In the presence of 3 different stresses (hyperkalemic cardio-
plegia [CPG], hypo-osmotic stress, metabolic inhibition),
cardiac myocytes demonstrate significant cell swelling and
associated reduced contractility and may reflect a mechanism
of myocardial stunning.1–5 These structural and functional
derangements are prevented by ATP-sensitive potassium
(KATP) channel opener, diazoxide (DZX). 1–5 However, the
exact mechanism of action of DZX remains undefined. It is
widely believed that DZX works through a mechanism inde-
pendent of the sarcolemmal KATP channel but through the pur-
ported mitochondrial K ATP channel (mK ATP).6–9 One proposed
hypothesis for the cardioprotective mechanism of action by
DZX is through mKATP channel regulation of the mitochon-
drial matrix volume.10–12

Mitochondria are dynamic structures, and the volume of
myocardial cell occupied by the mitochondria is correlated
with the rate of energy use.13 Mitochondrial matrix volume
composes up to 35% of cellular volume and represents a large
potential space.13,14 Under resting conditions, mitochondrial
matrix is expanded with a narrow average intermembrane
distance between the inner and outer mitochondrial mem-
branes.14,15 Under stress conditions, mitochondrial matrix
contracts, increasing the intermembrane distance.16 This con-
traction can be reversed and returned to near-normal state
with the opening of mK ATP channel.17 The primary regulator
of mitochondrial matrix volume homeostasis is potassium (K+).
K+ influx into the mitochondria carries with it inorganic phos-
phates, hydrogen, and water.7,16 This is counterbalanced by K+
eflux via the K+/H+ antiporter. The maintenance of mitochon-
drial matrix volume homeostasis preserves vesicular integrity
in the face of high inner mitochondrial membrane traffic of
ions and water.

Mitochondria are vital to oxidative phosphorylation and
the electron transport chain. Most enzymes that participate
in oxidative phosphorylation are bound to the mitochon-
drial membrane in highly ordered structures that facilitate
enzyme–membrane and enzyme–enzyme interactions.13
Within the electron transport chain, the generation of ATP

Background—Cardiac myocytes demonstrate significant swelling and associated reduced contractility in response to
stress that is prevented by the ATP-sensitive potassium channel opener, diazoxide (DZX) via an unknown mechanism.
One proposed mechanism of cardioprotection is mitochondrial matrix swelling. To establish the relationship between
mitochondrial and cellular volume during stress, this study examined the effect of DZX on mitochondrial volume.

Methods and Results—Isolated mouse mitochondria were exposed to the following solutions: Tyrode, isolation buffer,
cardioplegia (CPG)±DZX±ATP-sensitive potassium channel inhibitor, 5-hydroxydecanoate, and metabolic inhibition
(MI)±DZX±5-hydroxydecanoate. Mitochondrial volume was measured. DZX resulted in significant mitochondrial
swelling (P<0.0001 versus Tyrode). MI and CPG resulted in significant mitochondrial swelling compared with baseline
volume. The addition of DZX did not alter the response of mitochondrial volume to CPG (P=0.912) but increased swelling
in response to MI (P=0.036). The addition of 5-hydroxydecanoate to MI+DZX or CPG+DZX significantly reduced
mitochondrial swelling (P<0.003 MI+DZX versus MI+DZX+5HD; P<0.001 CPG+DZX versus CPG+DZX+5HD).

Conclusions—Both cellular and mitochondrial volume increased during exposure to MI and CPG. DZX did not alter
mitochondrial volume during CPG; however, it was associated with an increase in mitochondrial volume during MI.
5-Hydroxydecanoate reduced mitochondrial volume during exposure to both stresses with DZX, supporting
a role for a mitochondrial ATP-sensitive potassium channel in the mechanism of cardioprotection by DZX.
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Key Words: cardioplegia ■ myocardial ischemia ■ myocardial stunning ■ myocytes, cardiac ■ potassium channels

From the Division of Cardiothoracic Surgery, Department of Surgery (M.M.A., E.M.K., C.M.M., A.D.K., R.B.S., J.S.L.) and Departments of Cell
Biology and Physiology (H.Z., C.G.N.), Washington University School of Medicine, St. Louis, MO.
Presented at the 2012 American Heart Association meeting in Los Angeles, CA, November 3–7, 2012.
Correspondence to Jennifer S. Lawton, MD, Washington University School of Medicine, 660 S. Euclid Ave, Campus Box 8234, St. Louis, MO 63110.
E-mail lawtonj@wustl.edu

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uses the creation of a hydrogen gradient across the intermembrane space. ATP generated is then exported into the cytosol via a series of complexes (ATP/ADP translocator, mitochondrial creatinine kinase, and voltage-dependent anion channel). Mitochondrial creatinine kinase bridges the space between the inner and outer mitochondrial membranes and is in close contact with ATP/ADP translocator and voltage-dependent anion channel, allowing for low-conductance transfer of high-energy phosphates. For this low-conductance transfer to occur, there must be a narrow intermembrane space distance.

In the setting of ischemia, there is decreased mitochondrial K⁺ uptake resulting in an imbalance between K⁺ influx and efflux, resulting in mitochondrial matrix contraction. This results in an increase in intermembrane space, resulting in increased distance among the intermembrane enzymes necessary for cellular function, thereby compromising efficient energy transfer.

An increase in mitochondrial matrix volume has been demonstrated after exposure to mK_{ATP} openers by multiple investigators. The primary function of mK_{ATP} channels is proposed to be regulation of mitochondrial volume. Open mK_{ATP} channels result in mitochondrial uptake of potassium and associated inorganic phosphates, anions, and water and cells achieve a 15% to 20% increase in steady state matrix volume in the mitochondria. This effect is inhibited by 5-hydroxydecanoate (5-HD), an inhibitor of the mK_{ATP} channel. The ratio of matrix water to cytosol water is approximately 1:4, and the influx of water into the mitochondria could cause depletion in cytosolic inorganic phosphates, leading to an increased rate of reactive oxygen species production, thereby benefiting the myocyte. Therefore, most changes in the mitochondrial volume (as a result of mK_{ATP} channel opening) are reflected in a reciprocal change in the intermembrane space volume. The spatial relationship of enzymes and membranes in the mitochondria is not only reflective of cellular activity but is also vital to cellular function.

The relationship between cellular and mitochondrial matrix volume regulation has not been previously investigated. We hypothesize that the mechanism of action of DZX involves the mK_{ATP} channel and may involve mitochondrial matrix volume expansion as a compensatory mechanism after total myocyte volume increase during exposure to stress. Thus, by using the huge potential space provided by the mitochondria, the cell maintains volume homeostasis. We have documented significant myocyte swelling after exposure to 3 stresses in 3 species and, therefore, propose that adding a K_{ATP} channel opener would be associated with a subsequent increase in mitochondrial volume. This study was designed to investigate the relationship between myocyte and mitochondrial volume in response to stress (hyperkalemic CPG and metabolic inhibition) with and without DZX and 5-HD.

### Methods

All animal procedures were approved by the Animal Studies Committee at Washington University School of Medicine, and all animals received humane care in compliance with the National Institute of Health’s Guide to Care and Use of Laboratory Animals.

### Mitochondrial Isolation

Mitochondria were isolated from hearts of C57BL/6J mice. Mice (both sexes, 6- to 16-weeks old, average 24.8 g) were anesthetized with 3% Avertin (0.3 g 2,2,2-tribromoethanol, 0.186 mL 2-methyl-2-butanol, 9.814 mL sterile water) intraperitoneally, and rapid carotid ligation was performed. Whole heart tissue was rapidly minced in cold buffer (in mmol/L: 10 HEPES (N-[2-hydroxyethyl] piperazine-N’-[4-butanesulfonic acid]), 1 EDTA-potassium, 250 sucrose, adjusted to a pH of 7.1 with 20% KOH and transferred to a 10-mL glass tube with a Teflon pestle (Glasc-Col Homogenizer, Terre Haute, IN), and volume was adjusted to 7 mL with buffer. The tissue was mechanically homogenized with a Teflon pestle driven by a low-speed motor drive shaft set at 120 rpms. The homogenate was transferred to 6 microcentrifuge tubes and centrifuged at 900g for 10 minutes at 4°C. The supernatant was combined into a clean test tube and mixed to get a homogeneous solution that was divided equally between 6 clean microcentrifuge tubes and centrifuged at 5000g for 15 minutes. One pellet was resuspended in 100 µL buffer, and 10 µL was taken in duplicate for the Bradford protein assay (Thermo Scientific; Rockford, IL) to determine total protein. Each pellet was maintained on ice and was resuspended in test media volume to equal 0.3 µg/µL.

### Experimental Protocol

Isolation buffer (IB; in mmol/L: 10 HEPES, 1 EDTA-potassium, and 250 sucrose, buffered to pH 7.1 with 20% KOH) was used as a control solution. Solutions of IB with 100 µmol/L DZX (Sigma, St. Louis, MO), 50 µmol/L Pinacidil (nonspecific K_{ATP} channel opener; Sigma, St. Louis, MO), and 100 µmol/L 5-HD (Sigma, St. Louis, MO) were used to evaluate individual effects on mitochondrial matrix volume. In addition, mitochondria were perfused with 37°C physiological Tyrode solution (in mmol/L: 130 NaCl, 5 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 24 NaHCO₃, 1.75 Na₂HPO₄, and 10 glucose) buffered to a pH of 7.4 using 95% O₂,5% CO₂ for 20 minutes. Tyrode was used as a control solution and more closely represents the extracellular environment. In addition, 100 µmol/L DZX was added to Tyrode to evaluate the effect of DZX alone on mitochondrial matrix volume.

Mitochondria were exposed to stress solutions, including hyperkalemic CPG and metabolic inhibition (MI) solution, because these have been previously used to induce cellular stress. CPG is composed of Plegisol (Abbott Laboratories, North Chicago, IL) that contains (in mmol/L) 110 NaCl, 16 KCl, 16 MgCl₂, 1.2 CaCl₂, equilibrated with 95% O₂ and 5% CO₂, and titrated to a pH of 7.3 with 8.4% NaHCO₃. MI consisted of (in mmol/L) 130 NaCl, 5 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 24 NaHCO₃, 1.75 Na₂HPO₄, 5 2-deoxyglucose, bubbled with 95% O₂,5% CO₂ and adjusted to a pH of 7.4, and 5 sodium cyanide. Sodium cyanide is prepared as a stock solution of 250 mmol/L in 670 mmol/L HEPES. Experimental groups included the following: IB, CPG, MI, Tyrode, Tyrode+DZX, CPG+DZX, CPG+DZX+5-HD, MI+DZX, MI+DZX+5-HD, IB+DZX, IB+Pinacidil, and IB+5-HD.

### Mitochondrial Matrix Volume Measurements

Mitochondrial matrix volume measurements were obtained using a light scattering technique, where the absorbance, at 520 nm, of a solution of isolated mitochondria was obtained every 2 minutes during a 20-minute time frame using UV Probe 2.33 (Shimadzu Scientific Instruments, Columbia, MD) and a spectrophotometer (UV-1700 Spectrophotometer; Shimadzu Scientific Instruments, Columbia, MD). This method is a well-established method for the estimation of mitochondrial matrix volume changes. Each reaction was plotted as a change in absorbance (y axis) versus time elapsed in minutes (x axis).

### Statistical Analysis

Data were analyzed using SYSTAT 13 (SYSTAT Software, Inc, Point Richmond, CA). All data are presented as mean±SEM, with n equal to the number of measurements in each group. A repeated-measures ANOVA with 1 factor design was used to compare values of change of absorbance over time (repeated measure) and between experimental
solutions (factor). Post hoc multiple comparisons between different test groups were made separately for each test solution using contrast with a Dunn–Sidak correction when overall P values were significant for ANOVA. P values are for the 20-minute time point for comparisons between experimental groups. P<0.05 was considered significant.

### Results

Changes in mitochondrial matrix volume are represented as percent change in absorbance over time. Mitochondrial matrix swelling is inversely related to absorbance measured at 520 nm. Mitochondrial volume changes are summarized with previous findings in Figure 1.1-5,9,22

Mitochondria exposed to extracellular Tyrode solution demonstrated an average 15% change in absorbance from baseline, resulting in mitochondrial matrix swelling (Figure 2). The addition of DZX to Tyrode solution resulted in an 18% change in absorbance and increased matrix swelling compared with Tyrode alone (P<0.001).

Mitochondria exposed to IB demonstrated small changes in absorbance (average 4% change from baseline), representing nonsignificant changes in mitochondrial volume (Figure 2). The addition of DZX or Pinacidil produced a nonsignificant change in mitochondrial volume (P=0.077 and P=0.058, respectively, versus IB). There was significantly greater mitochondrial swelling in IB+DZX compared with IB+ Pinacidil (P<0.001). The addition of 5-HD resulted in an increase in matrix volume compared with IB alone (P=0.016) and IB+Pinacidil (P<0.001). There was no significant difference between IB+DZX and IB+5-HD (P=0.085).

Mitochondria exposed to CPG resulted in an increase in mitochondrial volume (average percent change in absorbance of 17.2% from baseline; Figure 3). The addition of DZX did not result in a significant change in mitochondrial volume compared with CPG alone (P=0.912). The addition of 5-HD to CPG+DZX, however, did result in a significant decrease in mitochondrial matrix swelling compared with CPG alone (P<0.001) and CPG+DZX (P<0.001).

All CPG groups (CPG, CPG+DZX, and CPG+DZX+5-HD) resulted in significant mitochondrial swelling compared with IB (P<0.001) and less swelling compared with Tyrode+DZX (P<0.0001), but no significant difference versus Tyrode alone (P=0.275; Figures 2 and 3).

Mitochondria exposed to MI resulted in an increase in mitochondrial volume, with an average percent change of 21% from baseline (Figure 4). The addition of DZX did result in a statistically significant increase in mitochondrial volume compared with MI alone (P=0.036). The addition of 5HD to MI+DZX resulted in a statistically significant decrease in matrix volume compared with MI+DZX (P=0.003) and MI alone (P=0.026). All MI groups (MI, MI+DZX, MI+DZX+5-HD) demonstrated a statistically significant increase in mitochondrial matrix volume compared with IB (P<0.001) and Tyrode alone (P<0.001). Only the group MI+DZX resulted in significantly

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**Figure 1.** Cellular and mitochondrial volume derangements as a result of stress (cardioplegia [CPG], metabolic inhibition [MI]) and the effect of diazoxide (DZX) and 5-hydroxydecanoate (5-HD).1-5,9,22 Up arrow indicates increase from baseline, down arrow indicates decrease from baseline, and flat arrow indicates return to baseline. *Divergence from myocyte volume effect. h indicates human; m, mouse; and r, rabbit.

**Figure 2.** The effect of diazoxide (DZX) and pinacidil on mitochondrial volume. The addition of DZX to Tyrode resulted in a significant increase in mitochondrial volume vs Tyrode alone. DZX did not result in a significant change in mitochondrial volume when added to isolation buffer (IB). However, the addition of DZX to IB resulted in greater mitochondrial volume swelling compared with the addition of Pinacidil. 5-HD indicates 5-hydroxydecanoate.
more mitochondrial volume swelling compared with the Tyrode+DZX group (P<0.001).

**Discussion**

We have documented derangements in myocyte volume and contractility secondary to exposure to stress (exposure to hyperkalemic CPG, osmotic stress, and MI), as well as the ability to ameliorate these derangements with the addition of K\textsubscript{ATP} channel opener DZX.\textsuperscript{1–5} These beneficial effects require the sulfonylurea receptor subunit 1 (SUR1) subunit of a proposed mK\textsubscript{ATP} channel,\textsuperscript{9} and recent work suggests that the Kir pore-forming subunit may be the renal outer medullary K\textsuperscript{+} inward-rectifying K\textsuperscript{+} channel.\textsuperscript{8} A proposed mechanism of cardioprotection after opening of a purported mK\textsubscript{ATP} channel is that of mitochondrial volume expansion.

The ability of mitochondria to compensate for volume change provides the cell with a potential reservoir or alternate location for handling of excess water. The mitochondria are known to make up ≈29% to 36% of cardiac myocyte volume,\textsuperscript{26} and a large volume change at the mitochondrial level may be sufficient to result in a change in total cellular volume. The relationship between myocyte and mitochondrial volume is unknown. Other investigators have proposed relationships between the 2 volumes and have suggested that mitochondrial swelling can comprise a significant portion of cell volume change and that the mitochondrial fraction increases to 40% of cytosolic volume in swollen cells.\textsuperscript{13,24} Ganote proposed 3 methods of how mitochondrial and cellular volume are related, noted an increase in both mitochondrial and cellular volume after ischemia, and documented that a reduction in either cytosolic or mitochondrial swelling would mediate decreased cell death.\textsuperscript{24}

The present study evaluated the relationship between cellular and mitochondrial volume in response to stress. Mitochondrial matrix volume remained unchanged during exposure to IB. The addition of DZX and Pinacidil to IB did not result in a significant change in mitochondrial matrix volume compared with IB alone. Physiological Tyrode has been used previously as a control solution in myocyte volume experiments and demonstrated no significant change in myocyte volume.\textsuperscript{1,2,4,5,22} Exposure of mitochondria to Tyrode resulted in significant mitochondrial volume swelling. The addition of DZX to Tyrode resulted in a significant increase in mitochondrial volume. Other investigators have documented significant mitochondrial volume expansion with exposure of K\textsubscript{ATP} channel openers and have used mitochondrial volume change as an mK\textsubscript{ATP} activity assay.\textsuperscript{7,8,19}

Tyrode likely resulted in tremendous mitochondrial swelling because it is a physiological extracellular and not an intracellular solution. The addition of DZX to both control solutions (Tyrode and IB) did not result in a significant change in mitochondrial matrix volume compared with IB alone. Physiological Tyrode has been used previously as a control solution in myocyte volume experiments and demonstrated no significant change in myocyte volume.\textsuperscript{1,2,4,5,22} Exposure of mitochondria to Tyrode resulted in significant mitochondrial volume swelling. The addition of DZX to Tyrode resulted in a significant increase in mitochondrial volume. Other investigators have documented significant mitochondrial volume expansion with exposure of K\textsubscript{ATP} channel openers and have used mitochondrial volume change as an mK\textsubscript{ATP} activity assay.\textsuperscript{7,8,19}

Figure 3. The effect of hyperkalemic cardioplegia on mitochondrial volume. Cardioplegia (CPG) resulted in an increase in mitochondrial volume. The addition of diazoxide (DZX) did not result in a significant change in mitochondrial volume. However, the addition of 5-hydroxydecanoate (5-HD) to CPG+DZX resulted in a significant decrease in mitochondrial volume swelling compared with CPG alone.

Figure 4. The effect of metabolic inhibition on mitochondrial volume. Metabolic inhibition (MI) resulted in an increase in mitochondrial volume. The addition of diazoxide (DZX) resulted in an additional significant increase in mitochondrial volume. The addition of 5-hydroxydecanoate (5-HD) to MI+DZX resulted in a significant decrease in mitochondrial volume compared with MI alone and MI+DZX.
significantly increase, which supports a hypothesis of a relationship between cellular volume homeostasis and mitochondrial volume.

The volume increase noted with MI was increased by the addition of DZX and decreased by the addition of 5-HD. In contrast, the mitochondrial volume increase observed with CPG was not increased by the addition of DZX; however, it was decreased by the addition of 5-HD. The differences observed in mitochondrial volume change in the present study may be as a result of the differences in the stress imposed on the mitochondria. CPG results in a hypo-osmotic extra-cellular potassium environment and depolarizes the cellular membrane; however, on exposure to isolated mitochondria it provides a lower potassium (16 mmol/L KCl) environment compared with the normal intracellular milieu (104–180 mmol/L potassium). MI results in a hyperosmotic intracellular environment, inhibits oxidative phosphorylation and electron transport, and provides a much lower environment for isolated mitochondria (5 mmol/L KCl). These solutions may, therefore, illicit different responses at the mitochondrial level.

The observed effects of 5-HD (decreased swelling) suggest that the mechanism behind mitochondrial matrix volume regulation involves a purported mK$_{ATP}$ channel. Interestingly, previous work evaluating 5-HD at the cellular level did not alter observed myocyte swelling because of stress or its amelioration by DZX. These findings lend support to a mitochondrial location of action for DZX.

Ideally, the cardioprotection provided by DZX could be exploited during ischemic stress (compounded by the stress of exposure to hyperkalemic CPG) imposed on the myocardium during cardiac surgery. We have shown that in the in vivo setting, the observed cardioprotection (maintenance of volume homeostasis and preserved contractility) requires K$_{ATP}$ channel subunit SUR1 and involves the inhibition of succinate dehydrogenase. The present study is consistent with a location of action at the mitochondrial level. The clarification of the exact mechanism of DZX will significantly aid in its acceptability and translation in the clinical setting.

Limitations

It is unknown whether observed responses of isolated mitochondria to stress are similar to the responses of mitochondria within intact cells when exposed to the same stress. Ideally, mitochondria would be observed after exposing isolated cells to stress to correlate observations. Unfortunately, adequate amounts of viable mitochondria cannot be obtained using this sequence.

Although the light scattering technique is the most commonly used technique for evaluating mitochondrial matrix volume changes, there is debate over its accuracy and reproducibility. Other investigators have reported changes in mitochondrial volume during a 3-minute period, and the present study encompasses ≤20 minutes, which may be responsible for the different observations.

Conclusions

The complex relationship between cellular and mitochondrial volume regulation is unknown. Both MI and CPG result in myocyte and mitochondrial volume swelling. There is divergence in the effect of DZX on myocyte and mitochondrial volume during stress. On a cellular level, DZX exerts a cardioprotective effect on both MI- and CPG-exposed myocytes by providing volume homeostasis. However, at the subcellular level, DZX did not exert an additional effect on mitochondria exposed to CPG. The addition of 5-HD resulted in a decrease in observed mitochondrial volume, supporting involvement of a purported mK$_{ATP}$ channel in mitochondrial volume regulation.

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Disclosures

None.

References

Relationship Between Mitochondrial Matrix Volume and Cellular Volume in Response to Stress and the Role of ATP-Sensitive Potassium Channel
Melissa M. Anastacio, Evelyn M. Kanter, Carol M. Makepeace, Angela D. Keith, Haixia Zhang, Richard B. Schuessler, Colin G. Nichols and Jennifer S. Lawton

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