$  nmol Ca<sup>2+</sup>/mg protein, respectively,  $n = 6$ , data not shown). However, the maximal Ca<sup>2+</sup>-accumulating capacity of mitochondrial subpopulations (determined from experiments with multiple Ca<sup>2+</sup> pulses, described in Materials and Methods) was significantly decreased in SSM and was  $230 \pm 13$  nmol Ca<sup>2+</sup>/mg protein as compared with  $450 \pm 46$  nmol Ca<sup>2+</sup>/mg protein in IFM (Fig. 2A and 2B,  $n = 6$ ,  $p < 0.05$ ).

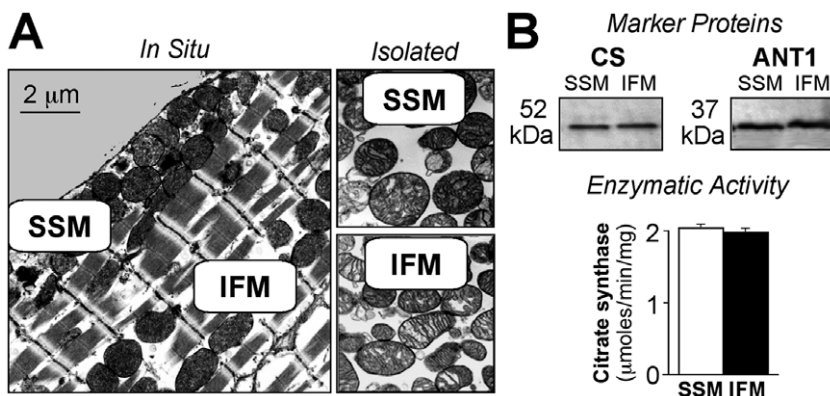
The capacity for oxidative phosphorylation assessed from ADP-mediated increase in mitochondrial respiration and membrane depolarization was also different in SSM and IFM (Fig. 2C and 2D). On average, ADP-stimulated respiration (State 3) in SSM was 49% lower than in IFM ( $267 \pm 15$  vs.  $518 \pm 37$  ng-atoms O<sub>2</sub>/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-induced membrane depolarization from the baseline ( $76 \pm 3$  sec in

SSM versus  $36 \pm 3$  sec in IFM; Fig. 2C, *lower panel*). Although the rates of respiration upon completion of ADP-to-ATP conversion (State 4) were different in these populations ( $67 \pm 5$  and  $123 \pm 11$  ng-atoms O<sub>2</sub>/min/mg protein in SSM and IFM, respectively), the resulting respiratory control ratio was not significantly different ( $3.2 \pm 0.7$  vs.  $4.2 \pm 0.5$ ,  $n = 6$ ), demonstrating similar coupling of oxidative phosphorylation in SSM and IFM.

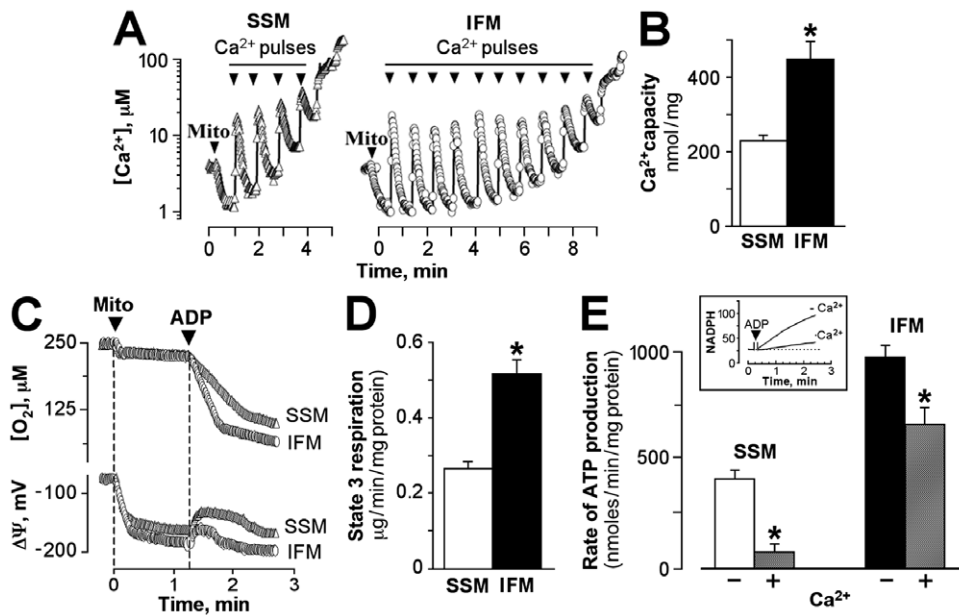
Loading of mitochondria with 150 nmol Ca<sup>2+</sup>/mg protein inhibited oxidative phosphorylation in both SSM and IFM, but the extent of inhibition was significantly higher in SSM. The rate of ATP synthesis in the presence of Ca<sup>2+</sup> was decreased from  $375 \pm 25$  to  $83 \pm 15$  nmol ATP/min/mg protein in SSM, and from  $875 \pm 39$  to  $583 \pm 45$  nmol ATP/min/mg protein in IFM (Fig. 2E,  $n = 6$ ,  $p < 0.05$ ). Thus, compared to IFM, SSM demonstrated lesser Ca<sup>2+</sup> handling capacity, enhanced susceptibility to MPT pore opening, decreased rate of oxidative phosphorylation and enhanced sensitivity to inhibition of mitochondrial energetics by excessive Ca<sup>2+</sup> loading.

### Diazoxide decreases Ca<sup>2+</sup> loading preferentially in SSM

Diazoxide, an opener of sarcolemmal ATP-sensitive K<sup>+</sup> channels with cardioprotective properties also known to target and depolarize isolated mitochondria [21,22,33–36], differentially affected mitochondrial membrane potential and Ca<sup>2+</sup> handling in these two mitochondrial subpopulations. The SSM-oxidizing succinate, demonstrated a higher sensitivity to diazoxide (100  $\mu$ M)-induced depolarization of the inner membrane ( $19 \pm 2$  mV) compared with IFM ( $10 \pm 2$  mV; Fig. 3A,  $n = 6$ ,  $p < 0.05$ ). Similarly, when added to mitochondria prior to Ca<sup>2+</sup> loading, diazoxide (100  $\mu$ M) suppressed the rate of Ca<sup>2+</sup> uptake preferentially in SSM (from  $347 \pm 9$  to  $137 \pm 7$  nmol Ca<sup>2+</sup>/min/mg protein) than in IFM (from  $503 \pm 13$  to  $326 \pm 10$  nmol Ca<sup>2+</sup>/min/mg protein), demonstrating a 61% vs. 35% inhibition of Ca<sup>2+</sup> 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 Ca<sup>2+</sup> during 20 min of observation (data not shown). However, diazoxide had a differential effect on Ca<sup>2+</sup> release from Ca<sup>2+</sup>-loaded mitochondria. In mitochondria preloaded with 100 nmol Ca<sup>2+</sup>/mg protein, the same concentration of diazoxide (100  $\mu$ M) induced faster release of accumulated Ca<sup>2+</sup> from SSM than from IFM (Fig. 3D). On average, the rate of diazoxide-induced Ca<sup>2+</sup> release was fivefold higher ( $10 \pm 2$  vs.  $2 \pm 1$  nmol Ca<sup>2+</sup>/min/mg protein) in SSM and



**Figure 1. Morphological and biochemical characteristics of two mitochondrial populations in heart muscle.** A, Electron micrographs of subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria within the heart cell (*In Situ*) and after isolation (*Isolated*). Magnification:  $\times 10,000$ . B, Expression of mitochondria-specific marker proteins in SSM and IFM. Top panel displays Western blots of citrate synthase (CS) and adenine nucleotide transporter 1 (ANT1). Bottom panel displays bar graphs of the activity of CS (SSM, *open bars*; IFM, *filled bars*). doi:10.1371/journal.pone.0044667.g001



**Figure 2. Ca<sup>2+</sup> handling and oxidative phosphorylation capacity of subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria.** A, Typical tracing of Ca<sup>2+</sup> loading in SSM (left panel) and IFM (right panel). Mitochondria were loaded with consecutive Ca<sup>2+</sup> pulses (arrows), and each pulse delivers 50 nmol of Ca<sup>2+</sup>. B, Average Ca<sup>2+</sup>-accumulating capacity of SSM (open bar) and IFM (filled bar), n=6, p<0.05. C, Typical tracing of ADP-induced changes in oxygen consumption (top) and changes in membrane potential from baseline (bottom) of SSM and IFM. D, Average ADP-stimulated (State 3) respiration of SSM and IFM (n=6, p<0.05). E, The rate of ATP production in SSM (left bars) and IFM (right bars) before (B/W bars) and after (gray bars) loading with 150 nmol Ca<sup>2+</sup>/mg protein, n=3, p<0.05. Inset: Alternative monitoring of ATP production in mitochondria using coupled enzymes. Asterisks signify statistical differences with p<0.05. doi:10.1371/journal.pone.0044667.g002

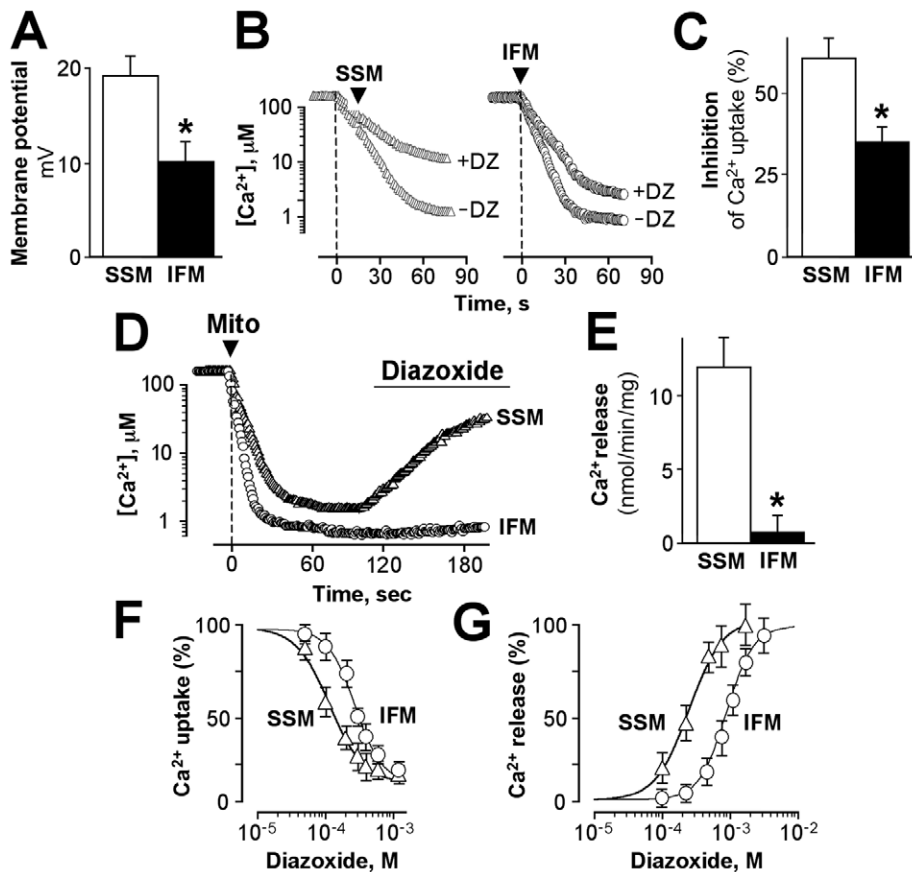
IFM, respectively (Fig. 3E, n=6, p<0.05). Overall, the effect of diazoxide on mitochondrial Ca<sup>2+</sup> uptake and release was dose-dependent, and the concentration of diazoxide causing 50% inhibition of Ca<sup>2+</sup> uptake (IC<sub>50</sub>) was estimated at 104±15 and 289±32 µM in SSM and IFM, respectively (Fig. 3F, n=6, p<0.05), whereas the concentration of diazoxide causing 50% Ca<sup>2+</sup> release was 114±21 and 377±44 µM in SSM and IFM, respectively (Fig. 3G, n=6, p<0.05). Thus, SSM and IFM exhibit differential responsiveness toward diazoxide-mediated membrane depolarization, Ca<sup>2+</sup> uptake, and release of accumulated Ca<sup>2+</sup>.

#### Diazoxide restores Ca<sup>2+</sup>-inhibited ATP production

Excessive mitochondrial Ca<sup>2+</sup> 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 Ca<sup>2+</sup>-inhibited State 3 respiration was more prominent in SSM compared to IFM (Fig. 4) and was dependent upon the level of mitochondrial Ca<sup>2+</sup> overload (Fig. 4B and 4C). In mitochondria loaded with 150 nmol Ca<sup>2+</sup>/mg protein, diazoxide restored Ca<sup>2+</sup>-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 Ca<sup>2+</sup>/mg protein, the protective effect of diazoxide was only 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 Ca<sup>2+</sup>-inhibited ATP production in SSM than IFM. In mitochondria loaded with 150 nmol Ca<sup>2+</sup>/mg protein, diazoxide restored the rate of ATP production by 48±5% in SSM compared with 5±3% in IFM. Thus, diazoxide restores Ca<sup>2+</sup>-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 Ca<sup>2+</sup>-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 Ca<sup>2+</sup> overload and inhibition of oxidative phosphorylation when compared to IFM, in line with previous observations [7,10,11,16–18]. While Ca<sup>2+</sup>-mediated inhibition of oxidative phosphorylation was more prominent in SSM compared to IFM [8,10], diazoxide was more effective in restoring Ca<sup>2+</sup>-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 Ca<sup>2+</sup> ions in the physiological range of Ca<sup>2+</sup> concentrations [39,40]. However, excessive Ca<sup>2+</sup> 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 Ca<sup>2+</sup> loading is accompanied by partial restoration of Ca<sup>2+</sup>-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

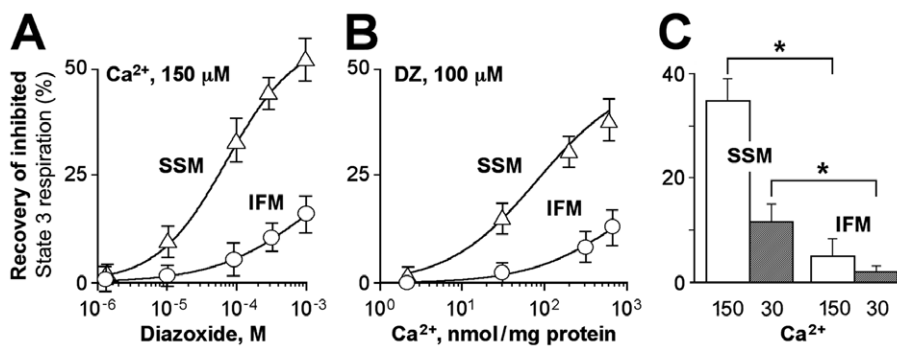


**Figure 3. Effect of diazoxide on the membrane potential and  $\text{Ca}^{2+}$  handling in subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria.** A, Depolarizing effect of diazoxide (100  $\mu\text{M}$ ) in SSM (open bar) and IFM (filled bar),  $n = 6$ ,  $p < 0.05$ . B, Mitochondrial  $\text{Ca}^{2+}$  uptake in SSM and IFM in the absence (-DZ) and presence (+DZ) of diazoxide (100  $\mu\text{M}$ ). C, Inhibition of the rate of  $\text{Ca}^{2+}$  uptake in SSM (open bar) and IFM (filled bar),  $n = 6$ ,  $p < 0.05$ . D, Diazoxide (100  $\mu\text{M}$ ) mediated  $\text{Ca}^{2+}$  release from preloaded SSM and IFM. E, Average rate of diazoxide-mediated  $\text{Ca}^{2+}$  release from mitochondria,  $n = 3$ ,  $p < 0.05$ . F and G, Dose-dependent effect of diazoxide on  $\text{Ca}^{2+}$  uptake (F) and  $\text{Ca}^{2+}$  release (G) in SSM (triangles) and IFM (circles),  $n = 6$ .

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(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  $\text{Ca}^{2+}$ -loaded mitochondria was greater in mitochondria with a higher level of  $\text{Ca}^{2+}$  load, indicating that the effect of diazoxide is condition-



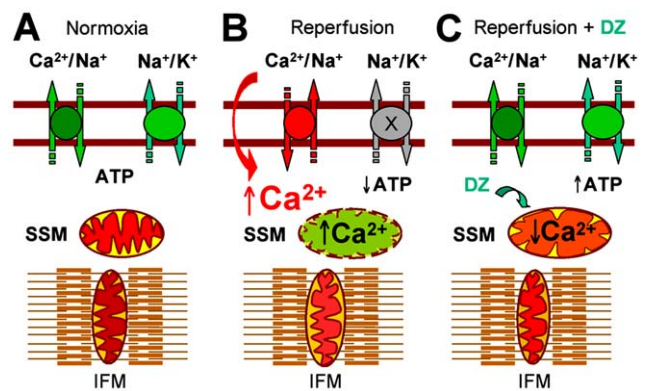
**Figure 4. Diazoxide-mediated recovery of  $\text{Ca}^{2+}$ -inhibited ATP synthesis.** A, Dose-dependent effect of diazoxide on restoration of  $\text{Ca}^{2+}$ -inhibited State 3 respiration in subsarcolemmal (SSM) (triangles) and interfibrillar (IFM) (circles) mitochondria. B, Effect of diazoxide on  $\text{Ca}^{2+}$ -inhibited State 3 respiration in SSM and IFM preloaded with 0–150 nmol  $\text{Ca}^{2+}$ /mg protein. C, Bar graphs of diazoxide (100  $\mu\text{M}$ )-mediated recovery of  $\text{Ca}^{2+}$ -inhibited State 3 respiration in SSM and IFM loaded with 150 (open bars) and 30 nmol  $\text{Ca}^{2+}$ /mg protein (hatched bars),  $n = 3$ . Asterisks signify statistical differences with  $p < 0.05$ .

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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  $\text{Ca}^{2+}$  loading, diazoxide had little effect on State 3 respiration in both populations, 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 protonophoric 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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  loading demonstrated in various cellular microdomains [54–58]. Our observation that SSM are more sensitive toward diazoxide-mediated protection from  $\text{Ca}^{2+}$  injury is consistent with reports on the higher vulnerability and reduced tolerance of this mitochondrial population toward  $\text{Ca}^{2+}$ -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 ionic pumps fail to maintain required gradients of  $\text{Na}^+$  and  $\text{K}^+$  ions across the sarcolemmal membrane, resulting in increased cytosolic  $\text{Ca}^{2+}$  (Fig. 5B). At reperfusion, oxygen availability quickly restores mitochondrial membrane potential and leads to excessive uptake of  $\text{Ca}^{2+}$  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 ionic 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  $\text{Ca}^{2+}$  overload by decreasing the rate of  $\text{Ca}^{2+}$  uptake or releasing accumulated  $\text{Ca}^{2+}$  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



**Figure 5. Schematic illustration of preferential targeting of subsarcolemmal mitochondria (SSM) by diazoxide.** A, Before ischemic insult (*Normoxia*), SSM and interfibrillar mitochondria (IFM) produce sufficient ATP to feed ionic pumps and maintain ionic homeostasis of cell. During ischemic period and decreased supply of substrates and oxygen, the mitochondrial ATP formation decreases and disables ionic pumps, resulting in increased cytosolic  $\text{Ca}^{2+}$ . B, At reperfusion (*Reperfusion*), restored supply of substrates and oxygen reenergizes the SSM, resulting in excessive  $\text{Ca}^{2+}$  uptake (from high  $\text{Ca}^{2+}$  cytosol) and inhibition of ATP production. C, In the presence of diazoxide during reperfusion (*Reperfusion + DZ*)  $\text{Ca}^{2+}$  uptake into SSM will be decreased, thus preserving mitochondrial ability to produce ATP, which is required for the activity of ionic pumps and restoration of normal cellular homeostasis.

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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  $\text{Ca}^{2+}$  uptake, reduction of mitochondrial  $\text{Ca}^{2+}$  loading and through release of excessively loaded  $\text{Ca}^{2+}$  and restoration of  $\text{Ca}^{2+}$ -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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