Expression and Activation of BK\textsubscript{Ca} Channels in Mice Protects Against Ischemia-Reperfusion Injury of Isolated Hearts by Modulating Mitochondrial Function

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**Aims:** Activation and expression of large conductance calcium and voltage-activated potassium channel (BK\textsubscript{Ca}) by pharmacological agents have been implicated in cardioprotection from ischemia-reperfusion (IR) injury possibly by regulating mitochondrial function. Given the non-specific effects of pharmacological agents, it is not clear whether activation of BK\textsubscript{Ca} is critical to cardioprotection. In this study, we aimed to decipher the mechanistic role of BK\textsubscript{Ca} in cardioprotection from IR injury by genetically activating BK\textsubscript{Ca} channels.

**Methods and Results:** Hearts from adult (3 months old) wild-type mice (C57/BL6) and mice expressing genetically activated BK\textsubscript{Ca} (Tg-BK\textsubscript{Ca}R\textsubscript{207Q}, referred as Tg-BK\textsubscript{Ca}) along with wild-type BK\textsubscript{Ca} were subjected to 20 min of ischemia and 30 min of reperfusion with or without ischemic preconditioning (IPC, 2 times for 2.5 min interval each). Left ventricular developed pressure (LVDP) was recorded using Millar’s Mikrotip\textsuperscript{®} catheter connected to ADInstrument data acquisition system. Myocardial infarction was quantified by 2,3,5-triphenyl tetrazolium chloride (TTC) staining. Our results demonstrated that Tg-BK\textsubscript{Ca} mice are protected from IR injury, and BK\textsubscript{Ca} also contributes to IPC-mediated cardioprotection. Cardiac function parameters were also measured by echocardiography and no differences were observed in left ventricular ejection fraction, fractional shortening and aortic velocities. Amplex Red\textsuperscript{®} was used to assess reactive oxygen species (ROS) production in isolated mitochondria by spectrofluorometry. We found that genetic activation of BK\textsubscript{Ca} reduces ROS after IR stress. Adult cardiomyocytes and mitochondria from Tg-BK\textsubscript{Ca} mice were isolated and labeled with Anti-BK\textsubscript{Ca} antibodies. Images acquired via confocal microscopy revealed localization of cardiac BK\textsubscript{Ca} in the mitochondria.

**Conclusions:** Activation of BK\textsubscript{Ca} is essential for recovery of cardiac function after IR injury and is likely a factor in IPC mediated cardioprotection. Genetic activation of BK\textsubscript{Ca}
INTRODUCTION

The large conductance calcium and voltage-activated potassium channels (MaxiK, BK<sub>Ca</sub>, K<sub>Ca</sub>1.1) encoded by <i>Kcnma1</i> gene are ubiquitously expressed in excitable and non-excitable cells (1, 2). The functional channel is comprised of four pore-forming α-subunits, each with seven transmembrane domains where S4 serves as a voltage sensor and C-terminus contains Ca<sup>2+</sup>-sensing RCK1 and RCK2 domains (3). Ca<sup>2+</sup> and voltage sensing allow activation of BK<sub>Ca</sub> (4), resulting in its physiological involvement in neurotransmitter release and secretion (2). Increasing evidence indicates that BK<sub>Ca</sub> channels are located in intracellular organelles in addition to the plasma membrane, extending their functional roles in cellular physiology from organelle to organ level (1, 2, 5–10).

Studies involving activation (10–15) and inactivation (11, 16) with pharmacological and genetic tools, including global (10), and tissue-specific knockouts (17), have implicated BK<sub>Ca</sub> channels in cardiac function, neuroprotection (18), and cardioprotection from ischemia-reperfusion (IR) injury, in addition to IR-induced inflammation and mucosal barrier disruption in the small intestine (19). Further, it was shown that BK<sub>Ca</sub> is present in the mitochondria of adult cardiomyocytes (10, 20). Tissue-specific knockouts in which BK<sub>Ca</sub> was ablated in adult cardiomyocytes showed that expression of mitochondrial BK<sub>Ca</sub> is responsible for its cardioprotective effect (17). It has been shown that agonists or antagonists have no effect on global (10) and cardiomyocytes-specific (17) knockouts. However, mice expressing activated BK<sub>Ca</sub> have not been tested for cardioprotection from IR injury (8). Genetically modifying BK<sub>Ca</sub> in mice by introducing a mutation responsible for its constitutive activation (8), independent of pharmacological agents, can further support the role of BK<sub>Ca</sub> in cardioprotection from IR injury.

One of the possible outcomes of pharmacological activation or inactivation of BK<sub>Ca</sub> is decrease/increase in the production of reactive oxygen species (ROS) (21–24). The reduction in the levels of ROS accompanied by “mild” mitochondrial uncoupling (25) by BK<sub>Ca</sub> agonists is assigned as a possible mechanism for organ and cellular protection from IR injury (26). As stated earlier, all of these studies rely on the use of pharmacological tools with possible non-specific effects. To understand the role of activation of BK<sub>Ca</sub> and its influence on mitochondrial ROS generation, studies need to be performed independent of the pharmacological agents. Non-specific and off-target effects of pharmacological tools have generated reservations (12) on the role of BK<sub>Ca</sub> in modulating levels of mitochondrial ROS as well as cardioprotection from IR injury.

In the current study, we have used genetically-activated mice where BK<sub>Ca</sub> is constitutively active due to incorporation of a gain of function mutation (Tg-BK<sup>R207Q</sup> or Tg-BK<sub>Ca</sub> (8)) to test the role of BK<sub>Ca</sub> activation in mitochondrial ROS generation and cardioprotection from IR injury. We have established that the activation of BK<sub>Ca</sub> is vital for a cardioprotective effect in both IR as well as IPC using an <i>ex vivo</i> isolated perfused heart model. We have further shown that activation of BK<sub>Ca</sub> attenuates ROS from complex I and complex II/III of mitochondria only after IR injury. Our results presented here further corroborate the role of BK<sub>Ca</sub> in cardioprotection.

METHODS

All of the experiments on mice were approved by the Institutional Animal Care and Use Committee at the Drexel University and the Ohio State University. Animals were housed in the vivarium with food and water available ad libitum. Experiments were carried out on 3 month-old male and female. Experimentalists were blinded for the genotype of mice used.

Materials

Horseradish peroxidase (Sigma-Aldrich # P6782), DC<sup>TM</sup> protein assay kit (BIO-RAD Laboratories, #500-0113, 500-0114, 500-0115), glutamate (Sigma-Aldrich # G1626), malate (Sigma-Aldrich # M6773), succinate (Fluka # 14160), pyruvate (Sigma-Aldrich # P2256), Amplex<sup>®</sup> Red (Invitrogen/Thermo Fisher Scientific # A12222), anti-BK<sub>Ca</sub> antibody (Alomone labs, APC-21 lot #5) were procured for the study.

Ischemia-Reperfusion Injury Model <i>ex vivo</i>

Wild-type mice or mice co-expressing genetically activated BK<sub>Ca</sub> (Tg-BK<sub>Ca</sub>) (8) were anesthetized with 87 mg/kg of ketamine and 13 mg/kg of xylazine by administering these agents intraperitoneally (i.p.). The hearts were rapidly excised, washed in ice-cold modified Krebs-Henseleit (KH, pH 7.4, concentrations in mM: 118 NaCl, 4.7 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 24 NaHCO<sub>3</sub>, 11.1 Glucose, 2 CaCl<sub>2</sub>, 1 sodium pyruvate) solution, mounted on a cannula and perfused with KH solution at 37°C at a constant volume (2 mL/min). A pressure transducer (Millar Mikrotip<sup>®</sup> catheter) was introduced to the left ventricle, and after achieving a stable baseline, hearts were subjected to 20 min of global ischemia and 30 min of reperfusion (27). A subgroup of hearts was subjected to IPC before IR to evaluate the role of BK<sub>Ca</sub> in IPC-mediated cardioprotection. Two sets of 2.5 min of ischemia and 2.5 min of reperfusion were used to precondition hearts before IR. Left ventricular developed pressure (LVEDP) was recorded using Powerlab<sup>®</sup> hardware data acquisition system.
along with LabChart data acquisition and analysis software (ADInstrument, USA). After recording cardiac function, hearts either were analyzed for myocardial infarction by TTC staining or used to rapidly isolate mitochondria and measure ROS. WT IR, WT IPC, Tg-BK$_{Ca}$ IR, and Tg-BK$_{Ca}$ IPC group had 7, 8, 6, and 7 mice, respectively, for studying cardiac function and myocardial infarction in section Measurement of Myocardial Infarction.

**Measurement of Myocardial Infarction**

Isolated hearts were thawed, cut into 5 horizontal 2-mm sections using a heart slicer matrix, and incubated for 20 min at room temperature in 2% (w/v) TTC solution in phosphate buffer saline at a pH of 7.4. Images were obtained using Nikon SMZ1000 microscope connected to Nikon digital sight DS-Fi2 camera and analyzed with Image J. WT IR, WT IPC, Tg-BK$_{Ca}$ IR, and Tg-BK$_{Ca}$ IPC group had 7, 8, 6, and 7 mice, respectively.

**Measurement of Cardiac Function by Echocardiography**

To ensure that the results obtained related to cardiovascular function and the myocardial infarction is not due to the altered cardiac function of transgenic mice, we recorded the echocardiograph of WT ($n$ = 6) and Tg-BK$_{Ca}$ ($n$ = 5) mice before they were used for IR or IPC study. Vevo2100™ imaging system (FUJIFILM VisualSonics) with MS400 probe was used to acquire images (28). Briefly, mice were anesthetized using 2% (v/v) isoflurane in carbogen (95% oxygen and 5% carbon dioxide) with heart rate was maintained at more than 450 bpm. Cardiac functions were measured in both B and M mode with the probe positioned in long axis. Mean and peak velocities of the ascending and descending aorta were also recorded using color Doppler. Images were analyzed using Vevo Lab 3.1.1 analysis software.

**Measurement of ROS**

In case of some hearts, after reperfusion for 10 min, the cardiac tissue was rapidly cut into pieces in 2 mL of ice-cold mitochondrial isolation buffer A (succrose 70 mM, mannitol 210 mM, EDTA 1 mM, Tris HCl 50 mM, pH 7.4) and homogenized using a hand-held glass homogenizer without using any detergent. The homogenates were centrifuged at 4°C, 2,500 g for 5 min and supernatants were collected and centrifuged at 4°C, 12,000 g for 10 min. The mitochondrial pellets were resuspended in 100 µL of mitochondria isolation buffer B (succrose 70 mM, mannitol 210 mM, EDTA 0.1 mM, Tris HCl 50 mM, pH 7.4) and again centrifuged at 4°C, 12,500 g for 5 min. The pellets were again resuspended in 100 µL of ROS buffer (EGTA 1 mM, EDTA 1 mM, Tris HCl 20 mM, sucrose 250 mM, pH 7.4, 0.15% BSA was added before use) and centrifuged at 4°C, 12,000 g for 5 min. The pellets were then resuspended in 55 µL of ROS buffer and stored on ice until used for quantifying the generation of ROS. Horse Radish Peroxidase (0.5 µL of 10 mg/mL in 0.1 M phosphate buffer, pH 6) solutions were added to 2 mL of ROS buffer in the cuvette and the solutions were continuously stirred with a magnetic stirrer at 37°C. Basal absorbance was recorded at 560 nm excitation and 590 nm emission wavelength using Hitachi F-2710 fluorescence spectrophotometer. After 1 min, 2 µL of 10 µM amplex red was added to the cuvette. After another 1 min, 25 µL of the mitochondrial suspension was added to the cuvette. Subsequently, mitochondrial substrates, either glutamate (5 mM) and malate (5 mM) (WT IR, $n$ = 3; WT IPC, $n$ = 4; Tg-BK$_{Ca}$ IR, $n$ = 4; Tg-BK$_{Ca}$ IPC, $n$ = 4) or succinate (3 mM) (WT IR, $n$ = 3; WT IPC, $n$ = 4; Tg-BK$_{Ca}$ IR, $n$ = 4; Tg-BK$_{Ca}$ IPC, $n$ = 4), were added after 90 s and absorbance was recorded for a total duration of 45 min. Glutamate and malate are substrates for complex I mediated ROS production, and succinate as a substrate for complex II result in ROS production from complex III and by backflow of electrons to complex I. ROS generated was normalized per µg of mitochondrial protein. The remaining mitochondrial samples were used for measuring the protein concentration using DC™ protein assay kit from BIO-RAD Laboratories, Inc. and SPECTRAmax® spectrometer from Molecular Devices. The level of ROS produced was recorded using FL solutions software (Hitachi, USA) and ROS produced in arbitrary fluorescence units (a.u.) per µg of mitochondria were measured (29). For baseline ROS measurements, hearts from WT ($n$ = 3) and Tg-BK$_{Ca}$ mice ($n$ = 3) were used without exposing the hearts to IR and IPC.

**Visualization of BK$_{Ca}$**

Adult mice cardiomyocytes were isolated and loaded with mitotracker (100 nM, excitation: 579 nm and emission: 599 nm) for 60 min at 4°C followed by fixation with 4% paraformaldehyde. Cardiomyocytes loaded with mitotracker ($n$ = 5) were labeled with anti-BK$_{Ca}$ antibodies (Alomone labs, APC21, 1:200) to study the localization of BK$_{Ca}$ in mitochondria. For studying the localization of BK$_{Ca}$ on plasma membrane, cardiomyocytes labeled with anti-BK$_{Ca}$ antibodies were also marked with plasma membrane marker, wheat germ agglutinin conjugated with Alexa Fluor 488 (WGA, 1:1,000, $n$ = 5) as described earlier (10). Isolated cardiac mitochondria from wild-type ($n$ = 6), Tg-BK$_{Ca}$ ($n$ = 5), and Kenmnetr$^{-/-}$ ($n$ = 5) mice were loaded with mitotracker and labeled with anti-BK$_{Ca}$ antibodies as described earlier (10). Atto647N anti-rabbit secondary antibody was used to label BK$_{Ca}$. Images were acquired using a confocal microscope (FV1000, Olympus) and median filtered (29).

**Statistical Analysis**

Student’s t-test (unpaired and one-tailed) and one-way ANOVA followed by Tukey’s multiple comparison tests were used to measure the statistical difference between groups. Values are presented as mean ± SEM of 3–8 observations. A value of $p < 0.05$ was considered to be statistically significant.

**RESULTS**

Overall, our results demonstrate that expression and activation of BK$_{Ca}$ are vital for cardioprotection from IR injury as well as IPC-mediated cardioprotection. Cardioprotection mediated by BK$_{Ca}$ is possibly modulated by mitochondrial ROS production.
Genetic Activation of BKCa Preserves Cardiac Function Recovery During Reperfusion

The role of activation of BKCa in cardioprotection has been demonstrated by usage of pharmacological tools (1, 10, 11, 13, 14, 30–32), and these drugs are known to have non-specific effects (12). Even though the expression of BKCa is vital for cardioprotection from IR injury (10, 17), the role of activation of BKCa in cardioprotection is not yet well-characterized. We have utilized Tg-BKCa mice expressing genetically activated BKCa in a DEC splice variant background (GenBank: JX429072.1) (8) and carried out IR injury assays with and without IPC (Figures 1A–D). Tg-BKCa mice are viable, normal in body weight (8), and exhibit increased BKCa protein expression in a wide variety of tissues as well as display increased BKCa channel currents (8). Tg-BKCa is under Period1 (Per1) promoter which is ubiquitously expressed in all tissues. Tg-BKCa mice express the R207Q mutation in the S4 voltage sensor of the BKCa α subunit which strongly augments voltage-dependent gating of the channel without affecting the Ca2+-dependent activation (33). In the Tg-BKCa mice, cardiac functional recovery was higher in comparison to the wild-type control (Figures 1B vs. A). Percentage recovery of LVDP after IR-injury was significantly higher (p = 0.02) for Tg-BKCa mice (60 ± 5%, n = 6), in comparison to WT mice (34 ± 7%, n = 7) (Figures 1A,B,E).

Genetic Activation of BKCa Confers Protection During Ischemic Preconditioning

In addition to protecting the heart from IR injury, pharmacological activation or blocking of BKCa has also been implicated in mediating cardioprotection through IPC (17, 32, 34). We have also tested whether activation of BKCa plays a role in cardioprotection mediated by IPC. Two brief IPC events before IR provided cardioprotection to wild-type (Figures 1A,C,E) as well as Tg-BKCa mice (Figures 1B,D,E). The percentage recovery of LVDP seen with the hearts from Tg-BKCa mice exposed to IPC (n = 7) was significantly higher than in Tg-BKCa mice hearts exposed to IR (p = 0.01, n = 6) and in WT mice hearts exposed to IPC (p = 0.001, n = 8). Percentage recovery of LVDP at the end of reperfusion was 51 ± 3% (n = 8) and 97 ± 12% (n = 7) for WT IPC and Tg-BKCa IPC groups, respectively.

Genetic Activation of BKCa Attenuates Myocardial Infarction Following IR

The degree of myocardial infarction was quantified following IR using TTC staining. Viable cells appeared red while dead cells appeared pale yellow (Figure 2). The degree of infarction was higher in WT IR group (n = 7) with 59 ± 3% cell death but infarction was significantly less in (p = 0.0001) in WT IPC group (n = 8) with only 25 ± 4% cell death (Figures 2A–C).
Hearts from Tg-BK\textsubscript{Ca} mice sustained less infarction [25 ± 3% (n = 6), p = 0.04] after IR in comparison to hearts from WT mice [59 ± 3% (n = 7)] (Figures 2A–C, p = 0.0001). IPC further protected myocardium of the Tg-BK\textsubscript{Ca} (n = 7) mice which exhibited the least infarction to the heart cells (13 ± 2%), (Figures 2A–C).

Taken together, our results (Figures 1, 2) implicate genetic activation of BK\textsubscript{Ca} in cardioprotection from IR injury as well as cardioprotection mediated by IPC.

Cardiac Functional Parameters of WT and Tg-BK\textsubscript{Ca} Mice Did Not Alter at the Baseline

Left ventricular ejection fraction (LVEF) and fractional shortening (LVFS) assessed by echocardiography (Figures 3A,B) did not demonstrate any differences. The LVEF of WT and Tg-BK\textsubscript{Ca} were 69 ± 2% (n = 6) and 75 ± 4% (n = 5), respectively (Figure 3C). The fractional shortening of WT and Tg-BK\textsubscript{Ca} were 37 ± 1% (n = 6) and 43 ± 4% (n = 5), respectively (Figure 3D). Similarly, we did not observe any difference in the mean and peak velocities in the ascending aorta and descending aorta of both the WT and Tg-BK\textsubscript{Ca} mice (Figures 3E–H). Mean ascending aorta velocities of WT and Tg-BK\textsubscript{Ca} were 347 ± 47 mm/s (n = 6) and 315 ± 88 mm/s (n = 5), respectively (Figure 3G). The mean peak ascending aorta velocities of WT and Tg-BK\textsubscript{Ca} were 611 ± 80 and 515 ± 145 mm/s, respectively (Figure 3G). The mean descending aorta velocities of WT and Tg-BK\textsubscript{Ca} were −398 ± 22 mm/s (n = 6) and −358 ± 56 mm/s (n = 5), respectively (Figure 3H). The peak descending aorta velocities of WT and Tg-BK\textsubscript{Ca} were −661 ± 38 mm/s (n = 6) and −616 ± 79 mm/s (n = 5), respectively (Figure 3H).

Genetic Activation of BK\textsubscript{Ca} Reduces the Production of Mitochondrial ROS

ROS produced during IR-mediated injury is well-characterized in cardiac cell death (35), and BK\textsubscript{Ca} has been pharmacologically implicated in the modulation of cardiac mitochondrial ROS generation (17, 19, 21, 22, 24, 26, 32, 36, 37). Given that BK\textsubscript{Ca} is present exclusively in mitochondria of adult cardiomyocytes (10) and plays a direct role in cardioprotection from IR injury (17), we tested whether activation of BK\textsubscript{Ca} can directly modulate mitochondrial ROS production. We quantified the amount of ROS produced by isolated mitochondria after 20 min of ischemia and 10 min of reperfusion (Figures 4A,B). With IPC (n = 4), the amount of ROS produced with glutamate and malate as substrates for mitochondria from hearts of WT mice were significantly (p = 0.004) reduced, in comparison to hearts only exposed to IR (n = 3) (234 ± 10 a.u./µg of mitochondria vs. 193 ± 4 a.u./µg of mitochondria, Figure 4C). The amount of ROS produced after IPC (n = 4) with glutamate and malate as substrates from the heart of Tg-BK\textsubscript{Ca} mice was also significantly (p = 0.003) reduced, in comparison to hearts only exposed to IR (n = 4) (188 ± 7 a.u./µg of mitochondria vs. 166 ± 6 a.u./µg of mitochondria, Figure 4C). Surprisingly, ROS produced from mitochondria isolated from hearts of Tg-BK\textsubscript{Ca} mice subjected to IPC showed similar levels of ROS as wild-type subjected to IPC (Figure 4C).

The ROS produced by succinate as a substrate for cardiac mitochondria isolated from Tg-BK\textsubscript{Ca} mice exposed to IPC (n = 4) was significantly (p = 0.027) decreased, in comparison to hearts exposed to IR (n = 4) (444 ± 6 a.u./µg of mitochondria vs. 417 ± 8 a.u./µg of mitochondria, Figures 4B,D). However, the
FIGURE 3 | Cardiovascular function of WT and Tg-BKCa mice. WT and Tg-BKCa mice were anesthetized and comprehensive echocardiography measurements were obtained to evaluate cardiac function. (A,B) Representative B-mode and M-mode of mice hearts from both groups. Left ventricular ejection fraction (C) and fractional shortening (D) were measured using the parasternal long axis view. There was no difference between WT (black) and Tg-BKCa (blue) mice. (E,F) Color Doppler of ascending (red) and descending (blue) aorta from both groups. The right panel shows representative images of peak velocities of ascending and descending aorta from both groups. (G) Mean (black and blue) and peak (gray and light blue) velocities of ascending aorta of WT and Tg-BKCa mice showed no difference. (H) Mean (black and blue) and peak (gray and light blue) velocities of descending aorta of WT and Tg-BKCa mice showed no difference. The values are presented as mean ± SEM of 5–6 readings.
ROS produced by succinate from the heart of WT mice exposed to IPC ($n = 4$) was similar ($p = 0.181$) to WT mice hearts exposed to IR ($n = 3$) alone (514 ± 45 a.u./µg of mitochondria vs. 471 ± 21 a.u./µg of mitochondria, Figures 4B,D). These results indicate that IPC decreases ROS levels from complex I (Figure 4C) with glutamate and malate as a substrate, and not from complex II/III (Figure 4D) where succinate was used as a substrate in wild-type mice. ROS produced in presence of glutamate and malate, or succinate was lower in Tg-BKCa mice (Figures 4C,D).

FIGURE 4 | Cardioprotection is inversely proportional to the amount of ROS produced during reperfusion. ROS produced by the mitochondria from the heart of WT (A) or Tg-BKCa (B) mice exposed to IR with or without IPC. The generation of ROS by succinate (black and gray) is higher than glutamate and malate (red and orange). (C) Quantification of total ROS produced with glutamate and malate as substrate. ROS produced by mitochondria from the heart of WT mice exposed to IR ($n = 3$) is higher, in comparison to heart exposed to IPC ($n = 4$). Similarly, ROS produced by mitochondria from the heart of Tg-BKCa mice exposed to IR ($n = 4$) is higher, in comparison to heart exposed to IPC ($n = 4$). ROS produced with glutamate and malate by mitochondria from the heart of WT mice exposed to IR is higher than Tg-BKCa heart exposed to IR. (D) Quantification of total ROS produced with succinate as substrate. Mitochondrial ROS from the heart of WT mice exposed to IR ($n = 3$) is not different, in comparison to heart exposed to IPC ($n = 4$). However, ROS produced by cardiac mitochondria isolated from the heart of Tg-BKCa mice exposed to IR ($n = 4$) is higher than heart exposed to IPC ($n = 4$). (E) The amount of ROS produced by the mitochondria isolated from the heart of BKCa and Tg-BKCa mice not subjected to IR are not different in presence of any substrate. Mitochondrial protein yield was measured, and it was between 0.3 and 0.6 µg/µL. ROS accumulated / µg of mitochondrial protein was expressed for 45 min continuously in (A,B). ROS produced a.u./µg of mitochondrial protein was expressed for 45th min in (C–E). Values are presented as mean ± SEM of 3–4 independent experiments.

We further measured ROS levels in mitochondria rapidly (29) isolated from hearts of wild-type ($n = 3$) and Tg-BKCa mice ($n = 3$) which were not subjected to any ischemic stress. Surprisingly, there were no differences observed in ROS produced by mitochondria in the presence of glutamate-malate or succinate (Figure 4E). Although the ROS produced by succinate in Tg-BKCa mice was ~30% higher, in comparison to WT mice, we did not see any statistical difference due to wide variation in the level of ROS even with 95% power. The cardioprotective effect shown here using transgenic Tg-BKCa mice is similar to that of pharmacological preconditioning using activators of BKCa (38, 39).

BKCa Co-localizes to Mitochondria of Mouse Cardiomyocytes

As stated earlier, in adult cardiomyocytes BKCa has been exclusively localized to the inner membrane of mitochondria (10, 20). Our results implicate BKCa in mitochondrial ROS.
BK channels are modulated by calcium, voltage, and several other cellular components, making them key pathophysiological targets. Auxiliary proteins, \( \beta \) and \( \gamma \) subunits also directly regulate the activity of BK\(_{\text{Ca}} \) channels, as well as, their sensitivity toward channel activators and blockers. One of the major breakthroughs in BK\(_{\text{Ca}} \)-centric research over the past two decades is the availability of small molecular openers for BK\(_{\text{Ca}} \) channels. These small molecules and pharmacological agents have provided ample opportunities to decipher the role of BK\(_{\text{Ca}} \) channels in physiological functions. The use of several pharmacological agents including, NS1619 and NS11021, to activate BK\(_{\text{Ca}} \) has been shown to play a vital role in cardioprotection and neuroprotection from IR injury. However, due to non-specific and off-target effects of drugs (40–44), clinical application of BK\(_{\text{Ca}} \) agonists is not yet initiated. Preclinical and basic research provides sufficient evidence for a translational aspect of BK\(_{\text{Ca}} \) activators but due to limitations related to specificity and the lack of potency (40–44), concerns have been raised on the role of activation of BK\(_{\text{Ca}} \) in pathological conditions (45). The need to revisit the issue related to the role of activation of BK\(_{\text{Ca}} \) in a pathological condition such as IR injury is critical in the light of recent findings that expression of BK\(_{\text{Ca}} \) is vital for cardioprotection using global (10, 32) and cardiac-specific (17) knockout mice.

In this study, we have used transgenic mice expressing the BK\(_{\text{Ca}}^{R207Q} \) mutant subunit driven by Per1 locus. Per1 is ubiquitously expressed in a wide variety of tissues and in a previous study, it was shown that Tg-BK\(_{\text{Ca}}^{R207Q} \) mice displayed overexpression of constitutively-activated BK\(_{\text{Ca}} \) in several different tissues including aorta. In adult cardiomyocytes and cardiac mitochondria isolated from Tg-BK\(_{\text{Ca}} \) mice, there were no differences observed in the localization of BK\(_{\text{Ca}} \), implying that overexpression of the BK\(_{\text{Ca}}^{R207Q} \) mutant does not interfere with its cellular or organelle localization. We have not observed any abnormal phenotype or behavior in Tg-BK\(_{\text{Ca}} \) knockout mice.
mice. Cardiovascular analysis using echocardiography also did not demonstrate abnormal cardiac function, in comparison to the wild-type mice. Hence, Tg-BK$_{Ca}$ mice provide an appropriate tool to test whether overexpression and activation of BK$_{Ca}$ play a role in cardioprotection as shown by global (10) and cardiomyocytes-specific (17) null mutant mice.

In the last two decades, several studies using pharmacological tools have suggested a protective role of BK$_{Ca}$ from IR injury and in IPC-mediated cardioprotection and are summarized in recent reviews (1, 5). The first study to implicate BK$_{Ca}$ in cardioprotection (i.e., improved LVDP and reduced infarction) from global IR injury was conducted using the agonist NS1619, the effects of which were blocked by paxilline (20). Improved LVDP by preconditioning with 3 µM NS1619 was confirmed by Stowe et al. (38) possibly by modulating mitochondrial ROS and Ca$^{2+}$ concentrations. The same group has proposed that opening of BK$_{Ca}$ elevates the level of K$^{+}$ in the matrix of mitochondria, which is exchanged for H$^{+}$ ion by a K$^{+}$/H$^{+}$ exchanger (46). Increase in the level of H$^{+}$ in the mitochondrial matrix stabilizes mitochondrial membrane potential ($\Delta$Ψ$_{m}$) so that unutilized electrons combine with oxygen to generate a small amount of mitochondrial oxygen radicals and other ROS, which then protects the cardiomyocytes by stimulating downstream protective signaling pathways. In addition to NS1619, studies involving other BK$_{Ca}$ channel openers, NS11021 (14) and naringenin (47), have also demonstrated that cellular and cardioprotection from IR injury further provide strong evidence for the role of BK$_{Ca}$ in cardioprotection. However, in primary rat cortical neurons, NS1619-preconditioning caused mitochondrial depolarization which could not be prevented by paxilline (48).

Pharmacological data using agonists and antagonists provides strong evidence for the role of BK$_{Ca}$ in cardioprotection. However, the same set of drugs have resulted in non-specific effects in cells and organs. Paxilline, although is a specific BK$_{Ca}$ blocker, abolished isoflurane-mediated cardioprotection in wild-type as well as Kcnma1$^{-/-}$ mice (12). NS1619 (>10 µM) is known to inhibit SERCA (49), mitochondrial respiratory chain (44, 50), and H$^{+}$/K$^{+}$ leak (46). These issues have largely been addressed by using low concentrations (<10 µM) of NS1619 in conjunction with global (10) and cardiomyocytes specific (17) BK$_{Ca}$ knockout mice. Our results using activated BK$_{Ca}$ reiterates and further supported the studies involving global (10, 12, 32) and cardiomyocytes specific (17) knockout mice.

Interestingly, we observed an increase in the LVDP amongst a few hearts from Tg-BK$_{Ca}$ mice exposed to IPC (4 out of 7) beyond 100% during the reperfusion stage, in comparison to the baseline. We used a constant flow approach for this experiment. We anticipate that in the presence of some degree of infarction, the remaining cardiomyocytes would contract more and beat faster to balance the pressure overload arising due to the constant flow of buffer solution to the isolated heart. An increase in LVDP and + dp/dt was also observed by Okazaki et al. after IR-injury (51). Similarly, Papanicolaou et al., also observed an increase in LDVP up to 150% in mitofusin-2 deleted hearts after 10 min of ischemia and 20 min of reperfusion ex vivo, using Langendorff model (52). Moreover, an increase in the LVDP is associated with an increase in the flow rate (51) in ex vivo IR study and BK$_{Ca}$ activator, rottlerin, is known to increase the flow rate post ex vivo IR (24).

As others have shown, we too provide evidence that BK$_{Ca}$ is exclusively present in mitochondria of adult cardiomyocytes and modulate mitochondrial function. In a recently published study, the notion of BK$_{Ca}$ in adult cardiomyocytes playing a role in cardioprotection was confirmed, and cardioprotective role via BK$_{Ca}$ in smooth muscle cells was ruled out by using tissue-specific knockouts (17). However, an argument regarding the role or necessity of activation of BK$_{Ca}$ to provide cardioprotection from IR injury is still valid. Our study involving mice expressing activated BK$_{Ca}$ without any pharmacological tools support the notion that in addition to the expression of BK$_{Ca}$, activation of BK$_{Ca}$ is also important in cardioprotection from IR injury. Expression of BK$_{Ca}^{R207Q}$ protected the heart from IR injury, as well, as improved the recovery of LVDP after IPC and IR injury. Further, we also noticed a remarkable decrease in myocardial infarction in hearts isolated from Tg-BK$_{Ca}$ mice as compared to wild-type mice. IPC as anticipated, reduced myocardial infarction in wild-type mice which was further augmented in Tg-BK$_{Ca}$ mice, implying that activation of BK$_{Ca}$ can further enhance the IPC-mediated cardioprotection from IR injury. Soltysinka et al. (32) demonstrated a cause-effect relationship between “IPC-mediated cardioprotection” and “BK$_{Ca}$” using global BK$_{Ca}$ knockout mice. Cardioprotective property of IPC was lost in the hearts collected from BK$_{Ca}$ knockout mice (31). Our results along with other studies using global or tissue-specific knockout mice imply that expression and activation of BK$_{Ca}$ play an important role in cardioprotection from IR injury.

In IR injury, ROS is well-characterized to be the major player as a second messenger involved in preconditioning (53). Complex III of the electron transport chain (ETC) is the main site for ROS production, and the ROS produced is directed away from the antioxidant defenses of the mitochondrial matrix (54). However, complex I mediated ROS products are released in the mitochondrial matrix in the proximity of defense enzyme systems and is known to change the redox state of proteins present in the mitochondrial matrix, which causes a deleterious impact on cellular physiology (55). Association of K$^{+}$ channels to mitochondrial ROS has been well-established (56, 57). Specifically, K$_{ATP}$ channels mediate influx of K$^{+}$ into the mitochondrial matrix resulting in a small augmentation of ROS production to induce cardioprotection (56, 57). In contrast, pharmacological tools aimed at BK$_{Ca}$ channels indicate that the opening of BK$_{Ca}$ reduces IR-induced large-scale ROS production whereas closing the BK$_{Ca}$ channel increases deleterious ROS production (22, 24, 58). Recently, using cardiac-specific BK$_{Ca}$ knockout mice, it was shown that the absence of BK$_{Ca}$ increases ROS (17) which has been proposed earlier to regulate endoplasmic calcium release (59). Increase in ROS in the knockout mice is independent of change in expression of ROS degrading enzymes such as CuZnSOD (SOD1) and MnSOD (SOD2) (17). We have observed that activation of BK$_{Ca}$ does not affect the ROS production at the basal levels, however, after IR injury, activation of BK$_{Ca}$ results in a reduction in ROS production. The decline was observed for both complexes of the
ETC in isolated mitochondria from Tg-BK$_{C_a}$ mice. The other factor to be taken into account is the amount in addition to the site of production of ROS available in the cell (55). IPC reduced ROS generated by complex I in wild-type mice which indicate that reducing the total amount of ROS can also protect the cardiac tissue.

Activation of BK$_{C_a}$ is also known to increase the Ca$^{2+}$ retention capacity of mitochondria (10). Our recent findings on Ca$^{2+}$ (10), and ROS in this study, and studies from other groups have indicated that BK$_{C_a}$-mediated cardioprotection involves an interplay between ROS, Ca$^{2+}$ and mitochondrial permeability transition pore (mPTP) (1, 5, 22, 31, 38, 46, 60–62). One possible mechanism is an increase in Ca$^{2+}$ retention capacity possibly by modulating a mitochondrial Ca$^{2+}$ pump on activation of BK$_{C_a}$, hence allowing more Ca$^{2+}$ uptake during ischemia-reperfusion. Also, blocking BK$_{C_a}$ channels either pharmacologically or genetically enhances ROS production, hence increasing myocardial infarction. The precise manner of how Ca$^{2+}$ modulates ROS generation is not well-understood. However, three-dimensional conformational changes induced by Ca$^{2+}$ in the ETC complexes, such as changes in complex IV have been reported (63, 64). Direct addition of BK$_{C_a}$ channel activator in isolated mitochondrial preparation results in depolarization (5, 46) of mitochondrial membrane potential which made it difficult to study mitochondrial BK$_{C_a}$ channels in isolated preparations. However, the use of genetic approaches clearly indicates that partial activation (Tg-BK$_{C_a}$) does not change mitochondrial ROS generation under physiological conditions. During stress, the increase in Ca$^{2+}$ influx could further increase the open probability of the channel which could result in a reduction of ROS as observed in our studies. Since the decrease in ROS levels has been associated with cardioprotection from IR injury (65, 66), we anticipate that activation of BK$_{C_a}$ reduces deleterious ROS production, hence decreases Ca$^{2+}$ release from endoplasmic reticulum and reduction in an influx of Ca$^{2+}$ to the matrix and prevents Ca$^{2+}$ overload in mitochondria (59). This complex interplay between Ca$^{2+}$ and ROS is known to result in apoptosis, possibly by opening the mPTP. Hence, activation of BK$_{C_a}$ can be linked to delay in the formation and/or closing of mPTP.

In summary, our study implicates overexpression of activated BK$_{C_a}$ in cardioprotection against IR injury, and cardioprotection is mediated, in part, by decreasing deleterious mitochondrial ROS generation.

LIMITATIONS OF THE STUDY

Even though, our study does not involve pharmacological tools, there are several limitations which should be mentioned. The Tg-BK$_{C_a}$ mice are not homozygous but are generated in the background of wild-type mice as the homozygous mouse is embryonic lethal (8). The animals are phenotypically normal. This finding is important as usage of heterozygous mice suggests that partial activation of BK$_{C_a}$ is sufficient for cardioprotection and reduction of mitochondrial ROS. The gain-of-function BK$_{C_a}$ is present in all the cells under Per1 locus. Since Per1 is present in most cells types, the effect we observed could also arise from non-cardiomyocytes. Non-cardiomyocyte cells such as cardiac neurons are known to play a role in cardioprotection as well as cellular protection as reported earlier (12). Our study does not rule out the role of non-cardiomyocyte BK$_{C_a}$ in cardioprotection from IR injury. Our ex vivo approach using the Langendorff method partially rules out non-cardiac BK$_{C_a}$ as the heart is excised and isolated from other organs during the experiment. Since hearts were not paced at a constant rate, ± dp/dt may not be a good index of cardiac function. Therefore, we did not report ± dp/dt. We further isolated mitochondria and measured ROS levels with and without IR. Our results also indicate that BK$_{C_a}$ channel activation can modulate mitochondrial ROS levels. We have observed statistical significance in between WT and Tg-BK$_{C_a}$ IR for LVDP, infarction, and ROS production but not in WT IR vs. IPC when one-way ANOVA followed by Tukey’s multiple comparison tests was used. This could be due to the shorter duration of reperfusion (30 min).

Tg-BK$_{C_a}$ construct is generated on DEC splice variant (8) which is known to facilitate localization of BK$_{C_a}$ to mitochondria (10), and we did not observe any change in localization of BK$_{C_a}$ in cardiomyocytes or isolated mitochondria. Therefore, we corroborate earlier findings involving pharmacological and global, as well as cardiac-specific null mutant mice, in addition to the expression of BK$_{C_a}$, activation of BK$_{C_a}$ plays an important role in cardioprotection from IR injury.

ETHICS STATEMENT

The study was carried out in accordance with the recommendations from National Institute of Health. All protocols involving animals were approved by the Drexel University College of Medicine and the Ohio State University IACUC.

AUTHOR CONTRIBUTIONS

SKG, DP, ATH, KS, PK, SGR, MK, and HS performed the research, analyzed data and wrote the manuscript. ALM generated Tg-BK and BK mutant mice.

FUNDING

This work was supported by a grant from the W. W. Smith Charitable Trust, American Heart Association National Scientist Development Grant 11SDG230059 (HS), American Heart Association Grant-in-Aid 16GRNT29430000 (HS), National Institute of Health R01-HL133050 (HS), R01-HL102758 (ALM), R01-HL136232 (MK), and Drexel University College of Medicine startup funds to HS.

ACKNOWLEDGMENTS

We sincerely thank Dr. Rajika Roy and Prof. Walter J. Koch at Center for Translational Medicine, Temple University,
Philadelphia, for their help in quantifying the level of myocardial infarction and discussions. We would also like to thank Dr. Chun-An (Andy) Chen, and Dr. Naresh Kumar from the Khan laboratory at the Ohio State University for their help with Langendorff system and microscopy, respectively. We thank Dr. Parmpreet Gill for proofreading the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.