Published in final edited form as:

J Am Coll Surg. 2015 August ; 221(2): 319–325. doi:10.1016/j.jamcollsurg.2015.02.014.

Diazoxide Cardioprotection Is Independent of Adenosine Triphosphate-Sensitive Potassium Channel Kir6.1 Subunit in Response to Stress

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Abstract

Background—The sarcolemmal adenosine triphosphate-sensitive potassium channel (sK_{ATP}), composed primarily of Kir6.2 and SUR2A subunits, has been implicated in cardiac myocyte volume regulation during stress; however, it is not involved in cardioprotection by the K_{ATP} channel opener diazoxide. Paradoxically, the sK_{ATP} channel subunit Kir6.2 is necessary for detrimental myocyte swelling secondary to stress. The Kir6.1 subunit may also contribute to K_{ATP} channels in the heart, and we hypothesized that this subunit may play a role in myocyte volume regulation in response to stress.

Study Design—Isolated cardiac myocytes from either wild type mice (WT) or mice lacking the Kir6.1 subunit (Kir6.1^{−/−}) were exposed to control Tyrode's solution (TYR) for 20 min, test solution (TYR, hypothermic hyperkalemic cardioplegia (CPG), or CPG + K_{ATP} channel opener diazoxide (CPG + DZX)) for 20 min, followed by TYR for 20 min. Myocyte volume and contractility were measured and analyzed.

Results—Both WT and Kir6.1^{−/−} myocytes demonstrated significant swelling during exposure to stress (CPG) that was prevented by DZX. WT myocytes also demonstrated reduced contractility during stress of cardioplegia that was prevented by DZX. However, Kir6.1^{−/−} myocytes did not show reduced contractility during stress.

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Disclosure Information: Nothing to disclose.

Abstract presented at American College of Surgeons 100th Annual Clinical Congress, Surgical Forum, San Francisco, CA, October, 2014.

Conclusions—These data indicate that K_{ATP} channel subunit Kir6.1 is not necessary for diazoxide's maintenance of cell volume during the stress of cardioplegia. The absence of Kir6.1 does not affect the contractile properties of myocytes during stress suggesting the absence of Kir6.1 improves myocyte tolerance to stress via an unknown mechanism.

Introduction

Adenosine triphosphate-sensitive potassium channels (K_{ATP}) in the heart are inhibited by ATP and are open during times of stress, thus providing a “unique electrical transducer of the metabolic state of the cell” (1, 2). Pharmacologic opening of K_{ATP} channels provides cardioprotection and mimics ischemic preconditioning in multiple animal models (3–10) and in human myocytes (11). Paradoxically, the cell surface (sarcolemmal) K_{ATP} channel (sK_{ATP}) has been implicated in myocyte swelling secondary to stress, and deletion of the sK_{ATP} channel subunit Kir6.2 provides resistance to myocyte swelling secondary to stress (9, 12).

Cell surface K_{ATP} channels are composed of 4 pore-forming subunits (Kir6.2 or Kir6.1) and 4 of the ATP binding cassette family of membrane proteins (SUR1 or SUR2) (13, 14). The genes encoding these subunits have been cloned, which allows for genetic manipulation.

K_{ATP} channel opener diazoxide does not provide cardioprotection via the sK_{ATP} channel (composed of Kir6.2 and SUR2A), and the location and mechanism of action of diazoxide remain elusive (15). Proposed mechanisms include non-K_{ATP} channel or mitochondrial K_{ATP} channel location of action (16, 17). Using an isolated myocyte model of myocardial stunning, we have provided evidence that the cardioprotection provided by diazoxide may involve the inhibition of succinate dehydrogenase (independent of K_{ATP} channel) and requires the K_{ATP} channel subunit SUR1 (15, 18, 19). However, the pore-forming subunits involved have yet to be identified.

The present study was conducted to determine whether the K_{ATP} channel subunit Kir6.1 has any role in detrimental myocyte swelling secondary to stress or in the cardioprotection afforded by diazoxide.

Methods

All animal procedures were approved by the Washington University Animal Studies committee and all animals received humane care in compliance with the “Guide to Care and Use of Laboratory Animals” prepared by the Institutes for Laboratory Animal Research.

Kir6.1 knockout mice were generated as previously described by Miki and Seino (20) by replacing a part of intron 2 and exon 3 of KCNJ8 in the 129Sv background, and then backcrossing to mouse strain C57BL6 (20).

Mouse Myocyte Isolation

Ventricular myocytes were isolated from both wild type (WT) and Kir6.1 (–/–) mice (20) (age 6 weeks - 5 months and 15–30 g weight) as previously described (18). Mice were anesthetized with 2.5% Avertin intraperitoneally. Heparin (0.1 ml) was administered

intraperitoneally. Rapid cardiectomy was performed and Solution A (as defined below) was perfused through the aorta for 5 min. The heart was then perfused at 37°C for 12–20 min with solution B (as defined below). Ventricles were removed and minced and placed into solution C (as defined below), and gently dispersed by glass pipette. Cells were allowed to centrifuge by gravity, and serial washings were performed every 10 min for 15–20 min. A typical yield of viable myocytes was 65% to 75%. Viable cells were used within 5 hours after isolation and kept in solution C at room temperature prior to the experimental protocol.

Solution A consisted of (in mmol/L, except as noted) 116 NaCl, 5.36 KCl, 0.97 Na₂HPO₄1.47 KH₂PO₄21.10 HEPES (*N*-[2-hydroxyethyl] piperazine-*N'*-[4-butanesulfonic acid]), 11.65 glucose, 26.50 μmol/L phenol red (Sigma, St. Louis, MO), 3.72 MgCl₂4.40 NaHCO₃essential vitamins (100×, 10 mL, GIBCO, Grand Island, NY), and amino acids (50×, 20 mL, GIBCO, Grand Island, NY). Solution B consisted of solution A plus 10 μmol/L CaCl₂ and 1.2 mg/mL collagenase (Type 2, Worthington Biochemical Corporation; Freehold, NJ). Solution C consisted of solution A plus 5 mg/mL bovine serum albumin (Sigma, St. Louis, MO), 1.25 mg/mL taurine, and 150 μmol/L CaCl₂.

Experimental Protocol

Both WT and Kir6.1(–/–) myocytes were exposed to 37°C Tyrode's solution (TYR) for 20 min to obtain baseline volume. Any changes in cell volume secondary to the isolation would be evident during this period. Myocytes were then exposed to test solution (20 min) including: TYR 37°C, hypothermic hyperkalemic cardioplegia (CPG) at 9°C, or cardioplegia + diazoxide (CPG + DZX), followed by TYR 37°C for 20 min (Figure 1). Eight to twelve myocytes were observed for each group.

CPG consisted of (in mmol/L): NaCl 110, NaHCO₃ 10, KCl 16, MgCl₂ 16, and CaCl₂ 1.2, and was equilibrated with 95% O₂ to 5% CO₂ and titrated to the pH of 7.3 with 10% NaHCO₃ solution.

Diazoxide (7-chloro-3-methyl-1,2,4-benzothiadiazine-1,1-dioxide [DZX]; Sigma, St. Louis, MO) dose of 100 μmol/L was utilized as it was effective in ameliorating cell swelling secondary to stress in previous studies (15). A stock solution of DZX was made by dissolving DZX in 0.1% dimethyl sulfoxide (DMSO), at which concentration DMSO has no effect on cell volume (21).

Myocyte Volume Measurement

Myocytes were visualized on an inverted microscope stage (IX-51, Olympus, Japan) as previously described (Figure 2) (22). After 5 min, the chamber was perfused at a rate of 3 mL/min with TYR (in mmol/L): NaCl 130, KCl 5, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 24, Na₂HPO₄ 1.75, and glucose 10 (buffered to a pH of 7.4 using 95% O₂ to 5% CO₂). After viability was confirmed, myocyte images were captured using video-based edge detection software (IonOptix, Milton, MA) and volume measured every 5 min as previously described (22).

Myocyte Contractility

Myocyte contractility was measured using a video-based edge detection system (IonOptix, Milton, MA) as previously described (22). Cells were paced using a field stimulator (MyoPacer; IonOptix, Milton, MA) at a voltage of 10% above threshold at a frequency of 1HZ with a 5-ms duration to avoid the occurrence of fusion beats. After myocytes underwent 5 min of stimulation, data were obtained from 12–30 consecutive beats and averaged. Contractility was measured at baseline and after 10 and 20 minutes of re-exposure to TYR. Parameters of contractility including percentage of cell shortening (PS), maximal velocity of shortening (VS), and percentage of cell relengthening (PR) were analyzed as previously described (22). Cells that showed less than 7% cell shortening at baseline were excluded.

Statistical Analysis

All data were presented as mean $\pm$ standard error of the mean, normalized to baseline. A Shapiro-Wilk test was used to test for normality. Cell volume was normally distributed so a repeated measures analysis of variance was used for sequential time-based measurements for each test solution against its own baseline and control values. Multiple comparisons between groups were done using contrasts with Bonferroni correction. Contractility data was not normally distributed, so a nonparametric Kruskal-Wallis test was used to test the null hypothesis. Multiple comparisons were made using Dwass-Steel-Critchlow-Fligner test. All reported probability values are two-sided and adjusted. Probability values <0.05 were considered significant. Statistical analysis was performed using Systat v13 (Systat Software, Inc., San Jose, CA).

Results

Myocyte Volume During Stress of Cardioplegia

Similar to previous results, exposure to hypothermic hyperkalemic cardioplegia resulted in significant swelling in wild type mouse myocytes at 5, 10, 15, and 20 minutes after exposure to cardioplegia compared to wild type baseline Tyrode's ($p<0.001$ for all). This significant swelling was reduced by the addition of diazoxide ($p=0.030, 0.315, 0.433$, and 0.030 at 5, 10, 15, and 20 minutes after exposure to cardioplegia and diazoxide compared to wild type baseline Tyrode's and $p=0.999, 0.285, 0.555$, and 0.999 at 5, 10, 15, and 20 minutes after exposure to cardioplegia and diazoxide compared to wild type cardioplegia; Figure 3).

Myocytes from Kir6.1(–/–) mice also exhibited significant swelling secondary to cardioplegia ($p<0.001, <0.001, =0.030$, and 0.044 at 5, 10, 15, and 20 minutes after exposure to cardioplegia compared to Kir(–/–) baseline Tyrode's) that was markedly reduced by the K_{ATP} channel opener diazoxide ($p=0.002, 0.006, 0.044$, and 0.240 at 5, 10, 15, and 20 minutes after exposure to cardioplegia and diazoxide compared to Kir6.1(–/–) cardioplegia; Figure 3).

Myocyte volume in the Tyrode's wild type and Tyrode's Kir6.1(–/–) groups was also significantly different from cardioplegia Kir6.1(–/–) and cardioplegia wild type groups, respectively, during stress. In addition, myocyte volume in the cardioplegia wild type group

during stress was significantly different from the cardioplegia with diazoxide Kir6.1(–/–) group.

Myocyte volume in all groups returned to baseline level by 10 min re-exposure to Tyrode's solution.

Myocyte Contractility During Stress of Cardioplegia

Similar to previous results, exposure to hypothermic hyperkalemic cardioplegia resulted in reduced myocyte contractility ($p=0.039$ compared to Tyrode's control peak velocity of shortening at 50 min) that was prevented by diazoxide in WT myocytes ($p=0.228$ compared to Tyrode's control peak velocity of shortening at 50 min; Figure 4). Interestingly, myocytes from Kir6.1(–/–) mice did not exhibit a significant reduction from baseline contractility during exposure to CPG with or without DZX (Figure 4).

Discussion

The adenosine triphosphate-sensitive K_{ATP} channel opener diazoxide provides volume homeostasis and preserves contractility in isolated myocytes of mice, rabbits, and humans in response to stress (exposure to hyperkalemic cardioplegia, metabolic inhibition, or hyposmotic stress) (7, 9, 11, 22). The mechanism of cardioprotection by diazoxide could be at a mitochondrial K_{ATP} channel location or via a K_{ATP} channel-independent location. Diazoxide does not elicit sK_{ATP} channel current in ventricular myocytes, and its protective benefits require the presence of the K_{ATP} channel subunit SUR1 and may involve inhibition of succinate dehydrogenase (15, 18, 19). Activity at a proposed mitochondrial K_{ATP} channel is supported only by indirect methods and no mitochondrial K_{ATP} channel has been identified at the molecular level or cloned (23, 24). Similarly, efforts to localize known K_{ATP} subunits in mitochondria have been inconsistent and controversial (25, 26). Cell surface K_{ATP} channels are composed of either Kir6.2 or Kir6.1 subunits, and controversy exists as to whether Kir6.2 or Kir6.1 subunits may contribute to a mitochondrial K_{ATP} channel (20, 23, 25).

Our own investigations have demonstrated that deletion of the Kir6.2 subunit, the major pore-forming sK_{ATP} channel subunit, results in a lack of detrimental swelling secondary to stress, and no protective effect of diazoxide (9). The Kir6.1 subunit combines with subunit SUR2B to form the predominant K_{ATP} channel in vascular smooth muscle (27). Mice lacking Kir6.1 demonstrate a loss of K_{ATP} function in vascular smooth muscle and can exhibit sudden death due to arrhythmias (heart block) secondary to coronary spasm and ischemia (20). The present study was conducted to determine whether the lack of the Kir6.1 subunit affects myocyte volume homeostasis secondary to stress and the protective effect of diazoxide in this pathology. Similar to previous work, WT myocytes demonstrated significant swelling and reduced contractility secondary to the stress of cardioplegia that was prevented by diazoxide (9). Myocytes lacking Kir6.1 demonstrated significant swelling secondary to stress that was also prevented by diazoxide. These data indicate no role of Kir6.1 in detrimental swelling secondary to the stress of cardioplegia (as was found with Kir6.2), and that cardioprotection by diazoxide remains intact in the absence of Kir6.1.

Similar to our previous findings with deletion of Kir6.2 myocytes, Kir6.1 knockout myocytes in the present study did not exhibit a reduction in myocyte contractility during stress (9). Despite the detrimental volume change observed in Kir6.1(−/−) myocytes exposed to stress, there was no subsequent decline in contractility. These findings are different from those in WT mice that have consistently demonstrated an inverse relationship between myocyte volume derangement (swelling) and reduced contractility (28), suggesting an improved tolerance to stress in the absence of K_{ATP} activity via unidentified, unique mechanisms.

Thus the mechanism by which the K_{ATP} channel opener diazoxide protects against cell swelling due to cardioplegia remains elusive. The present study indicates that Kir6.1 subunit does not play a role. These data will direct further investigations into other diazoxide targets that may be implicated in cardioprotection in an attempt to identify and subsequently exploit the potential tremendous benefits of K_{ATP} channel openers.

Limitations

Isolated myocytes were utilized because they allow for repeated observations and the independent evaluation of one stress at a time. This model is not intended to mimic the clinical situation of ischemia and reperfusion. Caution should therefore be taken before any extrapolation to the clinical setting.

Acknowledgments

Support: Drs Henn and Janjua received grants from NIH RO1 HL098182-01A1 (JSL) and NIH 5T32HL007776 (MCH, MBJ), and Dr Lawton received a grant from the Barnes Jewish Hospital Foundation.

Abbreviations and Acronyms

K_{ATP}	Adenosine triphosphate–sensitive potassium channel
CPG	Hypothermic hyperkalemic cardioplegia
DMSO	Dimethyl sulfoxide
DZX	Diazoxide
PR	Percentage of relengthening
PS	Percentage of cell shortening
TYR	Tyrodé's solution
VS	Velocity of cell shortening
WT	Wild type
SUR	Sulfonylurea receptor
Kir	Potassium inward rectifier

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Cell volume Measurement

Contractility Measurement

Figure 1. Experimental protocol. Myocytes were exposed to 37°C control TYR for 20 min, followed by stress test solution (per methods) for 20 min, then 20 min re-exposure to Tyrode's solution. Volume measurements were taken every 5 min (red V symbol). Contractility measurements were taken at times 5 min (baseline) and at 10 and 20 min after re-exposure to TYR (time 50 and 60 min) (green stars).

Figure 2.

Experimental setup. (A) Diagram and (B) photograph showing the experimental setup. Isolated myocytes (WT and Kir6.1 $(-/-)$) were placed on an inverted microscope stage and video images were captured on a computer. Test solution was perfused across the microscope stage and pacemaker wires were attached to the stage.

Figure 3.

Diazoxide prevents myocyte swelling secondary to stress in WT and Kir 6.1(-/-) mice.

Isolated myocytes (WT and Kir6.1 (-/-)) were exposed to control Tyrode's physiologic solution (TYR) for 20 min (time 0–20), stress (TYR, CPG, or CPG + DZX) for 20 min (time 20–40), followed by TYR for 20 min (time 40–60). Data are mean $\pm$ SEM. Stress (CPG) resulted in significant swelling in both WT and Kir 6.1(-/-) myocytes. Significant myocyte swelling was reduced by DZX in WT myocytes and reversed by DZX in Kir 6.1 (-/-) myocytes. * $p < 0.05$ vs TYR WT; € $p < 0.05$ vs TYR Kir 6.1 (-/-); Ψ, $p < 0.05$ vs Kir 6.1(-/-).

Figure 4.

Diazoxide prevents reduced contractility after stress in WT myocytes. Isolated myocytes (WT and Kir6.1 (-/-)) were exposed to control Tyrode's physiologic solution (TYR) for 20 min (time 0–20), stress (TYR, CPG, or CPG + DZX) for 20 min (time 20–40), followed by TYR for 20 min (time 40–60). Data are mean $\pm$ SEM at 50 minutes. Stress (CPG) resulted in reduced contractility (peak velocity of shortening at 50 min) in WT myocytes that was prevented by DZX. * $p < 0.05$ vs TYR WT at 50 min; Ψ , $p < 0.05$ vs TYR Kir6.1 (-/-) at 50 min.