

# Cardioprotective mechanisms of mitochondria-targeted S-nitrosating agent and adenosine triphosphate-sensitive potassium channel opener are mutually exclusive



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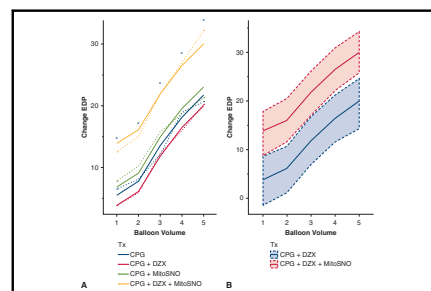
## ABSTRACT

**Background:** Myocytes exposed to stress exhibit significant swelling and reduced contractility. These consequences are ameliorated by adenosine triphosphate-sensitive potassium ( $K_{ATP}$ ) channel opener diazoxide (DZX) via an unknown mechanism.  $K_{ATP}$  channel openers also provide cardioprotection in multiple animal models. Nitric oxide donors are similarly cardioprotective, and their combination with  $K_{ATP}$  activation may provide synergistic benefit. We hypothesized that mitochondria-targeted S-nitrosating agent (MitoSNO) would provide synergistic cardioprotection with DZX.

**Methods:** Myocyte volume and contractility were compared following Tyrode's physiologic solution (20 minutes) and stress (hyperkalemic cardioplegia [CPG]  $\pm$  DZX;  $n = 5$ -20 each; 20 minutes) with or without MitoSNO ( $n = 5$ -11 each) at the end of stress, followed by Tyrode's solution (20 minutes). Isolated mouse hearts received CPG  $\pm$  DZX ( $n = 8$ -10 each) before global ischemia (90 minutes) with or without MitoSNO ( $n = 8$  each) at the end of ischemia, followed by reperfusion (30 minutes). Left ventricular (LV) pressures were compared using a linear mixed model to assess the impact of treatment on the outcome, adjusting for baseline and balloon volume.

**Results:** Stress (CPG) was associated with reduced myocyte contractility that was prevented by DZX and MitoSNO individually; however, their combination was associated with loss of cardioprotection. Similarly, DZX and MitoSNO improved LV function after prolonged ischemia compared with CPG alone, and cardioprotection was lost with their combination.

**Conclusions:** MitoSNO and DZX provide cardioprotection that is lost with their combination, suggesting mutually exclusive mechanisms of action. The lack of a synergistic beneficial effect informs the current knowledge of the cardioprotective mechanisms of DZX and will aid planning of future clinical trials. (JTCVS Open 2021;8:338-54)



Individually, but not in combination, diazoxide and mitochondrial S-nitrosating agent improve cardiac function following ischemia.

## CENTRAL MESSAGE

MitoSNO and diazoxide individually provide cardioprotection; however, their use in combination is detrimental. These findings inform the current knowledge of the cardioprotective mechanisms of diazoxide.

## PERSPECTIVE

Diazoxide (DZX) with hyperkalemic cardioplegia is cardioprotective via an unknown molecular mechanism of action; however, DZX combined with cardioprotective mitochondrial S-nitrosating agent is detrimental. These results clarify the mechanisms and conditions necessary for DZX cardioprotection. Elucidation of DZX's mechanism of action will allow for its safe use in clinical trials in humans.

See Commentaries on pages 355 and 357.

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Dr Ahmad and Jie Wang contributed equally to this work and should be considered co-first authors.

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**Abbreviations and Acronyms**

|                  |                                               |
|------------------|-----------------------------------------------|
| CPG              | = cardioplegia                                |
| DZX              | = diazoxide                                   |
| EDP              | = end-diastolic pressure                      |
| K <sub>ATP</sub> | = adenosine triphosphate-sensitive potassium  |
| KHB              | = Krebs–Henseleit buffer                      |
| LV               | = left ventricular                            |
| LVDP             | = left ventricular developed pressure         |
| MitoSNO          | = mitochondrial-selective S-nitrosating agent |
| NO               | = nitric oxide                                |
| ROS              | = reactive oxygen species                     |
| SDH              | = succinate dehydrogenase                     |
| SUR              | = sulfonylurea                                |

 Video clip is available online.

In a unique in vitro model of myocardial stunning, myocytes exposed to stress (hypo-osmotic, exposure to hyperkalemic cardioplegia [CPG], or metabolic inhibition) have significant swelling and subsequent reduced contractility that is prevented by the adenosine triphosphate (ATP)-sensitive potassium (K<sub>ATP</sub>) channel opener diazoxide (DZX; 7-chloro-3-methyl-2H-1,2,4-benzothiadiazine 1,1-dioxide [C<sub>8</sub>H<sub>7</sub>ClN<sub>2</sub>O<sub>2</sub>S]; molecular weight 230.7 g/M).<sup>1-4</sup> K<sub>ATP</sub> channels uniquely provide endogenous myocardial protection via coupling of cell membrane potential to myocardial metabolism, are inhibited by ATP, and open during times of metabolic stress.<sup>5</sup> Pharmacologic opening of K<sub>ATP</sub> channels mimics ischemic preconditioning in multiple animal models.<sup>6-11</sup> DZX was found to preserve diastolic function in a murine model of prolonged global ischemia and preserves systolic and diastolic function in a large animal model of prolonged cardioplegic arrest.<sup>11,12</sup> The molecular mechanism of this cardioprotection is unknown, but we have suggested that it involves a mitochondrial location<sup>13</sup> and requires the inhibition of succinate dehydrogenase (SDH)<sup>14</sup> and the K<sub>ATP</sub> channel sulfonylurea subunit 1 (SUR1).<sup>15</sup> Clarification of DZX's cardioprotective mechanism of action is vital to its future use in clinical trials as a component of hyperkalemic CPG.

Others have suggested that K<sub>ATP</sub> activation combined with nitric oxide (NO) donation (via protein kinase C activation) provides synergistic cardioprotection,<sup>16</sup> or that NO generated during ischemia triggers the opening of mitochondrial K<sub>ATP</sub> channels.<sup>17</sup> Murphy and colleagues<sup>18</sup> demonstrated that mitochondria-selective S-nitrosating

agent (MitoSNO) reduces reactive oxygen species (ROS) production and infarct size in a mouse model of myocardial infarction via reversible inhibition of mitochondrial complex I.<sup>18</sup>

Both DZX and MitoSNO have proposed cardioprotective mechanisms involving a mitochondrial location, alterations in oxidative phosphorylation, and activity involving succinate accumulation or complex II (SDH). The exploration of their potential synergistic effects would add to the current to knowledge regarding DZX's mechanism of action. We hypothesized that MitoSNO would provide synergistic cardioprotection when used with the K<sub>ATP</sub> channel opener DZX. The aim of this study was to evaluate the effect of MitoSNO with DZX on myocyte contractility following stress and on myocardial function following prolonged global ischemia, and to gain mechanistic knowledge regarding the cardioprotective mechanism of DZX.

**METHODS**

All animal procedures were approved by the Animal Care and Use Committee at Johns Hopkins University. Animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals.<sup>19</sup> Although both male and female animals were used in all experiments, subgroup analyses of results by sex were not performed, owing to the lack of power from the small number of animals in each group.

**Mouse Myocyte Stress Model**

**Myocyte isolation.** Mice (either sex; C57BL/6J) underwent rapid cardiectomy with aortic cannulation for extracellular tissue digestion and cellular isolation using solutions A and B (detailed below), as described previously.<sup>13</sup> The left ventricle was transferred into solution C (detailed below) and gently dispersed. The cells were allowed to centrifuge by gravity, and serial washings were performed. The typical yield of viable myocytes was 50% to 80% per mouse. Cells were selected for viability using the following criteria: normal rod shape, smooth edges, sharp borders, clear striations, absence of vacuoles or blebbing, and lack of spontaneous beating. Up to 3 myocytes were used per isolation. Myocytes were used in experiments within 4 hours. Investigators were blinded to test group during each experiment and during data analysis.

Solution A consisted of 116 mmol/L NaCl, 5.36 mmol/L KCl, 0.97 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 21.10 mmol/L Hepes (N-[2-hydroxyethyl]-piperazine-N'-[4-butanedisulfonic acid]), 11.65 mmol/L glucose; 26.50 μmol/L phenol red (Sigma-Aldrich, St Louis, Mo), 3.72 mmol/L MgCl<sub>2</sub>, 4.40 mmol/L NaHCO<sub>3</sub>, essential vitamins (100×, 10 mL; Gibco; Grand Island, NY), and amino acids (50×, 20 mL; Gibco). Solution B consisted of solution A plus 10 μM CaCl<sub>2</sub> and 1.2 mg/mL collagenase (type 2; Worthington Biochemical; Freehold, NJ). Solution C consisted of solution A plus 5 mg/mL bovine serum albumin (Sigma-Aldrich), 1.25 mg/mL taurine, and 150 μM CaCl<sub>2</sub>.

DZX (Sigma-Aldrich) was used at a dose of 100 μmol/L based on previous studies.<sup>1-3</sup> MitoSNO was a generous gift from Dr Michael Murphy, University of Cambridge. A dose-response assessment of MitoSNO was performed (0.1–10 μmol/L). Stock solutions of DZX and MitoSNO were made with 0.1% dimethyl sulfoxide, a concentration that has no effect on cell volume.<sup>20</sup> Hyperkalemic CPG solution (Plegisol; Pfizer, New York, NY) consisted of 110 mmol/L NaCl, 10 mmol/L NaHCO<sub>3</sub>, 16 mmol/L KCl, 32 mmol/L Mg, and 2.4 mmol/L CaCl<sub>2</sub>, titrated to pH 7.8 with 8.4% NaHCO<sub>3</sub> solution.

**Experimental protocol.** Myocytes were perfused with 37°C Tyrode's solution (130 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L CaCl<sub>2</sub>, 1.2 mmol/L MgSO<sub>4</sub>, 24 mmol/L NaHCO<sub>3</sub>, 1.75 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, and 10 mmol/L glucose), buffered to a pH of 7.4 for baseline measurements (Figure 1). Myocytes were then perfused for 20 minutes with test solution (n = 5-20 each): Tyrode's solution, 9°C CPG solution, or 9°C CPG + 100  $\mu$ M DZX. MitoSNO (1.0  $\mu$ M or 10  $\mu$ M) was administered at the beginning of reperfusion (n = 5-11 for each). Myocytes were then reexposed to 37°C Tyrode's solution for 20 minutes.

DZX was administered before stress because it has been demonstrated to be cardioprotective only when administered prior to stress or metabolic inhibition.<sup>21</sup> Similarly, Murphy and colleagues<sup>22</sup> determined that MitoSNO is cardioprotective only when given at reperfusion. Therefore, each was administered at the optimal time during the experiments.

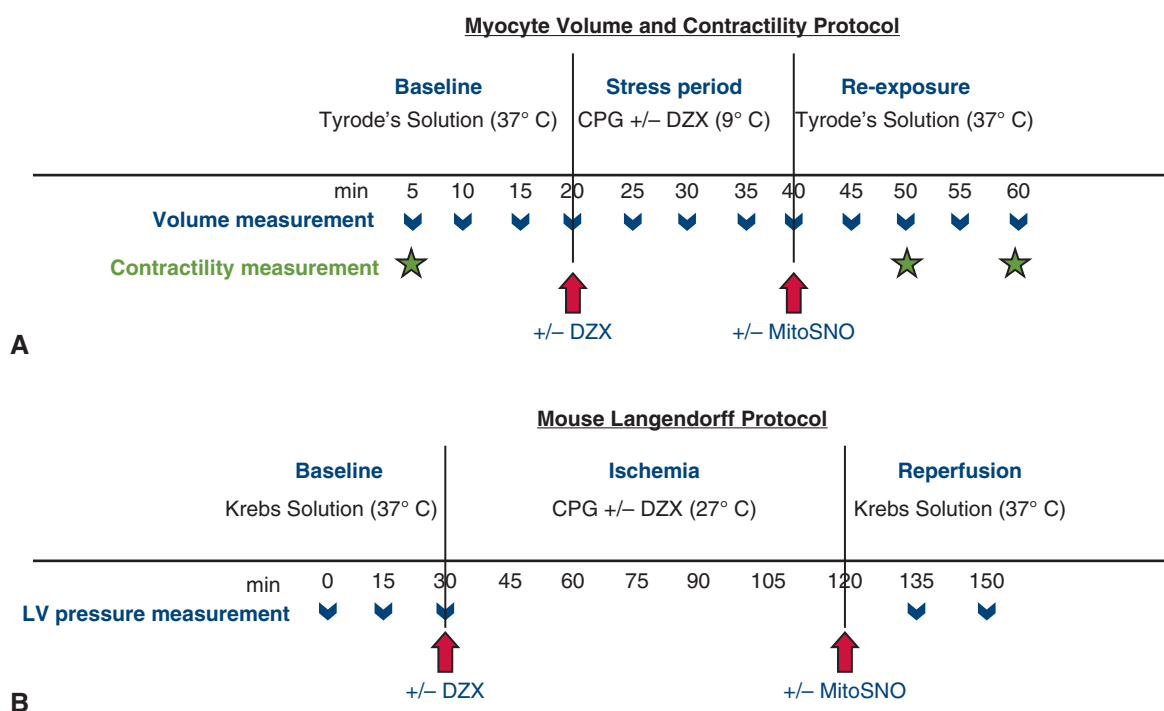
**Myocyte volume measurement.** Myocyte volume was measured as described previously.<sup>1</sup> Isolated myocytes were placed on an inverted microscope stage (model IX51; Olympus, Tokyo, Japan). After a 5-minute stabilization, the chamber was perfused (3 mL/min). Chamber temperature was controlled by a water bath system (37°C; HAAKE; Thermo Electron Karlsruhe, Karlsruhe, Germany or 9°C; Polystat; Cole-Parmer, Vernon Hills, Ill). Cell images were displayed on a video monitor using a charge-coupled device camera (IonOptix, Westwood, Mass). Digital images of viable cells were captured using a video frame grabber (Scion, Frederick, Md) every 5 minutes (Figure 1). Relative cell volume change was determined as described previously.<sup>1</sup>

**Myocyte contractility.** Myocyte contractility was measured using a video-based edge detection system (IonOptix) as described previously (Video 1).<sup>4,13</sup> Cells were paced using a field stimulator (MyoPacer; IonOptix), and data were recorded at baseline and at 10 minutes and 20 minutes after reexposure to Tyrode's solution (Figure 1). The following variables were computed using edge-detection software (IonOptix): percentage shortening, peak velocity of shortening, and peak velocity of relengthening. Myocytes that showed <7% cell shortening during baseline were excluded.

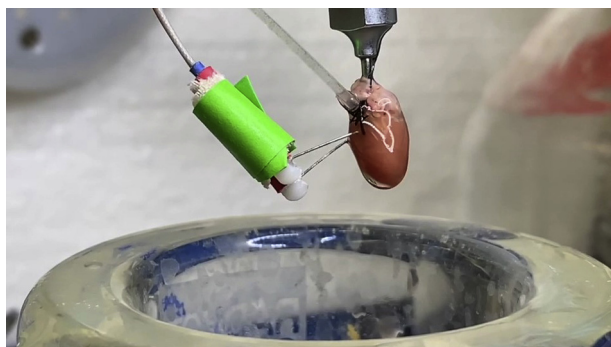
**Statistical analyses.** Continuous variables are presented as mean  $\pm$  SD. Hypothesis testing was conducted with one-way analysis of variance. *P* values of pairwise comparisons were adjusted using the Benjamini–Hochberg procedure. Statistical significance was defined as *P* < .05 (2-sided). Statistical analyses were done using R version 3.5.2 (R Core Team, Vienna, Austria), and illustrations were created using Prism version 8.0.2 (GraphPad Software, San Diego, Calif). Justification for the statistical analyses and additional alternative supplemental statistical analyses is provided in the [Online Data Supplement](#).

### Isolated Mouse Heart Model (Langendorff) of Global Ischemia

**Animals and anesthesia.** Mice (either sex; C57BL/6J) were anesthetized and underwent rapid cardiectomy as described previously (Video 2).<sup>11</sup> The heart was submersed in ice-cold Krebs–Henseleit buffer



**FIGURE 1.** Isolated myocyte and Langendorff isolated heart experimental protocols. A, Isolated mouse myocytes were exposed to 37°C control Tyrode's physiologic solution for 20 minutes (baseline), followed by a stress period (at 20 minutes; red arrow) with test solution (hypothermic hyperkalemic cardioplegia [CPG] with or without the adenosine triphosphate–sensitive potassium [K<sub>ATP</sub>] channel opener diazoxide [DZX]) for 20 minutes, followed by a 20-minute reexposure to Tyrode's solution. Mitochondria-targeted S-nitrosating agent (MitoSNO) was infused at the end of the stress period (at 40 minutes; red arrow). Volume measurements were obtained every 5 minutes (blue symbols). Contractility measurements were obtained at 5 minutes (baseline), 10 minutes, and 20 minutes after reexposure to Tyrode's solution (time, 50 and 60 minutes; green stars). B, Isolated mouse hearts underwent Langendorff baseline perfusion with Krebs–Henseleit buffer (KHB) solution for 30 minutes, followed by arrest (at 30 minutes; red arrow) with test solution (hypothermic hyperkalemic CPG with or without K<sub>ATP</sub> channel opener DZX) and 90 minutes of global ischemia, followed by infusion of MitoSNO 1  $\mu$ M or KHB (at 120 minutes; red arrow), then reperfusion with KHB for 30 minutes. Left ventricular pressure was recorded at baseline and at 15 minutes and 30 minutes following reperfusion at identical balloon volumes (blue symbols). LV, Left ventricular.

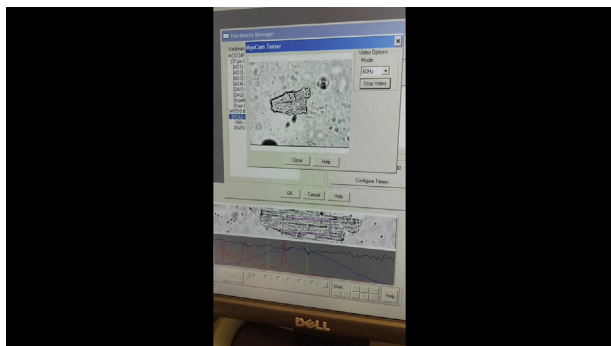


**VIDEO 1.** An isolated myocyte with field stimulation demonstrating myocyte contractility projected via a camera from an inverted microscope in our laboratory. Video available at: [https://www.jtcvs.org/article/S2666-2736\(21\)00229-1/fulltext](https://www.jtcvs.org/article/S2666-2736(21)00229-1/fulltext).

(KHB) during aortic cannulation. Hearts were perfused in a Langendorff fashion (retrograde via the aortic root) with KHB (118.5 mmol/L NaCl, 25.0 mmol/L NaHCO<sub>3</sub>, 3.2 mmol/L KCl, 1.2 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 1.2 mmol/L MgSO<sub>4</sub>, 1.2 CaCl<sub>2</sub>, and 35.0 mmol/L D-glucose). Hearts were excluded if aortic cannulation time exceeded 5 minutes. A balloon was introduced into the left ventricle. Hearts were epicardially paced at 450 bpm and surrounded by a warm (37°C) water-jacketed beaker. Coronary perfusion pressure was maintained at 80 mm Hg (the zone of autoregulation for coronary vasculature in murine hearts).

**Experimental protocol.** The left ventricular (LV) balloon was connected to a pressure transducer (HP1290C; ADInstruments, Colorado Springs, Colo) and an amplifier (20-4; Hugo Sachs Elektronik–Harvard Apparatus, March, Germany). Balloon volume was adjusted to an end-diastolic pressure (EDP) of 2.5 mm Hg. During a 30-minute baseline period, LV pressure was measured at increasing 1.4-μL balloon volume increments for baseline data collection. The LV balloon volume was then returned to the initial volume, and the heart was arrested for 90 minutes using test solution.

Randomly assigned test solutions included hyperkalemic CPG (Plegisol) (110 mmol/L NaCl, 10 mmol/L NaHCO<sub>3</sub>, 16 mmol/L KCl, 32 mmol/L Mg, and 2.4 mmol/L CaCl<sub>2</sub>, titrated to pH 7.8 with 8.4% NaHCO<sub>3</sub> solution) or CPG + DZX 100 μmol/L (Sigma-Aldrich). The DZX dose was chosen based on previous experiments.<sup>11</sup> After 90 minutes of global ischemia, hearts were assigned at random to receive MitoSNO 1 μmol/L (n = 8 with CPG, n = 10 with CPG + DZX) or KHB (n = 8



**VIDEO 2.** Technique for the mouse Langendorff model. Natalie Gaughan and Rosmi Thomas describe the technique of performing a mouse Langendorff model for the assessment of myocardial function in Dr Lawton's laboratory. Video available at: [https://www.jtcvs.org/article/S2666-2736\(21\)00229-1/fulltext](https://www.jtcvs.org/article/S2666-2736(21)00229-1/fulltext).

for each). The MitoSNO dose was chosen based on the dose response in isolated myocytes. KHB retrograde perfusion and epicardial pacing were then resumed. After 30 minutes, LV pressures were recorded at the same balloon volumes as at baseline. Investigators were blinded to test group during each experiment and during data analysis.

DZX was administered before stress because it has been demonstrated to be cardioprotective only when administered before stress or metabolic inhibition.<sup>21</sup> Similarly, Murphy and colleagues<sup>22</sup> identified that MitoSNO is cardioprotective only when given at reperfusion.<sup>22</sup> Therefore, each was administered at the optimal time during the experiments.

**Data acquisition and analysis.** LV end-systolic pressure, LV EDP, and LV developed pressure (LVDP) were determined from digitalized data files using LabVIEW 2014 (National Instruments, Austin, Tex). LVDP was defined as the difference between end-systolic pressure and EDP for each data point. Ventricular function and compliance were assessed through changes in LVDP and EDP, respectively, over a series of identical intracavitary balloon volumes. Fewer numbers at the 60-minute reperfusion time point indicates myocyte loss before 60 minutes.

Coronary flow rates were measured every 5 minutes by an inline N-series flow probe and a T206 flow meter (Transonic Systems, Ithaca, NY) and compared as described previously.<sup>11</sup>

**Statistical analysis.** A linear mixed model was used to obtain correct standard errors for estimates of treatment effects in the presence of subject-level correlation among measurements of LVDP or EDP (Online Data Supplement). Justification for statistical analyses used and additional alternative supplemental statistical analyses are provided in the Online Data Supplement.

## RESULTS

### Myocyte Volume

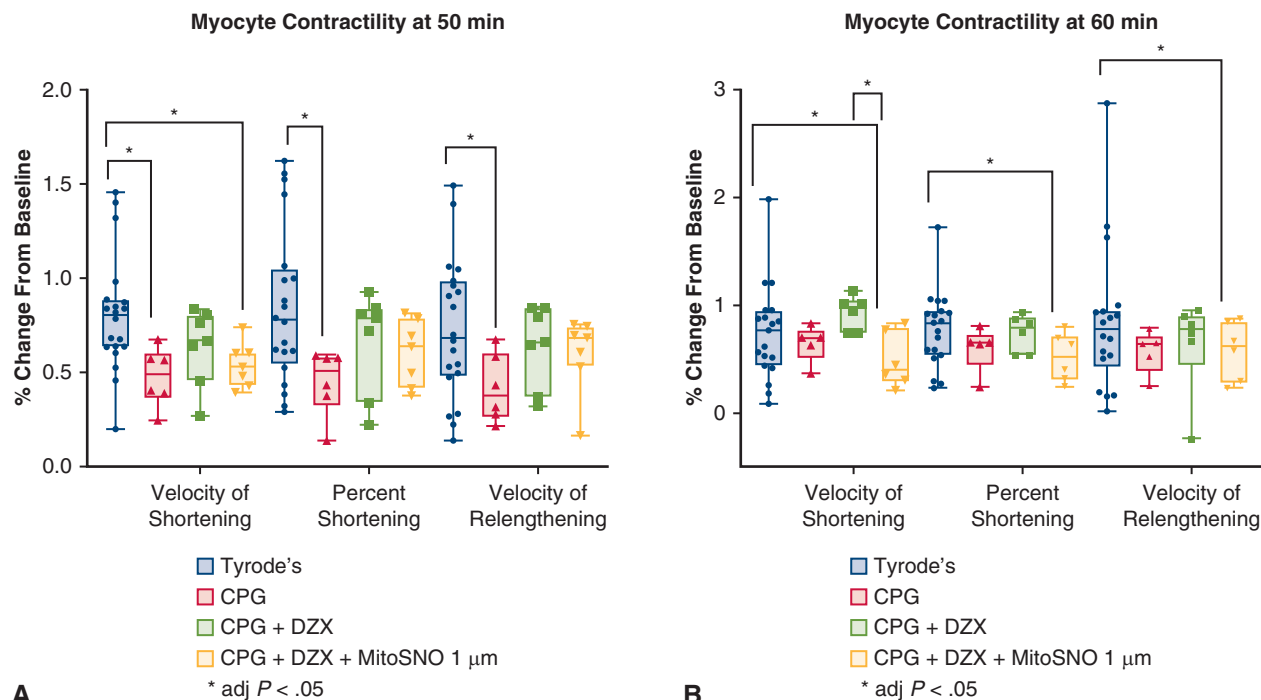
MitoSNO was administered at 40 minutes (end of stress) (Figure 1); therefore, comparisons of myocyte volume during baseline (time 0-20 minutes) and stress (time 20-40 minutes) were not relevant to the administration of MitoSNO. Administration of MitoSNO (1 μM) at 40 minutes was associated with a decrease in myocyte volume in Tyrode's group (vs Tyrode's solution alone) and in the hyperkalemic CPG group (vs CPG alone) ( $P < .05$  for both); however, no significant change was noted in the CPG + DZX group (Figures E1-E3).

Similar to previous work, exposure to CPG resulted in cell swelling during stress (time 20-40 minutes) (Figure E2), which was reduced by the addition of DZX (Figure E3).<sup>2</sup>

### Myocyte Contractility

**MitoSNO is cardioprotective.** MitoSNO alone (1 μM or 10 μM) increased contractility in myocytes exposed to either Tyrode's solution or CPG (Figure E4). The most significant improvement in contractility (2-fold increase) was observed in cells treated with Tyrode's solution plus MitoSNO 1 μM at 50 minutes (Figure E4, A). This improved contractility was less pronounced at 60 minutes (Figure E4, B). MitoSNO also increased contractility in myocytes exposed to CPG at 50 minutes (Figure E4, C); however, this difference was not statistically significant by 60 minutes (Figure E4, D).

**DZX is cardioprotective.** Similar to previous work, myocytes exposed to CPG during stress had reduced



**FIGURE 2.** Hyperkalemic cardioplegia (CPG) exposure results in reduced myocyte contractility that is prevented by the adenosine triphosphate-sensitive potassium ( $K_{ATP}$ ) channel opener diazoxide (DZX), and this protection is lost with the addition of mitochondrial-targeted S-nitrosating agent (MitoSNO). Myocytes were perfused with control 37°C Tyrode's solution (time 0-20 minutes), test solution (Tyrode's, CPG, or CPG + DZX;  $n = 5-20$  each group; time 20-40 minutes; stress), followed by administration of MitoSNO (1  $\mu$ M) or saline (at 40 minutes) and then 37°C Tyrode's solution (time 40-60 minutes). Contractility (velocity of shortening, percent shortening, or velocity of relengthening) was measured after 50 minutes (A) and 60 minutes (B) of reperfusion with Tyrode's solution. The middle horizontal line in the box represents the median  $\pm$  SD percent change from baseline. The upper and lower whiskers represent the maximum and minimum values of nonoutliers, and each data point is represented as a dot. \*Adjusted  $P < .05$ .

contractility compared with those exposed to Tyrode's solution (Figure 2), which was improved with the addition of DZX (which is not different from control Tyrode's solution).<sup>2</sup> This observed benefit with DZX was lost with the addition of MitoSNO (which is significantly worse than control Tyrode's solution).

Reduced contractility was also noted in the CPG + MitoSNO group compared with the Tyrode's + MitoSNO group (adjusted  $P < .05$ ; Figure 3). This reduction was more profound when DZX was added to the CPG + MitoSNO group and compared with the Tyrode's + MitoSNO group (adjusted  $P < .01$  or  $P < .001$ ; Figure 3). Contractility remained significantly worse compared with the Tyrode's + MitoSNO group at 60 minutes (Figure 3, B).

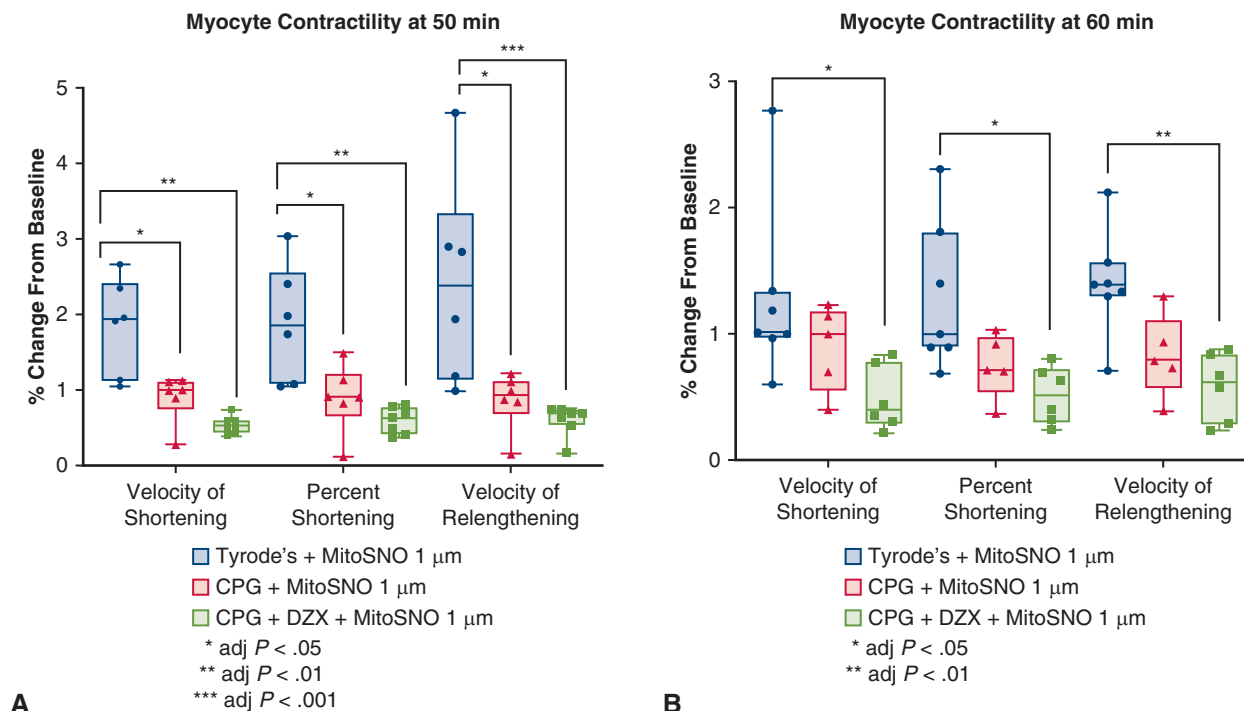
### LVDP and EDP After Global Ischemia

Coronary flow returned to 84% to 94% of baseline flow in all groups by 30 minutes of reperfusion and was not different among the groups (Figure E5). Similar to previous results, CPG + DZX improved LVDP and reduced EDP after prolonged global ischemia compared with CPG alone; LVDP was statistically significantly better in the CPG + DZX group compared with CPG alone at the highest balloon

volume, and EDP was significantly lower in the CPG + DZX group compared with the CPG group at the highest 2 balloon volumes (Figures E6 and E7).<sup>11</sup> The addition of MitoSNO to CPG did not significantly change LVDP or EDP compared with CPG alone ( $P$  not significant for both). The addition of MitoSNO to CPG + DZX was detrimental and associated with loss of the cardioprotection observed in the CPG + DZX group; EDP was significantly higher in the CPG + DZX + MitoSNO group compared with the CPG + DZX group at all balloon volumes (Figure 4). The lowest LVDP values observed following 90 minutes of global ischemia were noted in the MitoSNO + DZX + CPG group, and the lowest EDP values were noted in the CPG + DZX group (Figures 4 and E6-E8).

### DISCUSSION

DZX is thought to open a mitochondrial  $K_{ATP}$  channel, although the components of such a channel have not yet been definitively cloned or defined. The molecular mechanism of DZX cardioprotection remains unknown. Paggio and colleagues<sup>23</sup> have described potential components of a  $K_{ATP}$  channel using in vitro channel reconstitution and colocalization to mitochondria; however, the investigation of



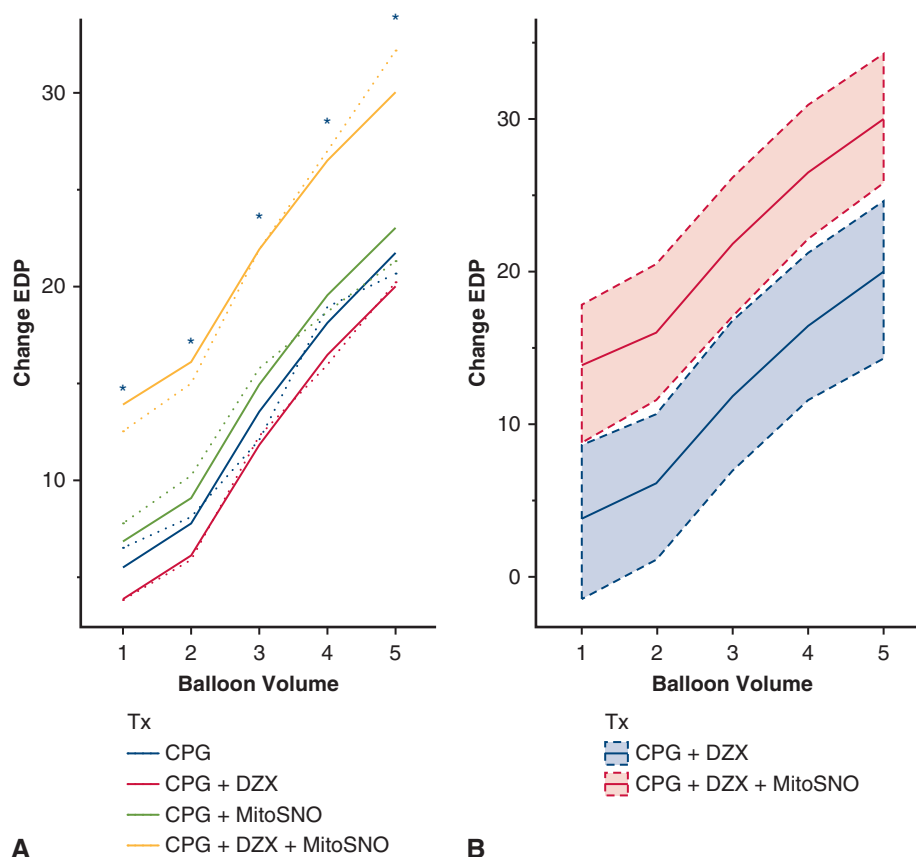
**FIGURE 3.** Hyperkalemic cardioplegia (CPG) with mitochondrial-targeted S-nitrosating agent (MitoSNO) results in reduced myocyte contractility compared with Tyrode's solution + MitoSNO that is worsened by the addition of adenosine triphosphate-sensitive potassium ( $K_{ATP}$ ) channel opener diazoxide (DZX). Myocytes were perfused with control 37°C Tyrode's solution (time 0-20 minutes), test solution (Tyrode's, CPG, or CPG + DZX;  $n = 5-20$  each group; time 20-40 minutes; stress), followed by administration of MitoSNO (1  $\mu$ M) or saline (at 40 minutes) and then 37°C Tyrode's solution (time 40-60 minutes). Contractility (velocity of shortening, percent shortening, or velocity of relengthening) was measured after 50 minutes (A) and 60 minutes (B) of reperfusion with Tyrode's. The middle horizontal line in the box represents the median  $\pm$  SD percent change from baseline. The upper and lower whiskers represent the maximum and minimum values of nonoutliers, and each data point is represented as a dot. \*Adjusted  $P < .05$ ; \*\*adjusted  $P < .01$ ; \*\*\*adjusted  $P < .001$ .

the mechanism of action of DZX remains largely dependent on direct methods, such as genetic deletion. We hypothesized that MitoSNO would provide synergistic cardioprotection when added to  $K_{ATP}$  channel opener DZX; however, this hypothesis was disproven. The new observation that the combination of MitoSNO and DZX was associated with a negative synergistic effect adds to the current knowledge regarding the cardioprotective mechanism of DZX (Figure 5).

### Isolated Myocyte Stress Model

We previously suggested an inverse relationship between increased myocyte volume during stress (hypo-osmotic stress, metabolic inhibition, or exposure to hyperkalemic CPG) and decreased contractility on reperfusion and suggested that this myocyte model could be a potential in vitro model of myocardial stunning.<sup>1,24</sup> Results from the present study are consistent with previous work demonstrating a significant decline in myocyte contractility following exposure to hypothermic hyperkalemic CPG that was prevented by DZX.<sup>1-4</sup> Interestingly, MitoSNO administration at 40 minutes (after stress) resulted in a

decrease in myocyte volume in the Tyrode's group, with an associated increase in contractility (Figures E1 and E4). Paradoxically, the increased volume in the CPG group during stress and decrease in volume during reperfusion (time of MitoSNO administration) was also associated with an increase in contractility (Figures 2 and E2). The observation of no myocyte volume change in the CPG + DZX + MitoSNO group during exposure to CPG + DZX during stress (due to the action of DZX) and no change in myocyte volume during reperfusion with MitoSNO administration, with an associated reduction in contractility, was also paradoxical (Figures 2 and E3). MitoSNO may provide increased contractility related to a reduction in myocyte volume during reperfusion regardless of myocyte volume change during stress; however, this was not seen in the CPG + DZX group. Therefore, myocyte swelling may represent only one mechanism of reduced contractility or stunning, as suggested previously.<sup>24</sup> In addition, MitoSNO may result in increased contractility because of NO donation during reperfusion. Further investigation is needed to determine the mechanisms of myocyte volume change and the relationship to contractility.



**FIGURE 4.** Both the adenosine triphosphate-sensitive potassium ( $K_{ATP}$ ) channel opener diazoxide (DZX) and mitochondrial-targeted S-nitrosating agent (MitoSNO) are associated with preserved left ventricular (LV) end-diastolic pressure (EDP) following prolonged global ischemia that is lost with their combination. Isolated mouse hearts underwent Langendorff perfusion for 30 minutes, followed by arrest with test solution (cardioplegia [CPG] or CPG + DZX) and 90 minutes global ischemia, infusion of MitoSNO 1  $\mu$ M or Krebs–Henseleit buffer, and then reperfusion for 30 minutes. LV EDP was recorded during baseline and following ischemia at identical balloon volumes;  $n = 8$ –10 for each. A, Change in LV EDP from baseline across all balloon volumes following global ischemia; fitted values (solid lines) of the linear mixed model with random intercept superimposed on a smoother (dotted lines) for each group. As balloon volumes increase, the average change of EDP in the CPG + DZX + MitoSNO group is separated from that in the other treatment groups. \*Adjusted  $P < .05$ , CPG + DZX + MitoSNO vs all other groups at each balloon volume. B, Change in LV EDP from baseline across all balloon volumes following global ischemia; fitted values (solid lines) of the linear mixed model with random intercept superimposed on a smoother (dotted lines) for the CPG + DZX and CPG + DZX + MitoSNO group. Bootstrapped 95% confidence intervals (500 times) per treatment and prediction intervals are depicted by shading. As balloon volumes increase, the average change of EDP in the CPG + DZX + MitoSNO shows no overlap with the CPG + DZX group. All other groups had some overlap of prediction intervals, as shown in Figure E8. \* $P < .05$  vs CPG. Exact  $P$  values at each balloon volume are listed in the Online Data Supplement. Confidence intervals are removed for clarity (shown in Figure E7).

The proposed  $K_{ATP}$  channel components suggested by Paggio and colleagues<sup>23</sup> may regulate organelle volume and function and stabilize the mitochondrial membrane potential. It is plausible that the pharmacologic opening of  $K_{ATP}$  channels during stress or ischemia stabilizes mitochondrial membrane potential, and that this potential is altered by the entry of MitoSNO during reperfusion, leading to cellular volume derangement, activation of mitochondrial permeability transition pores, and injury.

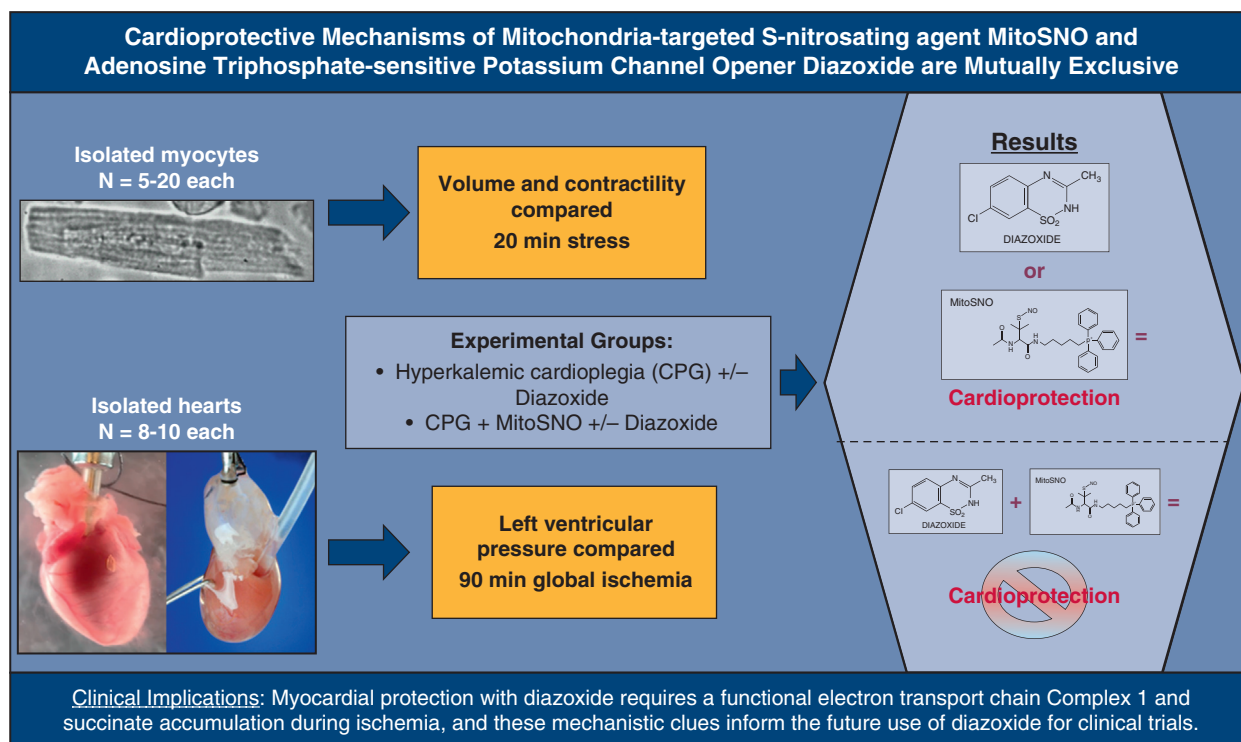
#### Isolated Mouse Heart Model (Langendorff) of Global Ischemia

Similar to previous work,<sup>11</sup> an improvement in LVDP and a reduction in EDP were noted when DZX was added

to hyperkalemic CPG in the present study. However, the cardioprotection provided by DZX was lost with the addition of MitoSNO, suggesting mutually exclusive mechanisms of action. In fact, this combination was detrimental and significantly worse than the CPG alone group.

#### Potential Mechanisms of Action of DZX

Previous work has suggested that DZX acts via a mechanism at the mitochondrial level.<sup>25</sup> Using genetic deletion, we noted that DZX cardioprotection involves the  $K_{ATP}$  channel SUR1 subunit at a nonsarcolemmal  $K_{ATP}$  channel location.<sup>13</sup> DZX is also a known inhibitor of mitochondrial enzyme complex II (SDH), a component of the electron transport chain (Figure 6).<sup>26–28</sup> The inhibition of SDH



**FIGURE 5.** Cardioprotective mechanisms of mitochondria-targeted S-nitrosating agent (*MitoSNO*) and adenosine triphosphate-sensitive potassium channel opener diazoxide (DZX) are mutually exclusive. Isolated myocytes (n = 5-20 each) underwent exposure to stress with or without DZX and with or without *MitoSNO*. Isolated mouse hearts (n = 8-10 each) underwent 90 minutes of global ischemia with or without DZX in cardioplegia (CPG) with or without *MitoSNO*. DZX and *MitoSNO* were each individually cardioprotective; however, the combination of the two was detrimental. These data inform the molecular mechanism of action of DZX to support future clinical trials.

by DZX has been proposed as a non- $K_{ATP}$  channel mechanism of DZX cardioprotection.<sup>27,28</sup> Consistent with this idea, other SDH inhibitors have been found to be cardioprotective, and the reversal of SDH during ischemia and succinate accumulation has been described as the primary driver of mitochondrial ROS production that underlies ischemia/reperfusion injury.<sup>27,29-33</sup>

In contrast, others have suggested that DZX cardioprotection may be related to an increase in ROS signaling and/or protein kinase C activation.<sup>32-36</sup> Consistent with this, ROS mimic ischemic preconditioning (attributed to activity at a  $K_{ATP}$  channel), and antioxidants block ischemic preconditioning.<sup>37-41</sup> Such a mechanism may involve the inhibition of SDH and a subsequent increase in ROS (Figure 6).<sup>26-28,32-34,36,37,42-44</sup>

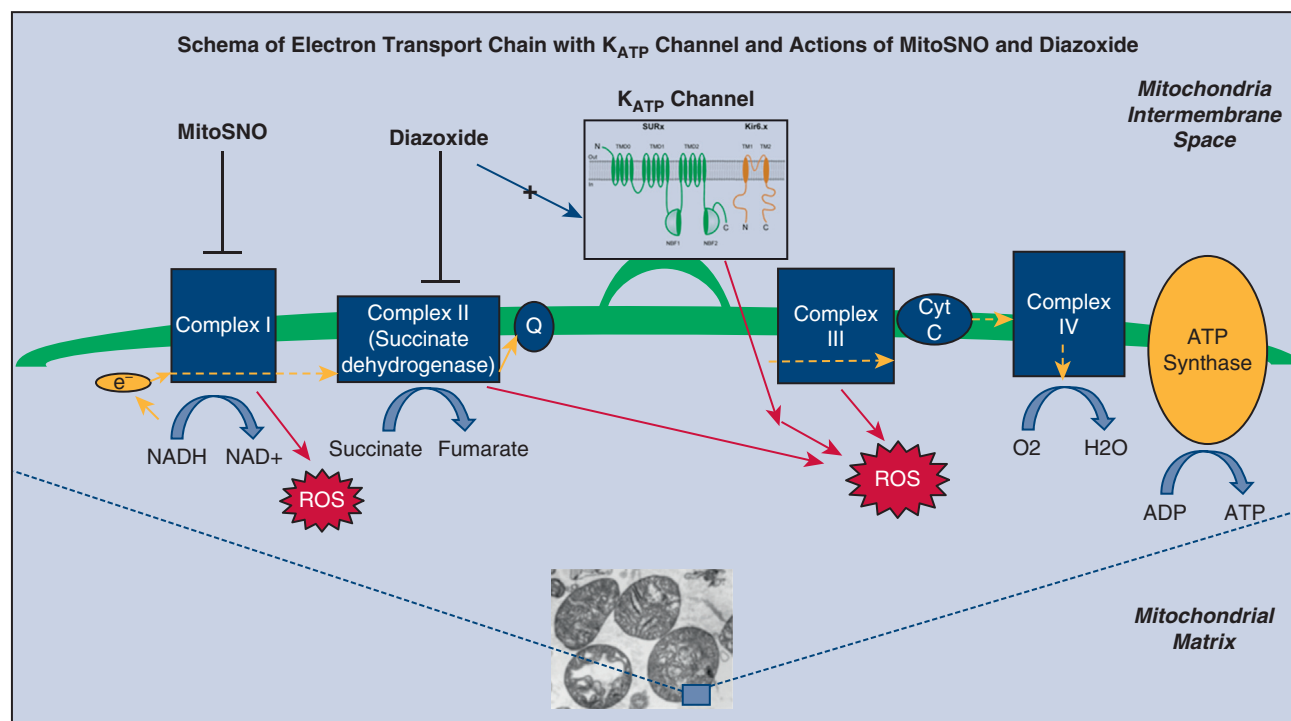
Others have suggested that DZX cardioprotection is mediated by generation of a moderate pro-oxidant environment, because it is associated with an increase in ROS that may be prevented by mitochondrial  $K_{ATP}$  channel blocker 5-hydroxydecanoate or antioxidants.<sup>37,42</sup> ROS may trigger  $K_{ATP}$  channel opening with resultant mitochondrial matrix volume change and uncoupling of electron transport and generation of additional ROS from complex III.<sup>37</sup> Consistent with this, we have shown that DZX does not inhibit

SDH in the presence of the antioxidant glutathione.<sup>14</sup> In addition, glutathione given during ischemia prevents the ability of DZX to prevent detrimental myocyte swelling during the stress of CPG, suggesting that the inhibition of SDH is important in DZX cardioprotection.<sup>14</sup> Thus, the present study is consistent with the suggested cardioprotective DZX mechanism of an increase in ROS, because antioxidant *MitoSNO* also prevented DZX cardioprotection.<sup>37,42</sup>

SDH and  $K_{ATP}$  channels have been linked structurally at the mitochondrial level. SDH may be a component of a mitochondrial  $K_{ATP}$  channel or a modulator of such a channel,<sup>33</sup> given the evidence of shared genetic information between  $K_{ATP}$  subunit SUR1 and SDH genes.<sup>45</sup> However, we have suggested that DZX inhibits SDH in the absence of  $K_{ATP}$  via genetic deletion of SUR1(-/-) or inhibition by 5-hydroxydecanoate, consistent with the effects of  $K_{ATP}$  activation being separable from SDH inhibition (Figure 6).<sup>15</sup>

#### MitoSNO Mechanism of Action

Murphy and colleagues<sup>29</sup> have proposed that succinate accumulation during ischemia leads to reverse electron transport at complex I and is responsible for mitochondrial ROS production during reperfusion. Succinate accumulation arises from reversal of SDH driven by fumarate



**FIGURE 6.** Schema of the mitochondrial electron transport chain with the adenosine triphosphate-sensitive potassium ( $K_{ATP}$ ) channel and actions of the  $K_{ATP}$  channel opener diazoxide (DZX) and mitochondria-targeted S-nitrosating agent (MitoSNO). An electron microscopy photograph of an isolated mitochondria from our laboratory shows that the figure is a simplified image of the mitochondrial electron transport chain and a proposed mitochondrial  $K_{ATP}$  channel on the inner mitochondrial membrane. DZX inhibits succinate dehydrogenase (SDH) and was administered during stress in both models in the current study. MitoSNO stalls complex I and prevents succinate accumulation and was administered at the onset of reperfusion in both models. DZX may provide cardioprotection via a mitochondrial  $K_{ATP}$  channel, via SDH (complex II) inhibition, or via another mechanism during ischemia. MitoSNO provides cardioprotection via reversible inhibition of complex I and prevention of SDH accumulation during the initial 30 minutes of reperfusion. Each is cardioprotective; however, together they have a synergistic negative effect. *NADH*, Nicotinamide adenine dinucleotide, reduced form; *NAD+*, nicotinamide adenine dinucleotide, oxidized form; *ROS*, reactive oxygen species; *ATP*, adenosine triphosphate; *ADP*, adenosine diphosphate.

overflow during ischemia and/or inhibition of SDH by reduction of the coenzyme Q pool during ischemia. On reperfusion, the accumulated succinate is rapidly reoxidized by SDH (within 2 minutes) and, with complexes III and IV at full capacity, drives extensive ROS by reverse electron transport at mitochondrial complex I.<sup>22</sup> MitoSNO reversibly inhibits complex I and thus reduces ROS production.<sup>18</sup> Similarly, various NO donors are cardioprotective via S-nitrosation, thereby preventing reverse electron transport at complex I and ROS production (Figure 6).<sup>22</sup> Increased ROS is responsible for oxidative damage, dysregulation of calcium levels, and induction of mitochondrial permeability transition and cellular death.<sup>46</sup>

Interestingly, SDH-mediated ischemic succinate accumulation and subsequent increase in ROS is inhibited by malonate (an SDH inhibitor) and has been associated with reduced oxidative damage and slowed mitochondrial repolarization on reperfusion.<sup>29</sup> Thus, malonate is cardioprotective, and this cardioprotection was suppressed by adding back succinate, suggesting that malonate acts only to blunt succinate accumulation and/or its oxidation on reperfusion.

In addition, malonate given during ischemia results in a reduction in nicotinamide adenine dinucleotide, with oxidation of complex I (or normal electron transport direction) on reperfusion and no increase in ROS.<sup>29</sup> We can propose that DZX would prevent reversal of SDH during ischemia, succinate accumulation, and reverse electron transport at complex I during reperfusion, thereby leading to reduced ROS levels. This may be an alternative mechanism of DZX cardioprotection. Murphy and colleagues<sup>29</sup> did not combine malonate during ischemia and MitoSNO at the start of reperfusion to determine whether the combination of inhibition of SDH and MitoSNO resulted in a negative synergistic effect.

### Negative Synergistic Effect With the Combination of DZX and MitoSNO: Potential Clinically Relevant Implications

In the present study, DZX was administered at the optimal time, just before or during ischemia or stress.<sup>21</sup> Consistent with the work of Murphy and colleagues,<sup>29</sup> DZX resulted in inhibition of SDH (and blocking of its

reversal and succinate accumulation, similar to malonate) while complex I is deactivated. Normally, during reperfusion, complex I is activated in 5 minutes and accumulated succinate is oxidized and restored to normal levels within 2 minutes<sup>22</sup>; however, with the addition of MitoSNO at the onset of reperfusion (at the optimal time<sup>22</sup>), complex I is inactivated for a short period. The inhibition of complexes I and II by MitoSNO and DZX, respectively, appears to be harmful. Perhaps excess electrons are driven to complex III or IV or oxygen to produce ROS during reperfusion in the presence of both DZX and MitoSNO.

Alternatively, complex I is susceptible to modification by MitoSNO (ie, selective S-nitrosation of Cys 39 on the ND3 subunit) only during ischemia, and this might not occur in the presence of DZX or SDH inhibition. MitoSNO also binds ATP synthase with no effect on ATP synthase function,<sup>29</sup> and this also may be altered in the presence of DZX, with potential detrimental consequences. These data suggest that DZX requires a functional complex I or ROS production during ischemia. The use of DZX as a cardioprotective agent in CPG may be detrimental in the presence of NO donors or antioxidants. Further research is needed to determine the molecular mechanism of cardioprotection to support its clinical use.

DZX has been approved by the Food and Drug Administration for clinical use in its oral form in primary persistent hyperinsulinemic hypoglycemia of infancy and other hypoglycemic syndromes and in its intravenous form for emergency reduction of blood pressure in severe hypertension.<sup>47-49</sup> Of note, the clinical use of DZX as an intracoronary agent (component of CPG) would be a fraction of the dose used as an intravenous or oral agent. In a large animal model of global ischemia, we did not find systemic effects of vasodilation at the doses used with intracoronary delivery (100-500  $\mu\text{mol/L}$ )<sup>12</sup>; therefore, systemic effects, such as vasodilation, are not likely.

### Limitations

The prolonged global ischemia model<sup>11</sup> used in this study was designed to mimic the clinical situation of global arrest during cardiac surgery. Therefore, a measurable discrete infarct is not created, and the functional results noted here cannot be correlated with structural myocardial changes. Similarly, histology, ATP levels, SDH, ROS mediators, lactate, protein content, and myocardial tissue perfusion were not measured owing to the prolonged period of ischemia. In addition, caution must be taken when extrapolating findings from isolated myocyte and heart models to humans.

Because our model is not a traditional infarct model, we were unable to quantify an infarct size. Histology and measurements of ATP and protein content of the mouse heart have not been routinely obtained using this model and were not done in these experiments owing to the prolonged

period of ischemia. In addition, this is a nonworking heart model with an ex vivo heart, and thus it is not possible to place piezoelectric crystals for measurement under loading or nonloading conditions.

We and others have questioned the selectivity and efficacy of  $\text{K}_{\text{ATP}}$  channel inhibitors, as well as the changes in drug efficacy with different metabolic states. We have used both purported mitochondrial and sarcolemmal channel inhibitors and have not found them to be selective or consistently effective (or informative as to mechanism).<sup>2,50</sup> Therefore, channel inhibitors were not used in these experiments.

### CONCLUSIONS

DZX and MitoSNO are individually cardioprotective in isolated myocyte and heart models; however, the combination of the two is detrimental. This negative synergistic effect suggests that MitoSNO cardioprotection requires succinate accumulation during ischemia, and that DZX cardioprotection requires a functional complex I or ROS production during reperfusion. These results inform the molecular mechanism of DZX cardioprotection, which will be vital to its use in clinical trials.

### Conflict of Interest Statement

The authors reported no conflicts of interest.

The *Journal* policy requires editors and reviewers to disclose conflicts of interest and to decline handling or reviewing manuscripts for which they may have a conflict of interest. The editors and reviewers of this article have no conflicts of interest.

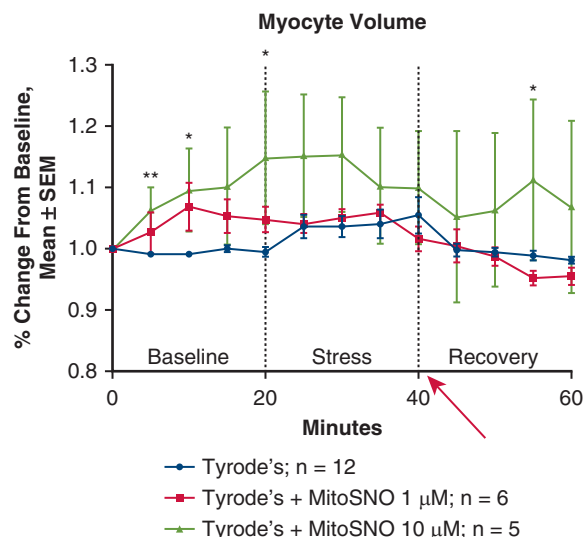
The authors thank Rosmi Thomas and Natalie Gaughan for creating the video on how to perform a mouse Langendorff model.

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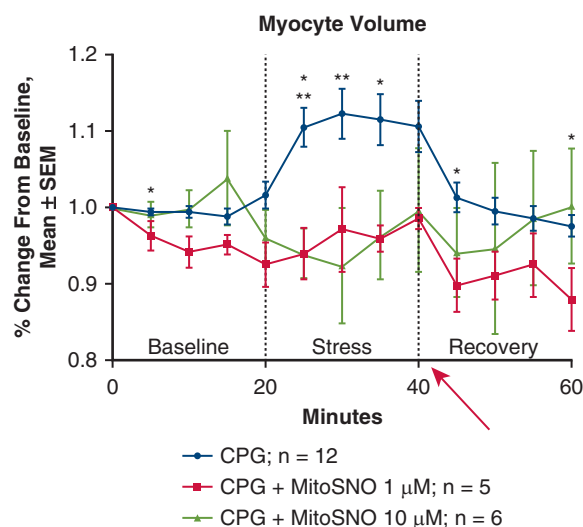
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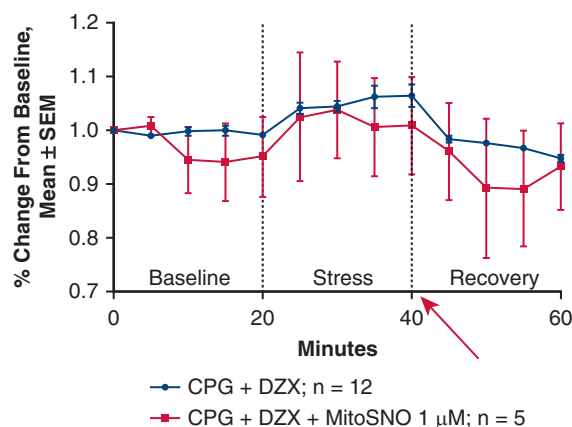
**Key Words:** ischemia, preconditioning, basic science, ion channels



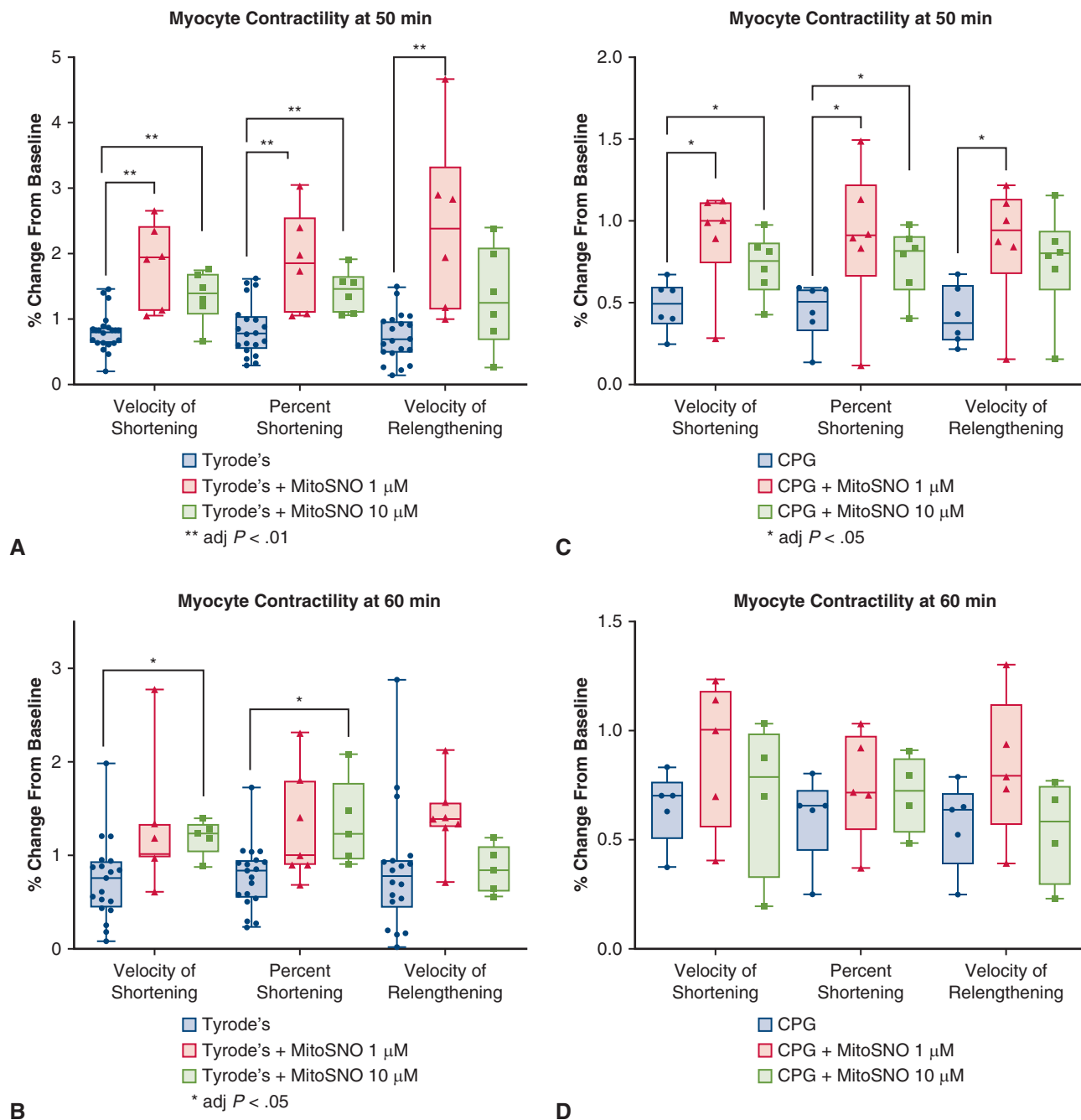
**FIGURE E1.** The addition of mitochondrial-targeted S-nitrosating agent (MitoSNO) to Tyrode's physiologic solution reduces myocyte volume. Myocytes were perfused with control 37°C Tyrode's solution (time 0-20 minutes) or test solution (Tyrode's), followed by administration of MitoSNO (1  $\mu$ M or 10  $\mu$ M) or saline (at 40 minutes; red arrow) and then reperfusion with 37°C Tyrode's solution (time 40-60 minutes). The mean  $\pm$  SEM percent change in myocyte volume from baseline is shown. \*Adjusted  $P < .05$  for Tyrode's vs Tyrode's + MitoSNO 1  $\mu$ M; \*\*adjusted  $P < .05$  for Tyrode's vs Tyrode's + MitoSNO 10  $\mu$ M. SEM, Standard error of the mean.



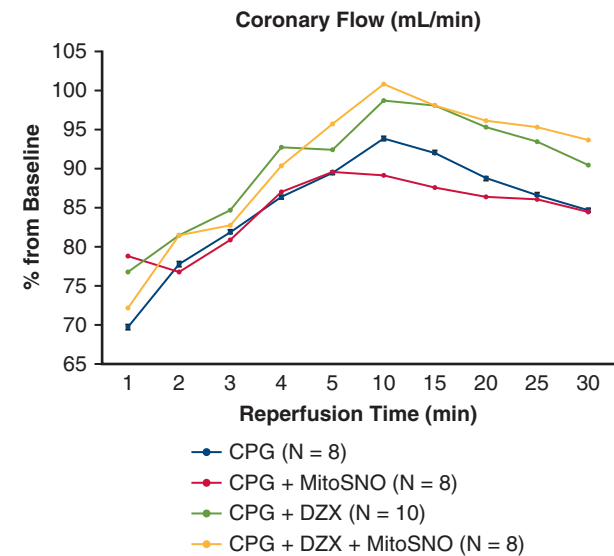
**FIGURE E2.** The addition of mitochondrial-targeted S-nitrosating agent (MitoSNO) following stress of hypothermic, hyperkalemic cardioplegia (CPG) reduces myocyte volume. Myocytes were perfused with control 37°C Tyrode's solution (time 0-20 minutes), then test solution (CPG) (time 20-40 minutes; stress), followed by administration of MitoSNO (1  $\mu$ M or 10  $\mu$ M) or saline (red arrow, at 40 minutes) and then reperfusion with 37°C Tyrode's solution (time 40-60 minutes). The mean  $\pm$  SEM percent change in myocyte volume from baseline is shown. \*Adjusted  $P < .05$  for CPG vs CPG + MitoSNO 1  $\mu$ M; \*\*adjusted  $P < .05$  for CPG vs CPG + MitoSNO 10  $\mu$ M. SEM, Standard error of the mean.



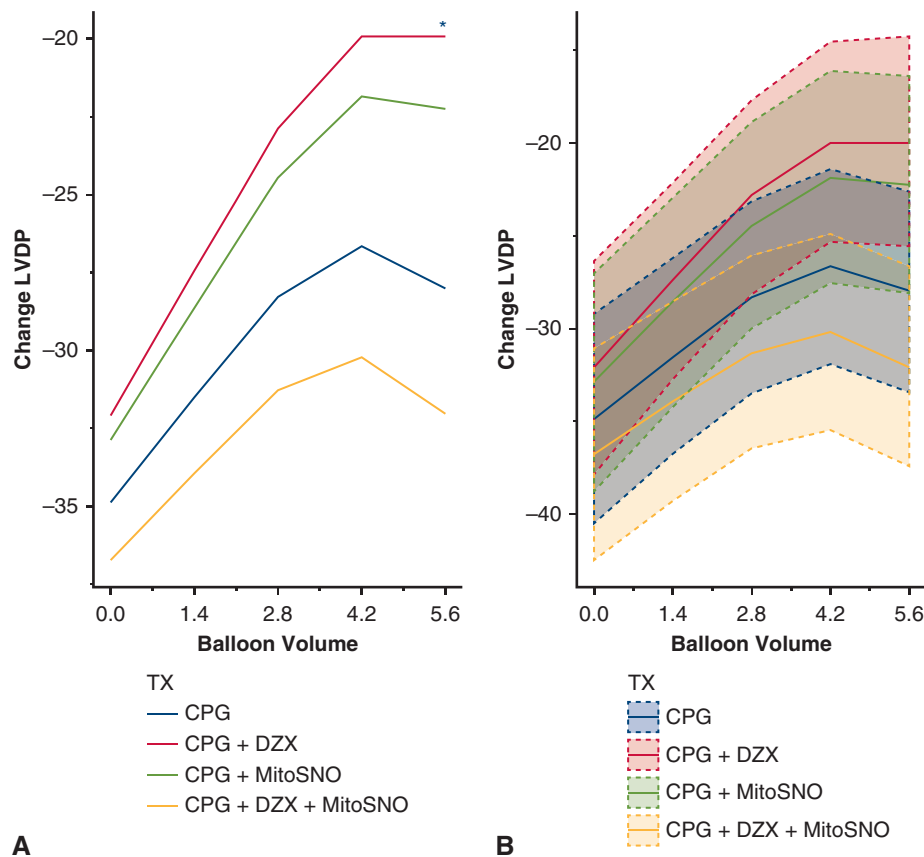
**FIGURE E3.** The addition of mitochondrial-targeted S-nitrosating agent (MitoSNO) following stress of hypothermic, hyperkalemic cardioplegia (CPG) with diazoxide (DZX) does not alter myocyte volume. Myocytes were perfused with control 37°C Tyrode's solution (time 0-20 minutes), test solution (CPG + DZX) (time 20-40 minutes; stress), followed by administration of MitoSNO (1  $\mu$ M or 10  $\mu$ M) or saline (at 40 min; red arrow), and then reperfusion with 37°C Tyrode's solution (time 40-60 minutes). The mean  $\pm$  SEM percent change in myocyte volume from baseline is shown. SEM, Standard error of the mean.



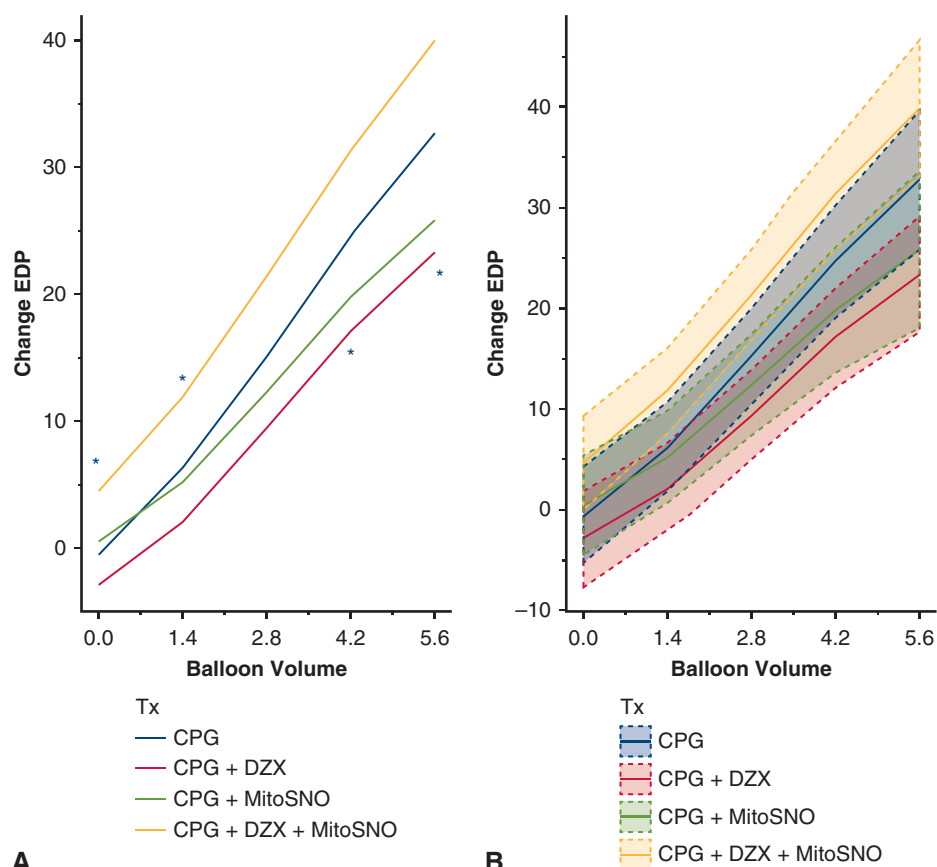
**FIGURE E4.** Mitochondrial-targeted S-nitrosating agent (*MitoSNO*) administered following exposure to Tyrode's physiologic solution or hyperkalemic cardioplegia (*CPG*) improves myocyte contractility. Myocytes were perfused with control 37°C Tyrode's solution (time 0-20 minutes) and test solution (Tyrode's or *CPG*) (time 20-40 minutes), followed by administration of *MitoSNO* (1  $\mu$ M or 10  $\mu$ M) or saline (at 40 minutes), then reperfusion with 37°C Tyrode's solution (at 40-60 minutes) ( $n = 5$ -20 each). Contractility (velocity of shortening, percent shortening, or velocity of relengthening) was measured after 50 minutes (A and C) and 60 minutes (B and D) of reperfusion with Tyrode's solution. Values are reported as mean  $\pm$  SEM percent change from baseline. \*Adjusted  $P < .05$ ; \*\*adjusted  $P < .01$ . *SEM*, Standard error of the mean.



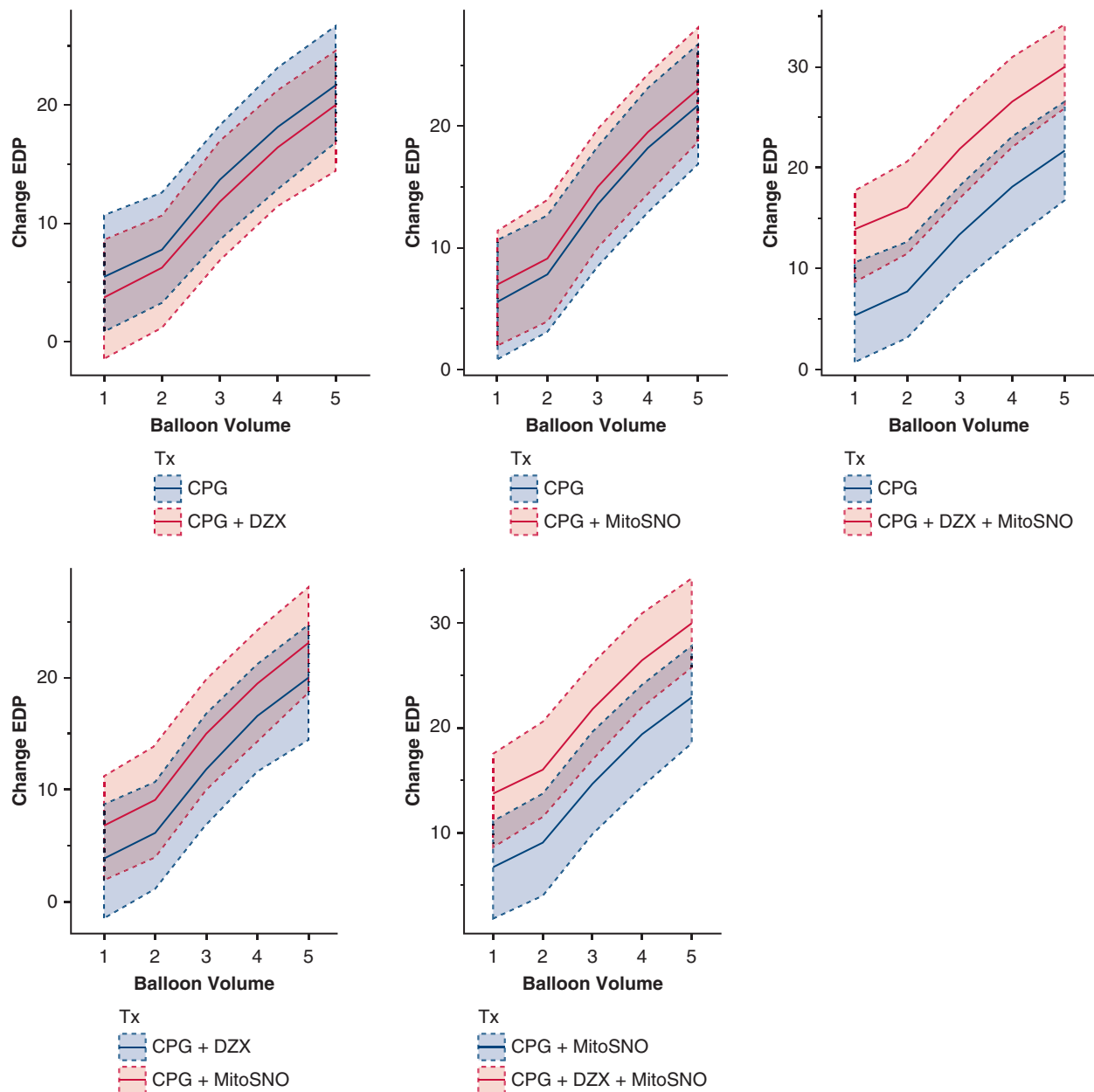
**FIGURE E5.** Coronary flow following global ischemia was similar between groups. Isolated mouse hearts underwent Langendorff perfusion for 30 minutes, followed by arrest with test solution (cardioplegia [CPG] or CPG + diazoxide [DZX]) and 90 minutes of global ischemia, then infusion of mitochondrial-targeted S-nitrosating agent (*MitoSNO*) 1  $\mu$ M or Krebs–Henseleit buffer, then reperfusion for 30 minutes. Coronary flow was measured by an in-line coronary flow probe and is represented as a percentage of baseline flow for each minute after reperfusion. Here confidence intervals are removed for clarity.



**FIGURE E6.** Both  $K_{ATP}$  channel opener diazoxide (*DZX*) and mitochondrial-targeted S-nitrosating agent (*MitoSNO*) are associated with improved left ventricular developed pressure (*LVDP*) following prolonged global ischemia that is lost with their combination. Isolated mouse hearts underwent Langendorff perfusion for 30 minutes, followed by arrest with test solution (cardioplegia [*CPG*] or *CPG* + *DZX*) and 90 minutes of global ischemia, then infusion of *MitoSNO* 1  $\mu$ M or Krebs–Henseleit buffer and reperfusion for 30 minutes. *LVDP* was recorded during baseline and following ischemia at identical balloon volumes. The change in *LVDP* at each balloon volume following 90 min global ischemia is represented for each group ( $n = 8-10$  for each). **A**, Change in *LVDP* depicted using a linear mixed model for each group with confidence intervals removed for clarity. \* $P < .05$  vs *CPG*. **B**, Confidence intervals for each group indicated by color shading for each.



**FIGURE E7.** Both the adenosine triphosphate–sensitive potassium ( $K_{ATP}$ ) channel opener diazoxide (*DZX*) and mitochondrial-targeted S-nitrosating agent (*MitoSNO*) are associated with reduced left ventricular (LV) end-diastolic pressure (*EDP*) following prolonged global ischemia that is lost with their combination. Isolated mouse hearts underwent Langendorff perfusion for 30 minutes followed by arrest with test solution (cardioplegia [*CPG*] or *CPG* + *DZX*) and 90 minutes of global ischemia, then infusion of *MitoSNO* 1  $\mu$ M or Krebs–Henseleit buffer and reperfusion for 30 minutes. LV *EDP* was recorded during baseline and following ischemia at identical balloon volumes. The change in LV *EDP* at each balloon volume following 90 minutes of global ischemia is represented for each group;  $n = 8$ –10 for each. A, Change in *EDP* depicted using a linear mixed model for each group with confidence intervals removed for clarity.  $*P < .05$  vs *CPG*. Exact  $P$  values at each balloon volume are listed in the [Online Data Supplement](#). B, Confidence intervals for each group indicated by color shading for each.



**FIGURE E8.** Change in left ventricular (LV) end-diastolic pressure (EDP) from baseline with single group comparisons. Isolated mouse hearts underwent Langendorff perfusion for 30 minutes and arrest with test solution (cardioplegia [CPG] or CPG + diazoxide [DZX]) and 90 minutes of global ischemia, followed by infusion of mitochondrial-targeted S-nitrosating agent (MitoSNO) 1  $\mu$ M or Krebs-Henseleit buffer, then reperfusion for 30 minutes. LV EDP was recorded at baseline and following ischemia at identical balloon volumes;  $n = 8-10$  for each. Shown are the changes in EDP from baseline across all balloon volumes following global ischemia; fitted values (solid lines) of the linear mixed model with random intercept superimposed on a smoother (dotted lines) for all groups. Bootstrapped 95% confidence intervals (500 times) per treatment and prediction intervals are depicted by shading. All groups shown had some overlap of prediction intervals.