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Cardiac *Slo2.1* is required for volatile anesthetic stimulation of K^+ transport and anesthetic preconditioning

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Abstract

Background—Anesthetic preconditioning (APC) is a clinically important phenomenon in which volatile anesthetics (VA) protect tissues such as heart against ischemic injury. The mechanism of APC is thought to involve potassium (K^+) channels encoded by the *Slo* gene family, and we showed previously that *slo-2* is required for APC in *C. elegans.* Thus, we hypothesized that a *slo-2* ortholog may mediate APC-induced cardioprotection in mammals.

Methods—A perfused heart model of ischemia-reperfusion (IR) injury, a fluorescent assay for K^+ flux, and mice lacking *Slo2.1* (Slick), *Slo2.2* (Slack) or both (double knockouts, *Slo2.x dKO*) were employed, to test whether these channels are required for APC induced cardioprotection, and for cardiomyocyte or mitochondrial K^+ transport.

Results—In wild-type (WT) hearts APC improved post-IR functional recovery (APC = 39.5 ± 3.7 % of pre-ischemic rate x pressure product, vs. 20.3 ± 2.3 % in controls, means ± SEM, p=0.00051, non-paired two-tailed t-test, n=8) and lowered infarct size (APC = 29.0 ± 4.8 % of LV area, vs. 51.4 ± 4.5 % in controls, p=0.0043, n=8). Protection by APC was absent in hearts from *Slo2.1*$^{-/-}$ mice (% recovery APC = 14.6 ± 2.6 vs. 16.5 ± 2.1 % in controls, p=0.569, n=8–9, infarct APC = 52.2 ± 5.4 % vs. 53.5 ± 4.7 % in controls, p=0.865, n=8–9). APC protection was also absent in *Slo2.x dKO* hearts (% recovery APC = 11.0 ± 1.7 vs. 11.9 ± 2.2 % in controls, p=0.725, n=8, infarct APC = 51.6 ± 4.4 % vs. 50.5 ± 3.9 % in controls, p=0.855, n=8).

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Meanwhile, *Slo2.2*$^{-/-}$ hearts responded similar to WT (% recovery APC = 41.9 ± 4.0 vs. 18.0 ± 2.5 % in controls, p=0.00016, n=8, infarct APC = 25.2 ± 1.3 % vs. 50.8 ± 3.3 % in controls, p<0.000005, n=8). Furthermore, VA stimulated K^+ transport seen in cardiomyocytes or mitochondria from WT or *Slo2.2*$^{-/-}$ mice, was absent *Slo2.1*$^{-/-}$ or *Slo2.x dKO*.

Conclusion—Slick (*Slo2.1*) is required for both VA stimulated K^+ flux and for the APC induced cardioprotection.

Introduction

Clinically-relevant doses of halogenated volatile anesthetics (VA) can protect tissues such as the heart from ischemia and reperfusion (IR) injury,[1–3] a phenomenon known as "anesthetic preconditioning" (APC). APC is evolutionarily conserved from *C. elegans*[4] to humans,[3] and current AHA/ACC guidelines specifically recommend the use of VAs during cardiac surgery for their protective effects.[5]

The mechanism of APC is complex, and involves many of the same effector signaling pathways as ischemic preconditioning (IPC), including protein kinases C[6] and A,[7,8] inhibitory G-proteins,[9] adenosine,[10,11] nitric oxide,[12] and mitochondrial ROS generation.[13,14] These signals are thought to converge at the level of mitochondria through distinct K^+ channels, but the molecular identity of these channels is a subject of debate.

Several K^+ channel types have been proposed to exist in mitochondria, including: ATP sensitive (K_{ATP}),[15] voltage sensitive (K_V)[16] and large conductance (BK) channels of the *Slo* gene family,[17] as well as a K^+/H^+ exchanger (KHE)[18] (see [19,20] for review). Current evidence favors an involvement of mitochondrial K_{ATP} channels in IPC,[21] while a mitochondrial *Slo* channel is thought to underlie APC.[7,22,23]

The mammalian *Slo* channel family comprises *Slo1* (*KCNMA1*), *Slo2.1* (*KCNT2, Slick*), *Slo2.2* (*KCNT1, Slack*) and *Slo3* (*KCNU1*).[24] *Slo3* expression is germline restricted[24,25] but the others are widely expressed. Since pharmacologic *Slo1* activators can protect the heart against IR injury,[17,26] it was widely assumed that a mitochondrial Slo1 channel (also termed BK, K_{Ca} or BK_{Ca}) was responsible for APC. However, we recently showed that both *C. elegans* and mice lacking *Slo1* can still be protected by APC, and mitochondria from these organisms contain a K^+ channel activated by VA.[22]

We have also previously demonstrated that the K_{Ca} channel Slo2 is required for VA induced protection of *C. elegans* against IR injury, with *Slo2* mutant nematodes also lacking VA-stimulated mitochondrial K^+ flux.[22] Thus, we hypothesized that one of the mammalian *Slo2* orthologs, Slick or Slack (which are K_{Na}, not K_{Ca} channels[24,24,27]), may underlie APC in mammals. Herein we used novel gene deleted mice (*Slo2.1*$^{-/-}$, *Slo2.2*$^{-/-}$, and *Slo2.x* double knockout) to conclusively demonstrate that *Slo2.1* is the channel required for APC and for VA stimulated K^+ flux in cardiomyocytes and mitochondria.

Methods

(*Cut from 5.5 to 4 pages. Changes too numerous to list. Only requested additions are noted in red).

Animals

Mice were housed in an AAALAC-accredited pathogen-free facility with food and water available *ad libitum*. All procedures were approved by the University of Rochester's Committee on Animal Resources (protocol #2010-030) in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (2011 revision). A *Slo2.x dKO* strain containing both mutant Slo2.x alleles[28] was provided by Dr. Chris Lingle, Ph.D (Washington University, St. Louis MO). Mice were out-crossed >6x to a C57/BL6 background, and conventional breeding was employed such that experimental WT and knockout mice (males, 8–10 weeks old) were littermates. All animals were genotyped by tail clip PCR (Kapa Biosystems, Woburn MA)[29] with the following primers: *Slo2.1* (Slick), C523-24C 5′-AACTTTATGAGTTCCTCTTCCATG-3′, C320-24F 5′-GAGCATCATACTTTGCTTTTTGGG-3′, KO=269bp, WT=579bp; *Slo2.2* (Slack) C311-30 5′-CCCATTCCACACTGCAGCCCTGTCTCTTTC-3′, C315-30 5′-TGTTTACTAGGGTCCAGGGAGAACCTATGA-3′, KO=200bp, WT=607bp. Due to personnel limitations (i.e., same person handling mice and doing experiments), it was not feasible to blind the experimenter to animal genotype. However, for all experiments mice of varying genotypes were randomly assigned to experimental groups, with treatments in randomized order across experimental days.

Ex-vivo perfused heart

Mouse hearts were perfused as previously described.[22,26,29] Briefly, following tribromoethanol anesthesia (100mg/kg ip) the aorta was rapidly cannulated and perfused without pacing at a constant flow of 4 ml/min/100mg with Krebs-Henseleit buffer (KH, in $mmol \cdot L^{-1}$: 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 10 D-glucose, 1.2 mM $MgSO_4$, 1.2 KH_2PO_4, and 2.5 $CaCl_2$, gassed with 95% O_2, 5% CO_2 at 37°C). Left ventricular pressure was measured via a water-filled transducer-linked LDPE balloon. LV and coronary root pressures were monitored and digitally recorded at 1 kHz (DATAQ, Akron OH). Parameters calculated from the LV balloon throughout perfusion included: heart rate, systolic and end diastolic pressures, LV developed pressure (systolic minus diastolic), rate x pressure product, dP/dT_{MAX} (contraction) and dP/dT_{MIN} (relaxation).

Hearts were equilibrated for 20 min. prior to initiating data acquisition. 163 total hearts were analyzed, and none were excluded from the study. A 30 min. normoxic perfusion was followed by 30 min. of no flow global ischemia, then 60 min. of reperfusion (see Figure 1). Treatment groups were: (i) Control IR injury. (ii) IPC comprising 3 cycles of 5 min. ischemia plus 5 min. reperfusion, to replace the 30 min. perfusion. (iii) 10 min. isoflurane (Henry Schein animal health, Dublin OH) infusion (100 $\mu mol \cdot L^{-1}$ or ~0.35 MAC for a C57/BL6 mouse.[30,31]), (iv) 20 min. 7-Chloro-3-methyl-4H-1,2,4-benzothiadiazine 1,1-dioxide (Diazoxide, a K^+ channel activator[32]; Sigma, St. Louis MO) infusion (30 $\mu mol \cdot L^{-1}$). (v) 10 min. 2,2′-sulfanediylbis(4,6-dichlorophenol) (Bithionol a Slo channel activator[33]; TCI

America, Portland OR) infusion (2.5 nmol·L^{-1}). Pharmacologic agents were delivered via syringe pump just above the perfusion cannula for the indicated times, followed by 30 sec. washout prior to index ischemia. At the end of IR protocols, hearts were sliced, stained in 1% (wt/vol) 2,3,5-triphenyltetrazolium chloride (TTC) for 20 min., and fixed in 10% neutral buffered formalin for 24 hr. Heart slices were imaged and infarct size analyzed by planimetry as previously described.[22,26,29]

Plasma membrane thallium (Tl^+) flux assay on isolated mouse cardiomyocytes and HEK293 cells

Mouse adult ventricular cardiomyocytes were isolated by collagenase perfusion as previously described[26]. Briefly, following anesthesia (tribromoethanol as above), hearts were cannulated and perfused with isolation buffer (IB) at 37 °C (IB; in mmol·L^{-1}: 120 NaCl, 15 KCl, 0.6 Na_2HPO_4, 0.6 KH_2PO_4, 1.2 $MgSO_4$, 10 HEPES, 4.6 $NaHCO_3$, 30 taurine, 5.5 D-glucose, and 10 2,3-butanedione monoxime (BDM), pH =7.4) for <2 minutes before switching to digestion buffer (35 ml of IB plus $CaCl_2$ 12.5 μmol·L^{-1} final, trypsin 400 μL at 2.5% (w/v), collagenase A 6.525 units, collagenase D 15.375 units), and 8 minutes later placed in 2–3 ml of stop buffer (IB plus $CaCl_2$ 12.5μmol·L^{-1}, 10% heat inactivated FBS). Cardiomyocytes were dissociated and filtered through 75 μm mesh, then gravity sedimentation and resuspension were used to bring [Ca^{2+}] step-wise to 1.8mmol·L^{-1}, and the final pellet was placed into 1 ml of Minimal Essential Media (MEM; GIBCO, Grand Island NY). Cell viability and yield were determined using Trypan blue and a hemocytometer, and preparations with >85% viable rod-shaped cells were used experimentally. Cells were seeded (2500 cells/well) in a Falcon 96 well plate and incubated at 37 °C for 1 hr, then loaded with Thallos reagent (2 μmol·L^{-1}; Teflabs, Austin TX) in 65 μL Hanks Buffered Salt Solution (HBSS; GIBCO, Grand Island NY), and monitored for thallium (Tl^+) uptake by addition of 4 mmol·L^{-1} final Tl_2SO_4 in stimulus buffer (SB, in mmol·L^{-1}: 276 Na^+ gluconate, 2.6 $CaSO_4$, 1.6 $MgSO_4$, 11.2 D-glucose, 40 HEPES, pH 7.3) in a BioTek Synergy plate reader in kinetic mode measuring every 7 s. (λex 488 nm; λem 525 nm) in the presence or absence of channel modulators. N-benzyl-N-(3-isobutoxy-2-pyrrolidin-1-yl-propyl)aniline (Bepridil, Sigma, St. Louis MO) is a calcium channel blocker that also inhibits Slo2 channels[33]. Eight measurements were taken prior to additions. Paired control wells contained Tl^+ free SB, enabling determination of Tl^+-specific fluorescence changes.

HEK 293 cells were seeded on glass 12-mm coverslips and transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) with the expression vector pKT122 expressing an mCherry::rat Slick cDNA fusion in a pcDNA5/TO vector backbone (Invitrogen, Carlsbad CA). After 24 hours, cells were incubated in 1 ml of Hanks Buffered Saline Solution containing 2 μmol·L^{-1} Thallos reagent (TefLabs, Austin TX) for 30 min., then transferred to an open flow perfusion rig attached to a Nikon TE2000 microscope equipped with a monochromator (Till Photonics, Germany), CCD camera (PCO-TECH, Romulus MI) and appropriate filter sets. Transfected cells were identified via mCherry fluorescence and perfused with SB (as described above), with or without Slo2 channel modulators, then SB with 4 mmol·$L^{-1}$$Tl_2SO_4$, and images acquired every 5 s. (λex 488 nm; λem 535 nm).

Changes in fluorescent emission were quantified for all cells in the field of view, both transfected and untransfected, using TILLvisION software (TILL Photonics).

Mitochondrial Thallium (Tl^+) flux assay on isolated mouse heart mitochondria

Following anesthesia (tribromoethanol as above), mitochondria were isolated from 3 pooled mouse hearts, loaded with BTC-AM (Life Technologies, Carlsbad CA) and monitored for Tl^+ uptake (a surrogate for K^+ channel flux) as previously described.[22,29,34]

Isoflurane dosing

In perfused hearts, isoflurane infusion via syringe pump afforded no possibility for volatilization before entering the heart. For C57BL/6 mice, 100 μmol·L^{-1} isoflurane equates to ~0.35 MAC.[30,31] For cell and mitochondrial experiments, incubations generally lasted <1min. such that isoflurane volatilization was considered negligible. As such, isoflurane concentrations are denoted as “initial”.

Statistical analysis

Significance was determined using a two-way ANOVA; and when warranted post-hoc pair-wise t-testing (two-tailed) analysis using Bonferroni multiple comparisons correction. P values reported in the results are from t-tests, with $p<0.05$ (before multiple comparisons correction) considered statistically significant. Sample size was determined based upon experience and previous studies.

Results

Cardiac anesthetic preconditioning requires *Slo2.1* (Slick)

In *C. elegans*, *Slo2* is encoded by a single gene, and contributes to VA-stimulated mitochondrial potassium flux and APC.[22] However, in mammals *Slo2* has diverged into two paralogs (*Slo2.1* and *Slo2.2*) with differing ion sensitivity to the *C. elegans* channel. Using a recently developed mouse strain with the genes coding for Slick (*Kcnt2* or *Slo2.1*) and Slack (*Kcnt1* or *Slo2.2*) both deleted,[28] our goal herein was to determine if either or both of these mammalian gene products are orthologous to the worm *Slo2* channel, and facilitate K^+ flux across the mitochondrial inner membrane in response to VA. First, we verified using genomic PCR that the *Slo2.x* double knockout (dKO) contained the expected lesions in each of the two genes, and that each allele was unambiguously detectable (Figures 2A and 2B). Next, we used Western blot analysis to query whether the channels themselves were similarly ablated. Slick and Slack are abundantly expressed in neural tissue, and our results demonstrate that we can readily detect both paralogs in brain lysates as well as their absence in the dKO background (Figure 2C). Finally, we report that *Slo2.x dKO* mice were viable and had normal cardiac electrical function (Table S1).

To interrogate the individual contribution of each Slo2.x paralog to APC, the mutant alleles were genetically separated through extensive backcrossing to a C57BL/6J background. *Ex-vivo* perfused hearts from wild type (WT) and *Slo2* ablated mice (both the *Slo2.x dKO* and single mutant alleles) were then subjected to IR injury with optional APC (isoflurane in perfusion media). Injury was assessed by measuring post-IR functional recovery (Figure 3A)

and infarct size (Figure 3B). APC was protective in WT hearts, with a 95% improvement in the post-IR recovery of Rate Pressure Product (RPP; heart rate x left ventricular developed pressure, p=0.0005) and a 44% reduction in infarct size (p=0.0043), vs. control hearts. *Slo2.2*$^{-/-}$ mice exhibited a similar degree of protection by APC (132 % improvement in recovery of RPP, p=0.00016, 50% reduction in infarct size, p=0.000005) vs. control hearts. However, APC in *Slo2.1*$^{-/-}$ mice resulted in no protection (12% worse recovery of RPP, p=0.057, 2% reduction in infarct size, p=0.817, compared to IR alone). As expected, APC in *dKO* mice was similarly ineffective (8% worse recovery of RPP, p=0.725, 2% increase in infarct size, p=0.854, compared to IR alone). None of the genotypes exhibited significant differences in baseline susceptibility to ischemia.

Cardioprotection by ischemic preconditioning (IPC) or the mK$_{ATP}$ channel opener diazoxide (DZX) are independent of Slo2.x channel genotype

To discount the possibility that lack of protection by APC in the *Slo2.1*$^{-/-}$ or *dKO* hearts was due to an inability of these hearts to be protected at all, we examined two additional cardioprotective treatments that are unrelated to VA: namely IPC (Figure 4) and the mitochondrial K_{ATP} channel opener diazoxide (DZX) (Figure 5).

The improvement in functional recovery (RPP) induced by IPC for each genotype was: WT 148%, *dKO* 184%, *Slo2.1*$^{-/-}$ 108%, *Slo2.2*$^{-/-}$ 193%, compared to IR alone, p<0.025 for all genotypes (Figure 4A). The reduction in infarct size induced by IPC was: WT 53%, *dKO* 34%, *Slo2.1*$^{-/-}$ 45%, *Slo2.2*$^{-/-}$ 49%, compared to IR alone, p<0.0025 for all genotypes (Figure 4B). Similar to IPC, DZX delivered consistent cardioprotection across all genotypes. Namely, the improvement in functional recovery (RPP) induced by DZX: WT 98%, *dKO* 171%, *Slo2.1*$^{-/-}$ 125%, *Slo2.2*$^{-/-}$ 143%, compared to IR alone, p<0.01 for all genotypes (Figure 5A). The reduction in infarct size induced by IPC was: WT 37%, *dKO* 45%, *Slo2.1*$^{-/-}$ 55%, *Slo2.2*$^{-/-}$ 48%, compared to IR alone, p<0.001 for all genotypes (Figure 5B). Thus, cardioprotection by either IPC or DZX was consistent regardless of genotype, suggesting *Slo2.1*$^{-/-}$ hearts are still capable of being protected by these interventions and that cardioprotection, even via mechanisms thought to involve mitochondria, does not invariably require Slick.

In addition to functional recovery measured as rate x pressure product, data for dP/dT_{MAX} anddP/dT_{MIN}, LV developed pressure, and LV end diastolic pressure, for all genotypes and treatment regimens, are provided in Supplemental Tables 2, 3, and 4 respectively.

VA stimulated K^+ transport in cardiomyocytes and mitochondria requires *Slo2.1* (Slick)

Given the proposed role of a mitochondrial large conductance K^+ channel in APC,[7,22,23] we examined VA-stimulation of K^+ flux in both primary cardiomyocytes and isolated cardiac mitochondria, using a thallium (Tl^+) based fluorescence assay.[22,34] All four genotypes were studied at the isolated mitochondrial level, but due to our initial results (vide supra) indicating that Slick but not Slack is involved in APC, studies in cardiomyocytes were limited to WT and *Slo2.1*$^{-/-}$.

The VA isoflurane was found to stimulate K^+ flux in cardiomyocytes (Figure 6A) as well as mitochondria (Figure 6B) from WT mice, and this effect was blocked by the inhibitor

bepridil. Bepridil is thought to target cardiac K_{Na}[35] and other channels, [36] and has recently been shown to function as an Ebola anti-viral.[37] Its use here was motivated by its ability to inhibit Slo2 channels[33], which we validated using recombinant Slick expressed in HEK 293 cells (Figure S1). Notably, VA stimulation of K^+ flux was absent in both cardiomyocytes and mitochondria from *Slo2.1*$^{-/-}$ hearts (Figure 6A/B). At the mitochondrial level, the Tl^+ flux pattern in *Slo2.2*$^{-/-}$ was similar to WT, and the pattern in *dKO* was similar to *Slo2.1*$^{-/-}$. These data indicate that isoflurane induces a *Slo2.1* dependent K^+ flux in both cardiomyocytes and mitochondria.

To substantiate the hypothesis that K^+ flux and cardioprotection occur through Slick activation, we turned to a second distinct reagent. Bithionol has been shown to block Slo type channels,[33] and inhibits recombinant Slick expressed HEK293 cells (Figure S1). At the cardiomyocyte level, bithionol induced a bepridil-sensitive K^+ flux in WT cells that was absent in *Slo2.1*$^{-/-}$ cells (Figure 6C). In mitochondria, bithionol induced a bepridil-sensitive K^+ flux in WT that was absent in the *dKO* mitochondria and reduced in both the *Slo2.1*$^{-/-}$ and *Slo2.2*$^{-/-}$ mitochondria (Figure 6D). The cell data fully support our hypothesis. However, the mitochondria data suggest that both Slick and Slack partially contribute to bithionol-stimulated Tl^+ uptake, which is potentially interesting (see Discussion). However, it is likely that bithionol stimulates other channel activities or even mitochondrial function through unknown mechanisms of action, and very low levels of *Slo2.2* are expressed in the heart.[28]

Finally, additional support for our hypothesis came from the observation that bithionol was cardioprotective in WT hearts (Figure 7A/B: 129% improvement in RPP recovery, p=0.0098, 57% reduction in infarct size, p=0.00008, compared to IR alone). This protection was partially lost in *Slo2.1*$^{-/-}$ hearts (bithionol induced 97% improvement in RPP recovery, p=0.028, 14% reduction in infarct size, p=0.15). Overall these data support a K^+ transport pathway with the genetic and pharmacologic characteristics of Slick, being required for the cardioprotective effects of APC.

Discussion

Herein we demonstrated that the K_{Na} channel Slick, encoded by the *Slo2.1* gene, is required for cardioprotection by APC in mice. We also showed that Slick is responsible for VA-stimulated K^+ flux at both the cardiomyocyte plasma and mitochondrial membranes, and that pharmacologic Slick openers are cardioprotective.

While Slo2 is a K_{Ca} channel in *C. elegans*, in mammals it has diverged into two paralogs, Slick and Slack, that are K_{Na} channels.[24,24,27] These channels may contribute to various physiologic functions attributed to K_{Na} channels, including metabolic sensing and neuronal plasticity,[24,27,38] and although the major focus of K_{Na} channel studies to date has been neuronal, K_{Na} channels are found in cardiac myocytes.[39] Notably, they have not been reported in mitochondria. While these results cement a cardioprotective role for Slick, the relative contribution of plasma membrane vs. mitochondrial Slick channels to this effect remains unclear, and is a topic of ongoing research.

Many signaling pathways such as anesthetic preconditioning (APC) and ischemic preconditioning (IPC) are evolutionarily conserved,[22] and there are many redundancies in their signaling cascades. For example, the mechanism of APC and IPC share commonalities such as protein kinase C activation,[12,40] reactive oxygen species (ROS) generation,[14,41–43] and mitochondrial K^+ channels.[44–46] While the precise mechanism of APC is not well understood, evidence suggests that APC involves both altered mitochondrial function and K^+ channel activity (reviewed in [47]).

Studies have implicated the mitochondrial ATP-sensitive K^+ channel (mK_{ATP}) in APC largely through the use of non-specific measures of channel activity (e.g., flavoprotein fluorescence) and drugs that are known to have off-target mitochondrial effects (e.g., diazoxide or 5-hydroxydecanoate).[44–46,48,49] While these pharmacologic approaches suggest the involvement of the mK_{ATP}, the effects could have also been due to another K^+ channel such as Slick. The current lack of a molecular identity for the mK_{ATP} channel precludes a definitive answer to this dilemma.

Moreover, the data presented herein do not rule out a potential cross-talk between the mK_{ATP} and Slick. For example, since both diazoxide- and IPC-mediated cardioprotection were preserved in *Slo2.1*$^{-/-}$ mice, Slick activation may be upstream of mK_{ATP} channel activation. Overall, while there are many similarities between APC and IPC, there is also evidence for distinct signaling pathways[50], and herein we show that Slo2.1 appears to be involved only in APC, not IPC.

In the field of APC, the canonical BK channel Slo1 which may be present in cardiac mitochondria[51] emerged as an early candidate effector of APC protection.[52–54] However, the importance of this channel was recently questioned by evidence that both IPC and APC protection are robust in *Slo1* ablated organisms.[22] While the role of Slo1 in the heart remains to be fully elucidated, it is important to emphasize that our data do not preclude co-existence of Slo1 and Slick in cardiomyocytes or cardiac mitochondria. Rather, they conclude that only Slick is required for APC in the heart.

Our results are consistent with findings in *C. elegans* which showed that SLO-2 was required for APC.[22] Despite the large sequence similarity (~74% identical) [38] between Slick and Slack, we found no role for Slack in cardiac APC or IPC. This is in agreement with the low expression levels of *Slo2.2* in the heart.[27] However, this does not rule out a role for *Slo2.2* in protecting other tissues from ischemia. Both *Slo2.1* and *Slo2.2* are highly expressed in the nervous system[24] and there is evidence that they may co-assemble into channels.[55] In this respect, it is intriguing that both Slick and Slack appeared to contribute to bithionol-induced K^+ flux in purified mitochondria (Figure 6D). However, this was the only condition where both Slick and Slack were additive in our hands. Moreover, the mechanism of action of bithionol is unknown, but its activity as an antihelmintic may rely on suppressing succinate oxidation.[56] Hence, it is premature to conclude that Slack contributes to mitochondrial function. Nevertheless, it is possible that in other tissues such as the brain, *Slo2.2* may play a role protecting against stress.

It is also interesting to speculate that Slick did not evolve to be opened by volatile anesthetics, and given our results we speculate that it may have a physiologic role in the mitochondrion. While some mitochondrial K^+ channels are demonstrated to play roles in patho-physiology (e.g., protection from IR injury),[19] most are only reported at the phenomenological level as mitochondrial swelling or K^+ flux sensitive to activators/inhibitors, with very little known about the normal function of these channels. The activation of mitochondrial K^+ channels is hypothesized to modulate mitochondrial matrix volume, Ca^{2+} uptake capacity, and ROS production,[57] and while these events are involved in the pathophysiology of IR injury, they also have effects on regular mitochondrial function and metabolism. For example, Ca^{2+} stimulates oxidative phosphorylation, and matrix volume can determine the efficiency of high energy phosphate transport between the mitochondrion and cytosol. Ongoing research in our lab is aimed at determining the role of Slick in regulating physiologic mitochondrial function.

A potential limitation of this study is the low dose of isoflurane (~0.35 MAC) used to elicit cardioprotection. It is unclear if higher doses, or other volatile anesthetics (e.g., desflurane, sevoflurane) will exhibit similar *Slo2.1* dependent cardioprotective effects. Nevertheless, we note that the current widespread clinical use of VAs, and the ACC/AHA recommendations for their use during cardiac surgery,[5] suggests that the identification of Slick as a mediator of APC may drive development of novel cardioprotective drugs targeting this channel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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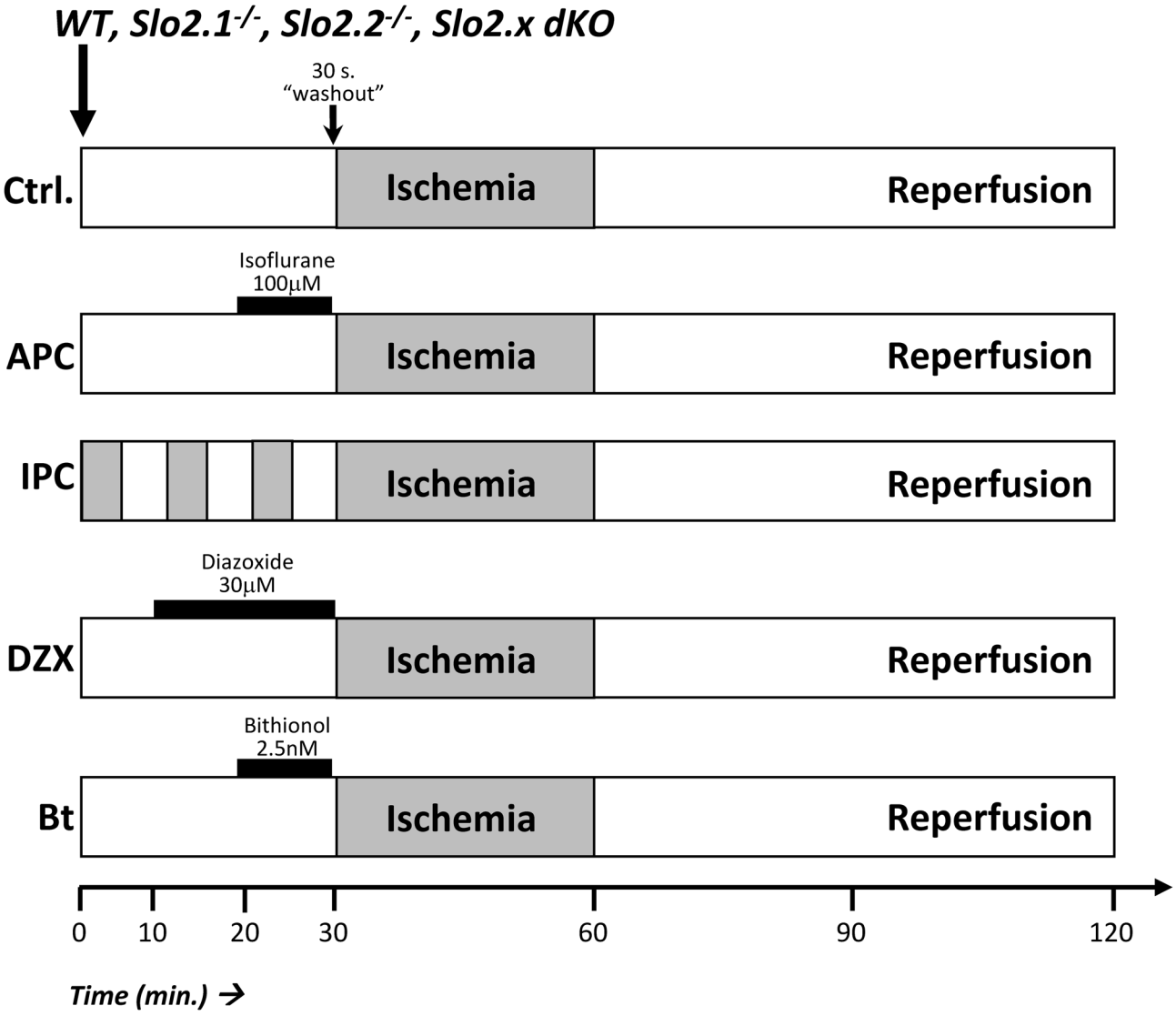

Figure 1. Experimental Protocol

Schematic shows the 5 perfused heart experimental conditions tested. Gray shading represents ischemia. 30s. washout was performed after administration of pharmacologic agents, before ischemia.

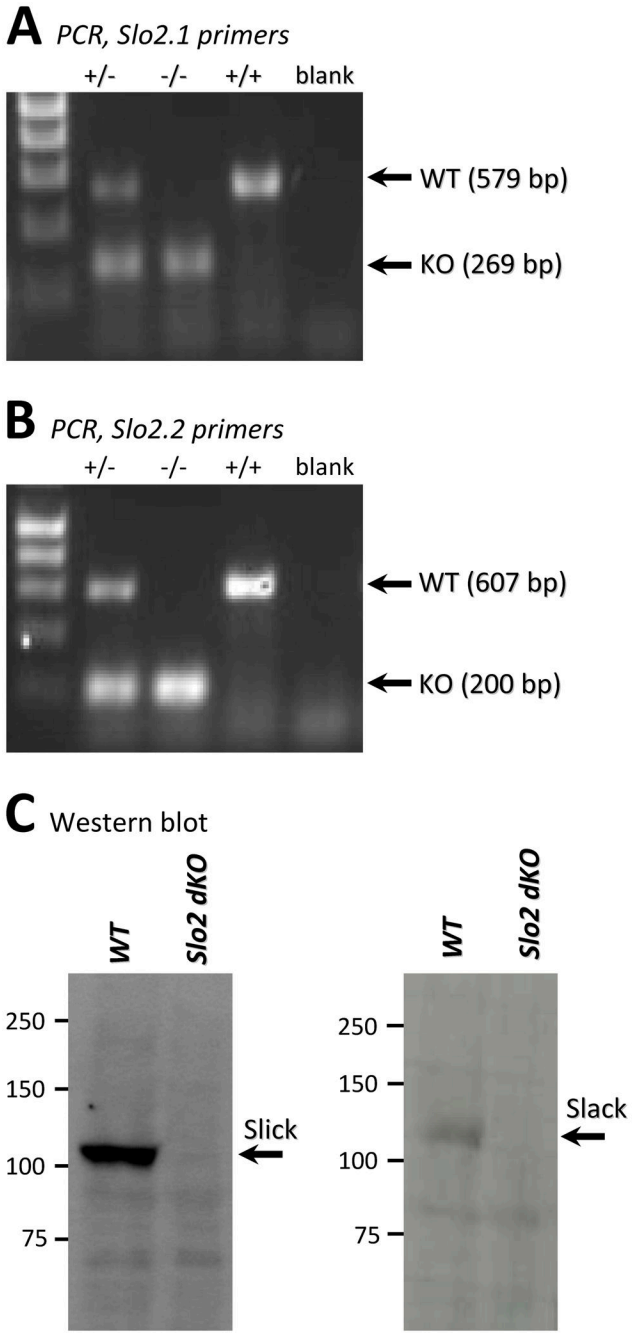

Figure 2. *Slo2.1* and *Slo2.*2 genotyping and protein expression analysis of Slick and Slack in *Slo2.x dKO* mice

(A): Tail clip PCR showing *WT* or knockout *Slo2.1* products at the indicated mass, for WT (+/+), knockout (−/−), or heterozygous (+/−) animals. Primers were as described in the methods. Left lane = DNA ladder. **(B):** As in panel A, but with *Slo2.2* primers. **(C):** Western blot for Slick and Slack (Neuromab antibody) on brain tissue lysates from wild-type (WT) and *Slo2.x dKO* mice.

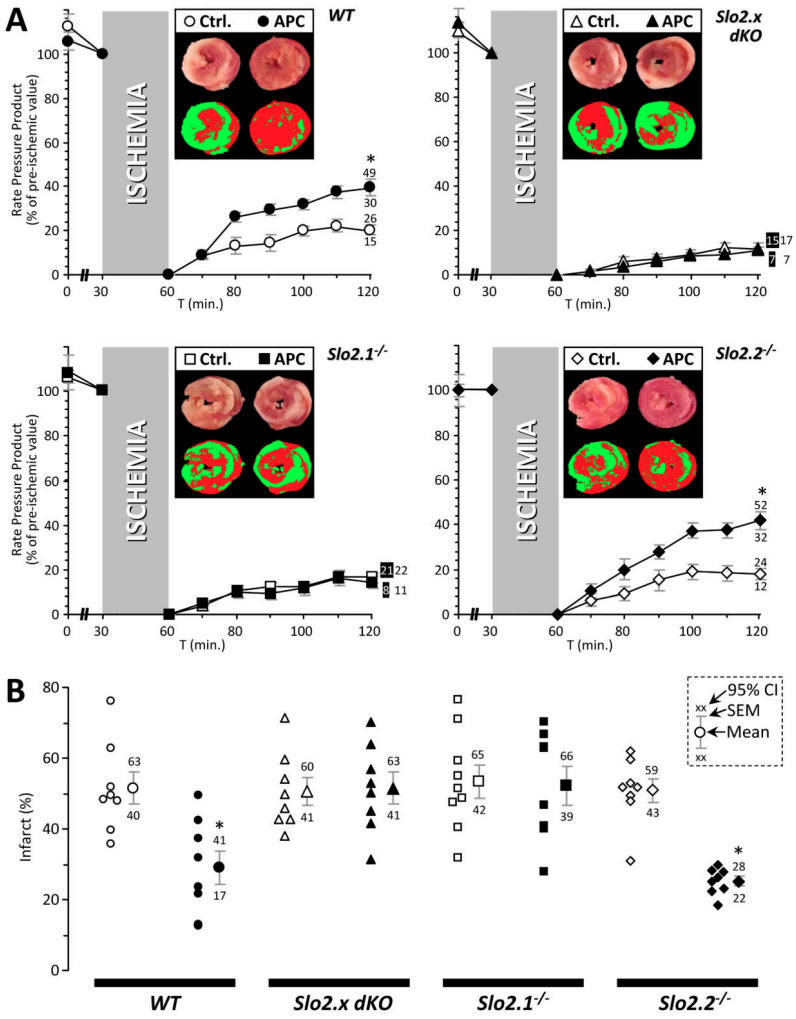

Figure 3. Anesthetic preconditioning in wild-type (WT) and *Slo2.x* knockout mouse hearts

Langendorff perfused hearts from *WT* (circles), *Slo2.x dKO* (triangles), *Slo2.1*$^{-/-}$ (squares) and *Slo2.2*$^{-/-}$ (diamonds) were subjected to ischemia-reperfusion (IR) injury alone (open symbols) or IR with anesthetic preconditioning (APC) comprising 100 μmol·L^{-1} isoflurane infusion for 10 min. prior to ischemia (filled symbols). **(A):** Cardiac function data (RPP, product of heart rate x left ventricular developed pressure) throughout perfusion. RPP is expressed as a percentage of the value immediately prior to ischemia. Data are means ± SEM, with 95% confidence intervals for the 60 min. reperfusion time point shown adjacent to error bars (numbers shaded where appropriate to indicate which data set they belong to – see inset to panel B). **Insets:** representative heart cross-sections stained with TTC (upper images), and threshold pseudo-colored images used to quantify infarct size (lower images). **(B):** Infarct size for control IR and APC+IR treated hearts from each genotype. Within each group, individual data points are on the left, thereby indicating the number of replicates (N). Means ± SEM are on the right, with 95% confidence intervals shown adjacent. Inset key shows position of mean, SEM, and 95% CIs on the graphs. *Statistically significant difference between control IR and APC+IR groups at 60 min. of reperfusion (ANOVA, with post-hoc unpaired t-test).

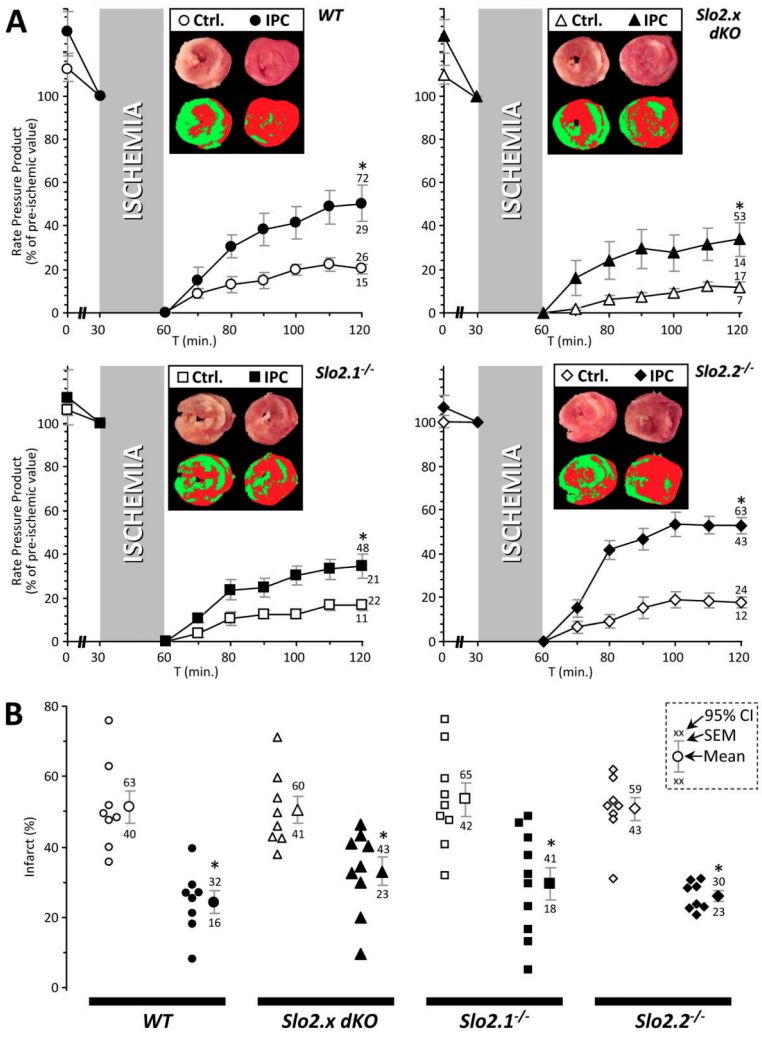

Figure 4. Cardioprotection by ischemic preconditioning (IPC) in wild-type (WT) and *Slo2.x* knockout mouse hearts

Langendorff perfused hearts were subjected to control ischemia-reperfusion (IR) injury alone (open symbols) or IR+IPC (filled symbols). Symbols for genotypes are the same as Figure 3. Data for controls (IR alone, no treatment) are reproduced from Figure 3 and shown for comparative purposes. **(A):** Cardiac function data (RPP, product of heart rate x left ventricular developed pressure) throughout perfusion. RPP is expressed as a percentage of the value immediately prior to ischemia. Data are means ± SEM, with 95% confidence intervals for the 60 min. reperfusion time point shown adjacent to error bars (see inset to panel B). **Insets:** representative heart cross-sections stained with TTC (upper images), and threshold pseudo-colored images used to quantify infarct size (lower images). **(B):** Infarct size for control IR and IPC+IR treated hearts from each genotype. Within each group, individual data on the left, thereby indicating the number of replicates (N). Means ± SEM are on the right, with 95% confidence intervals shown adjacent. Inset key shows position of mean, SEM, and 95% CIs on the graphs. *Statistically significant difference between control IR and IPC+IR groups at 60 min. of reperfusion (ANOVA, with post-hoc unpaired t-test).

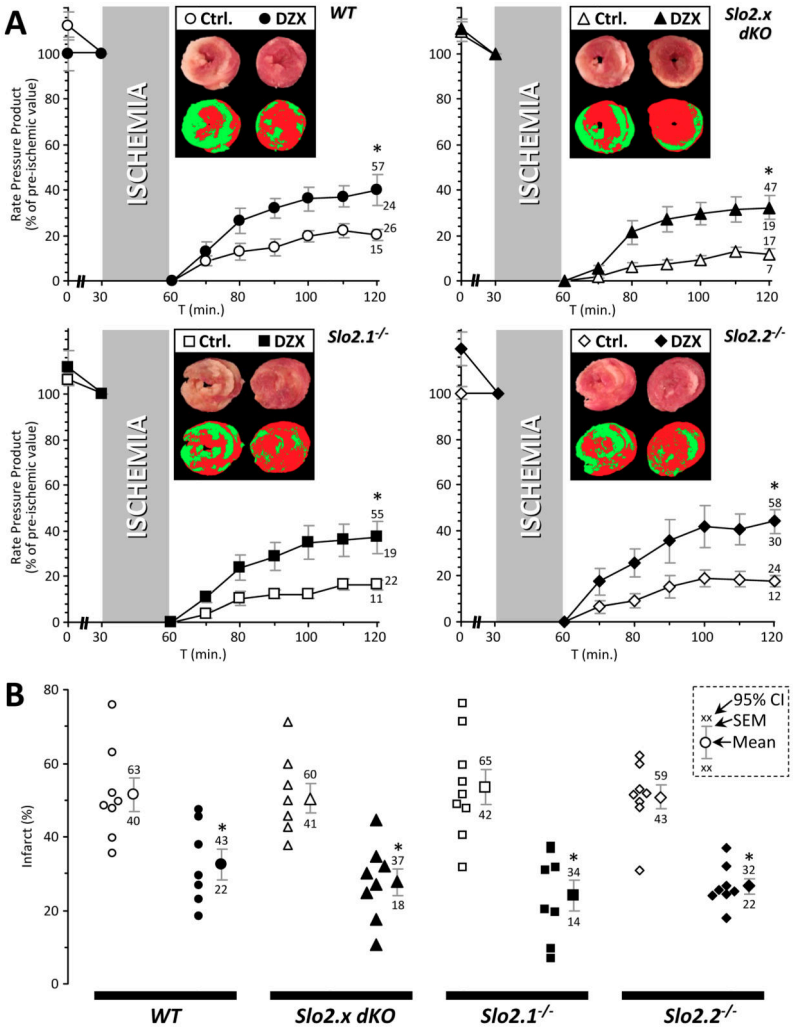

Figure 5. Cardioprotection by diazoxide in wild-type (WT) and Slo2.x knockout mouse hearts

Langendorff perfused hearts were subjected to control ischemia-reperfusion (IR) injury alone (open symbols) or diazoxide+IR (DZX, filled symbols). Symbols for genotypes are the same as Figure 3. Data for controls (IR alone, no treatment) are reproduced from Figure 3 and shown for comparative purposes. **(A):** Cardiac function data (RPP, product of heart rate x left ventricular developed pressure) throughout perfusion. RPP is expressed as a percentage of the value immediately prior to ischemia. Data are means ± SEM, with 95% confidence intervals for the 60 min. reperfusion time point shown adjacent to error bars (see inset to panel B). **Insets**: representative heart cross-sections stained with TTC (upper images), and resulting pseudo-colored images calculated via threshold masks and used to quantify infarct size (lower images). **(B):** Infarct size for control IR and DZX+IR treated hearts from each genotype. Within each group, individual data are shown on the left, thereby indicating the number of replicates (N). Means ± SEM are on the right, with 95% confidence intervals shown adjacent. Inset key shows position of mean, SEM, and 95% CIs on the graphs. *Statistically significant difference between control IR and DZX+IR groups (ANOVA, with post-hoc unpaired t-test).

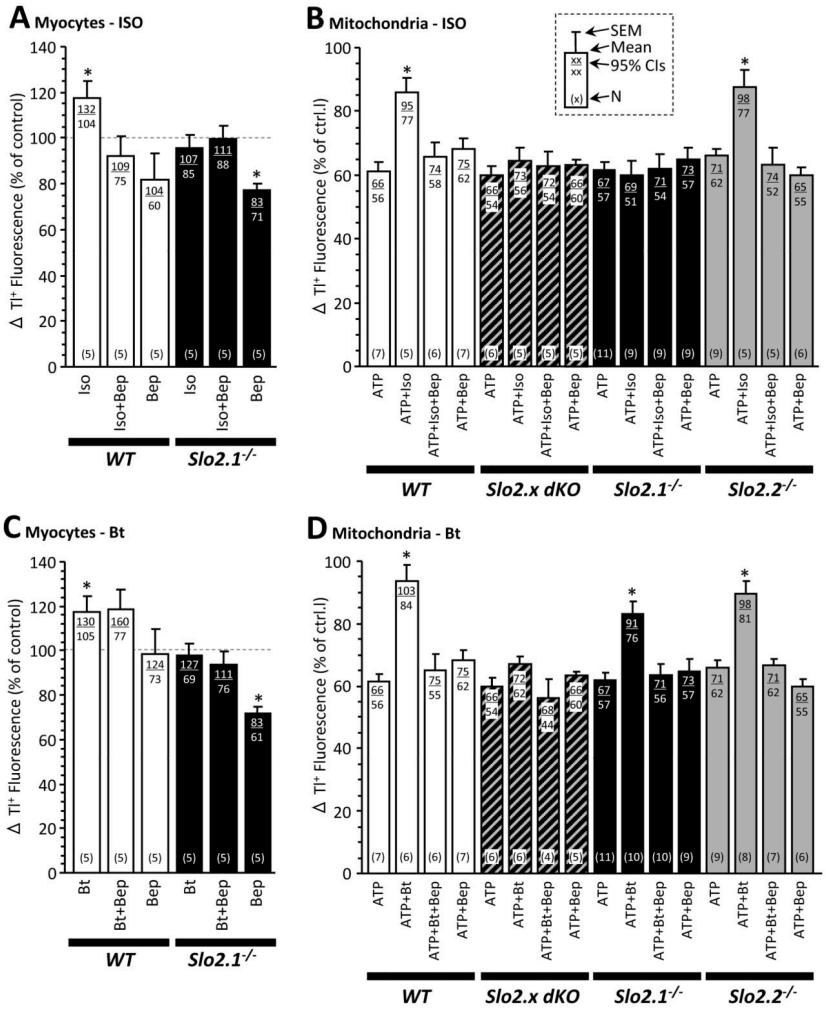

Figure 6. Thallium (Tl^+) flux in cardiomyocytes and mitochondria from mice of varying Slo2 genotype

(A): Cardiomyocytes were isolated from wild-type (WT) (white bars) and *Slo2.1*$^{-/-}$ (black bars) mice, and surface K^+ channel activity assayed via thallium (Tl^+) uptake. Where indicated, isoflurane (Iso, 150 μmol·L^{-1} initial) and/or bepridil (Bep, 10 μmol·L^{-1}) were added. Data are normalized to control (no addition, dashed line), and are means ± SEM. 95% confidence intervals are shown at top, number of replicates (N) at bottom, of each graph bar – see inset key in panel B. *Statistically significant difference versus control within a given genotype (ANOVA, with post-hoc unpaired t-test). **(B):** Mitochondria were isolated from WT (white bars) *Slo2.x dKO* (hashed), and *Slo2.1*$^{-/-}$ (black), and *Slo2.2*$^{-/-}$ (gray) hearts, and K^+ channel activity assayed via Tl^+ uptake. ATP was present throughout to block mK_{ATP} channels. Where indicated, isoflurane (Iso, 300 μmol·L^{-1} initial) and/or bepridil (Bep, 10 μmol·L^{-1}) were added. Data are normalized to control (no addition), and are means ± SEM. 95% confidence intervals are shown at top, number of replicates (N) at bottom, of each graph bar – see inset key. *Statistically significant difference versus ATP condition within a given genotype (ANOVA, with post-hoc unpaired t-test). **(C):** Cardiomyocyte Tl^+ flux, as in panel A, but stimulated with bithionol (Bt, 0.25 μmol·L^{-1})

instead of isoflurane. Data are normalized to control (no addition, dashed line), and are means ± SEM. 95% CIs and N as per inset key in panel B. *Statistically significant difference versus control within a given genotype (ANOVA, with post-hoc unpaired t-test). **(D):** Mitochondrial Tl^+ flux as in panel B, but stimulated with bithionol (Bt, 2.5 μmol·L^{-1}) instead of isoflurane. Data are normalized to control (no addition), and are means ± SEM. 95% CIs and N as per inset key in panel B. *Statistically significant difference versus ATP condition within a given genotype (ANOVA, with post-hoc unpaired t-test).

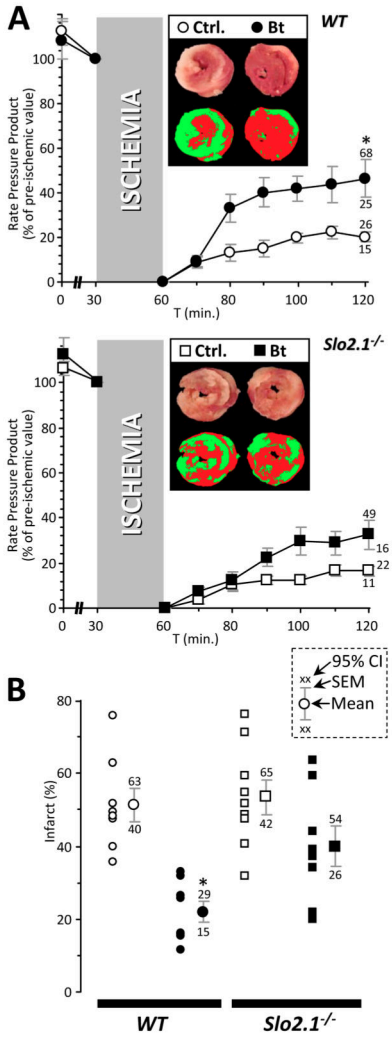

Figure 7. Effect of bithionol on IR injury outcomes in wild-type (WT) and *Slo2.1* knockout mouse hearts

(A): Langendorff perfused hearts from *WT* (circles) and *Slo2.1*$^{-/-}$ (squares) were subjected to ischemia-reperfusion (IR) injury alone (open symbols) or IR with bithionol (Bt, 2.5 nM for 20 min. prior to the onset of ischemia, filled symbols), as detailed in the methods and in Figures 3–5. The control groups are reproduced from Figure 3 and shown here for comparative purposes. RPP is expressed as a percentage of the value immediately prior to ischemia. Data are means ± SEM, with 95% confidence intervals for the 60 min. reperfusion time point shown adjacent to error bars (see inset, panel B). **Insets**: representative heart cross-sections stained with TTC (upper images), and resulting pseudo-colored images calculated via threshold masks and used to quantify infarct size (lower images). **(B):** Infarct size for control IR and Bt+IR treated hearts from each genotype. Within each group, individual data points are shown on the left, thereby indicating the number of replicates (N). Inset key shows position of mean, SEM, and 95% CIs on the graphs. *Statistically significant difference between control IR and Bt+IR groups at 60 min. of reperfusion (ANOVA, with post-hoc unpaired t-test).