

Impact of Administration Time and Kv7 Subchannels on the Cardioprotective Efficacy of Kv7 Channel Inhibition

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Purpose: The mechanism of cardioprotection by Kv7.1–5 (KCNQ1-5) channels inhibition by XE991 is unclear. We examined the impact of administration time on the cardioprotective efficacy of XE991, the involvement of key pro-survival kinases, and the importance of the Kv7 subchannels.

Methods: Isolated perfused rat hearts were divided into five groups: 1) vehicle, 2) pre-, 3) post- or 4) pre- and post-ischemic administration of XE991 or 5) chromanol 293B (Kv7.1 inhibitor) followed by infarct size quantification. HL-1 cells undergoing simulated ischemia/reperfusion were exposed to either a) vehicle, b) pre-, c) per-, d) post-ischemic administration of XE991 or pre-, per- and post-ischemic administration of e) XE991, f) Chromanol 293B or g) HMR1556 (Kv7.1 inhibitor). HL-1 cell injury was evaluated by propidium iodide/Hoechst staining. Pro-survival kinase activation of Akt, Erk and STAT3 in XE991-mediated HL-1 cell protection was evaluated using phosphokinase inhibitors. Kv7 subtype expression was examined by RT-PCR and qPCR.

Results: XE991, but not Chromanol 293B, reduced infarct size and improved hemodynamic recovery in all isolated heart groups. XE991 protected HL-1 cells when administered during simulated ischemia. Minor activation of the survival kinases was observed in cells exposed to XE991 but pharmacological inhibition of kinase activation did not reduce XE991-mediated protection. Kv7 subchannels 1–5 were all present in rat hearts but predominately Kv7.1 and Kv7.4 were present in HL-1 cells and selective Kv7.1 did not reduce ischemia/reperfusion injury.

Conclusion: The cardioprotective efficacy of XE991 seems to depend on its presence during ischemia and early reperfusion and do not rely on RISK (p-Akt and p-Erk) and SAFE (p-STAT3) pathway activation. The protective effect of XE991 seems mainly mediated through the Kv7.4 subchannel.

Keywords: cardioprotection, Kv7 channels, myocardial ischemia reperfusion injury, myocardial infarction

Introduction

Pharmacological and interventional treatment modalities together with optimized prehospital triage have reduced mortality from acute myocardial infarction (MI).^{1–3} However, ischemic heart disease remains a major cause of mortality and morbidity in the Western world. Adjunctive strategies to improve outcome after MI addressing ischemia-reperfusion (IR) injury are needed. Ischemic preconditioning (IPC), introduced in 1986 by Murry et al, reduces IR injury by four 5 min cycles of coronary

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occlusions interspersed by reperfusion prior to sustained ischemia.⁴ The protective mechanisms of IPC are complex and involve the activation of multiple pathways converging on mitochondria.^{5,6} Among the best-described pathways are the Reperfusion Injury Salvage Kinase (RISK) pathway⁷ and the Survivor Activating Factor Enhancement (SAFE) pathway.⁸ Cardioprotection can also be achieved by pharmacological conditioning targeting the protective signaling pathways evoked by ischemic conditioning.^{9–11}

The Kv7.1 voltage-gated potassium channel is involved in cardiac excitability.^{12,13} The potential role(s) of other Kv7 family members (Kv7.2–5) for cardiac function is subtle. Recently, we discovered that pharmacological inhibition of Kv7.1–5 by XE991 and linopirdine protects the heart from IR injury.¹⁴ Flupirtine, an activator of Kv7.2–5, has also been reported to protect against cardiac IR injury when administered prior to the onset of ischemia.¹⁵ Both studies implicate that Kv7 channels are involved in IR injury. However, the results are not in agreement, which may be explained by the difference in the timing of Kv7 channel modulation. We therefore investigated whether Kv7 channel inhibition by XE991 is cardioprotective by pre-, per-, post- and combined pre-, per- and post-ischemic administration.

The protective effects of Kv7 channel inhibition is additive to protection by IPC suggesting that Kv7 channel inhibition may act independently of the interactive pathways of protection by IPC.¹⁴ We therefore investigated whether the RISK and SAFE pathways are involved in cardioprotection by Kv7 channel inhibition.

Furthermore, the importance of each Kv7 subchannels in cardioprotection is poorly defined. Chromanol 293B and HMR1556 selectively inhibits Kv7.1 but selective inhibition of the other Kv7 subchannels is not feasible due to lack of inhibitors. We investigated Kv7 subchannel expression and the effects of Kv7.1 inhibition in ischemia/reperfusion in both cardiac tissues and HL-1 cells (mouse atrial cardiomyocytes).¹⁶

Methods

Study Design

Isolated perfused rat hearts (n=8–10 in each group) and HL-1 cells were used in the study (Figure 1). The hearts were randomized into 5 groups; 1) vehicle, 2) pre-, 3) post-, 4) pre-, per- and post-ischemic administration of XE991 in addition to 5) pre-, per- and post-ischemic administration of Chromanol 293B. Hearts were allowed to stabilize for 20 min and then exposed to perfusion according to group

allocation. All hearts were subjected to 40 min ischemia and 2 hrs reperfusion prior to biochemical staining with 2,3,5-triphenyl-2H-tetrazolium chloride (TTC). In the pre-, per- and post-ischemic administration groups, Kv7 channels were inhibited from 25 min prior to ischemia and throughout the perfusion protocol (Figure 1A).

In the HL-1 cell experiments, we compared a) vehicle with the effect of XE991 administration b) pre-ischemic, c) per-ischemic, d) post-ischemic, and e) pre-, per- and post-ischemic on cell survival (Figure 1B). Additionally, selective inhibition of Kv7.1 with f) Chromanol 293B and g) HMR1556 was evaluated with pre-, per- and post-ischemic administration. Furthermore, we tested whether the effect of pre-, per- and post-ischemic XE991 administration on the IR injury in HL-1 cells was affected by concomitant administration of the phosphokinase inhibitors PD98059, AG490 and Wortmannin (groups h-m) (Figure 1B). Cells were allowed to stabilize for 1 hr prior to 5.5 hr of simulated ischemia and reperfusion. The cells were harvested for Western blotting after 1 hr of simulated reperfusion or after 2 hrs for cell death evaluation by propidium iodide (PI) and Hoechst staining.

Langendorff Perfused Rat Heart

We used 10–12 weeks old, male Wistar rats (Taconic Biosciences, Ry, Denmark) weighing approx. 300 g. The animals were fed a standard chow (Altromin 1324), provided with free access to water and food, and kept in a pathogen-free environment with a 12 hr light/dark cycle. The Danish Animal Experiments Inspectorate, Copenhagen, Denmark approved the study, which followed the Guide for the Care and Use of laboratory Animals published by the National Institutes of Health (NIH publication no. 85–23, revised 1996). Rats were anaesthetized by a mixture of Fentanyl citrate (0.158 mg/kg body weight (bw)), Fluanisone (7.5 mg/kg bw) and Midazolam (0.5 mg/kg bw). The surgical procedure was initiated when the pedal withdrawal and righting reflexes were absent. A tracheotomy was performed followed by sternotomy and in situ cannulation of the aorta. Perfusion of the heart with 37°C Krebs-Henseleit (KH) buffer solution (NaCl: 118.5 mM, KCL: 4.7 mM, NaHCO₃: 25 mM, Glucosemonohydrate: 11.1 mM, CaCl: 2.4 mM, KH₂PO₄:1.2 mM) continuously saturated with 5% CO₂/95% O₂ was immediately commenced to ensure continuous perfusion of the heart. The heart was quickly excised and connected to the perfusion setup. The left atrial appendage was removed to allow for the insertion of a fluid-filled latex

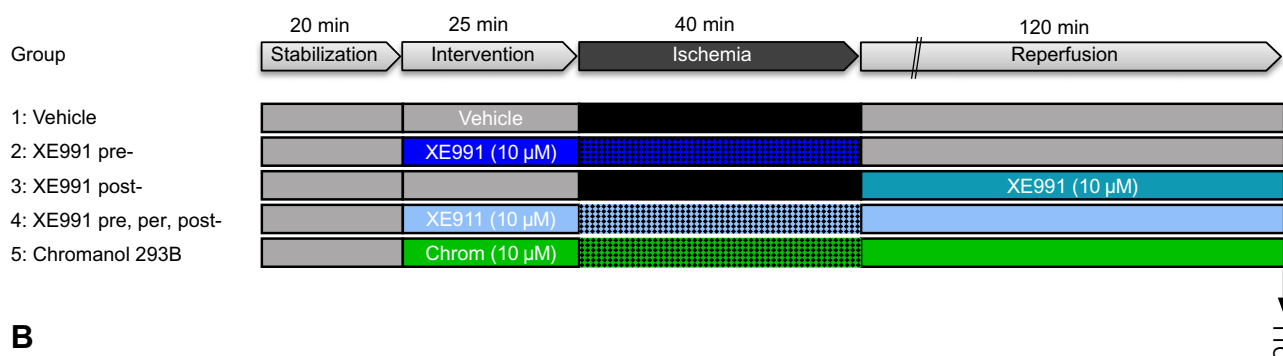
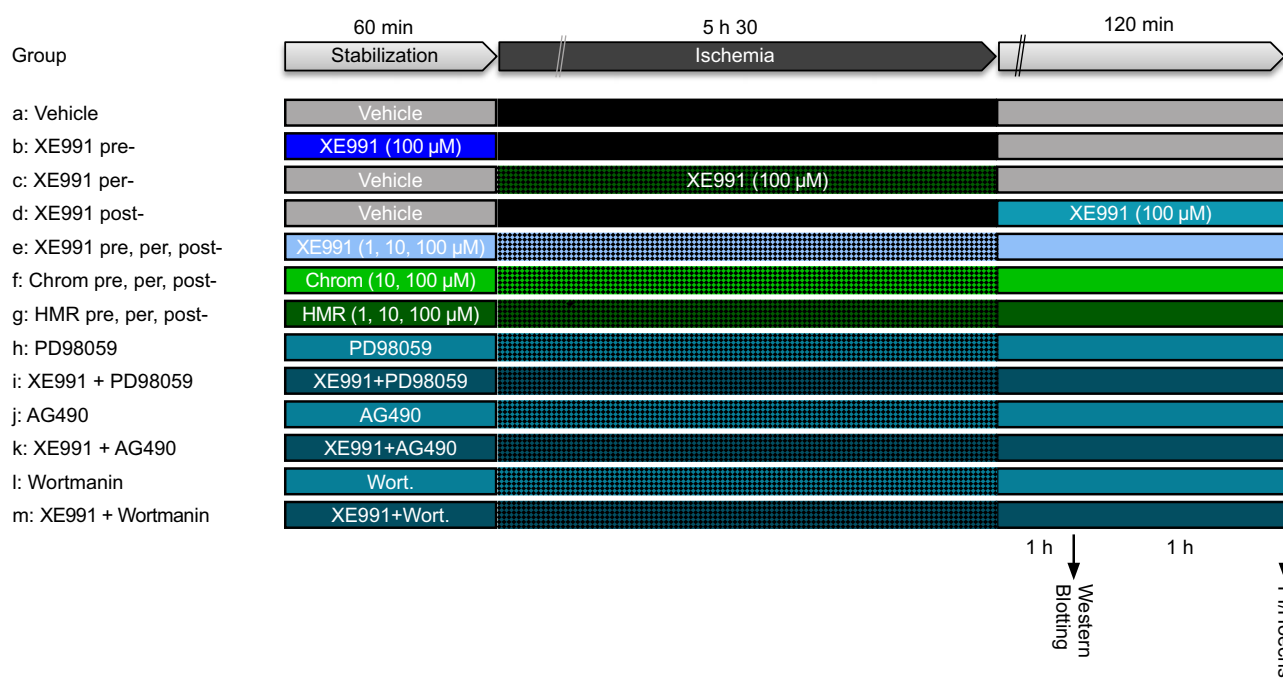
A**Evaluation of IR-injury in isolated rat hearts (n=8-10)****B****Evaluation of IR-injury in HL-1 cells**

Figure 1 Perfusion protocols for the perfused rat heart experiments (**A**) and protocol for the HL-1 cell experiments (**B**). In the isolated heart study (**A**), hearts were exposed to 20 min stabilization followed by perfusion with vehicle, 10 µM XE991 or 10 µM Chromanol 293B according to group allocation. All hearts were subjected to 40 min ischemia and 2 hrs reperfusion with vehicle, XE991 or Chromanol 293B. In the HL-1 cell experiments (**B**) cells were allowed to stabilize for 1 hr prior to 5.5 hrs of anoxia and 2 hrs of reoxygenation. Inhibitors of the Reperfusion Injury Salvage Kinase (RISK) and Survivor Activating Factor Enhancement (SAFE) pathways were co-administered according to the group. Cells were harvested for Western blotting after 1 hr or cell death quantification (PI/Hoechst) after 2 hrs of reoxygenation. Vehicle: DMSO 1 mL/L. XE991: Kv7.1-5 blocker. HMR1556 and Chromanol 293B (Chrom): Specific Kv7.1 blocker. PD98059: Erk1/2 inhibitor. AG490: JAK-STAT inhibitor. Wortmannin (Wort.): PI3K inhibitor.

balloon (Hugo Sachs Elektronik, size 7) connected to a pressure transducer. The ventricular balloon was filled to obtain a diastolic pressure of 8 mmHg. The coronary flow rate was measured by an inline flow-probe (Transonic System Inc., Ithaca, NY, USA). The heart was submerged in 37°C KH buffer through the perfusion protocol.

Baseline hemodynamics and coronary flow rate were evaluated after 19 min of stabilization and hearts were excluded if the heart rate was $<180 \text{ min}^{-1}$, the left ventricle

developed pressure (LVDP) was $<130 \text{ mm Hg}$ and/or the coronary flow rate was $<10 \text{ mL min}^{-1}$ or $>20 \text{ mL min}^{-1}$.

Infarct Size and Area-at-Risk

At the end of the perfusion protocol, hearts were immediately frozen in a -80°C freezer. A phosphate buffer containing 1% TTC was prepared and kept at 37°C. Frozen hearts were cut into 1.5 mm thick slices using a Rat Heart Slicer (Zivic Instruments, Pittsburgh, USA) and submerged for 3 min in

the TTC solution. Subsequently, slices were kept in 4% formaldehyde for 36 hrs and scanned on a flatbed photo scanner (EPSON V600, Suwa, Japan) to obtain high-resolution TIFF-images. The images were analyzed using imageJ software and manual delineation of infarcted and total slice areas was performed in a blinded manner. Total infarct size (IS) was calculated as infarct area/area-at-risk in each heart slice weighted by the relative mass of the heart slice.

Measurement of Hemodynamics

The left ventricular pressure was continuously measured using a fluid-filled intraventricular balloon connected through a water link to a pressure transducer (Micron Instruments, Simi Valley, CA, USA) providing real-time registration of isovolumetric pressures. LVDP was calculated as systolic – diastolic pressure. Data were digitally converted (DT9804 – Data Translation, Marlboro, MA, USA) and continuously monitored/stored using Notocord Hem Software (Notocord Systems, Croissy sur Seine, France).

HL-1 Cell Experiments

The HL-1 cells used in this study maintained the ability to undergo spontaneous contraction which is a special feature of the HL-1 cell phenotype as described by Claycomb.¹⁶ Cells were cultivated and subjected to 5.5-hrs simulated ischemia and 2 hrs reperfusion as previously described.¹⁷ In short, the cells ($\sim 1 \times 10^6$ cells/well) were seeded into six-well plates (n=3-5 wells for each group) and cultivated for 48 hrs prior to induction of ischemia. Upon induction of ischemia, the growth medium was substituted by a stabilizing buffer solution (CaCl₂: 1.25 mM, MgCl: 1mM, NaCl: 137 mM, KCl: 6 mM, Glucosemonohydrate: 10 mM, HEPES: 6 mM and NaH₂PO₄: 0.9 mM). During simulated ischemia, the buffer was changed to an acidic (pH 6.8) ischemia buffer containing lactate and deprived of glucose and oxygen (CaCl₂: 1.25 mM, MgCl: 1 mM, NaCl: 120 mM, KCl: 8 mM, HEPES: 6 mM, NaH₂PO₄ 0.9 mM, Na-Lactate 20 mM). The respective blockers of the RISK pathway (PD98059 and Wortmannin) and SAFE pathway (AG490) and insulin were added to the buffers according to the treatment group. Insulin served as a positive control of the Akt component of the RISK pathway.^{18–22}

Cell death was quantified with propidium iodide (PI) and Hoechst staining and excitation/emission were measured using a PHERAstar FS microplate reader (BMG LABTECH, Ortenberg, Germany). Optical modules

measured PI excitation/emission at 530/620 nm and Hoechst excitation/emission at 350/460nm.

Chemicals

XE991 (10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone), Chromanol 293B (trans-N-[6-Cyano-3,4-dihydro-3-hydroxy-2,2-dimethyl-2H-1-benzopyran-4-yl]-N-methyl-ethanesulfonamide) and HMR1556 (N-(6-Cyano-3-hydroxy-2,2-dimethyl-3,4-dihydrochromen-4-yl)-N-methylethanesulfonamide) were used to inhibit Kv7 channels in the Langendorff and cell experiments. XE991 is reported to have an IC₅₀ of approximately 1–3 μ M against Kv7 channels^{23,24} and Chromanol 293B to have an IC₅₀ of approximately 2 μ M.²⁵ PD98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) was used to inhibit mitogen-activated protein kinase (MEK) 1/2 and extracellular signal-regulated kinase 1/2 (Erk1/2) phosphorylation. Wortmannin was used to block phosphatidylinositol (4,5)-bisphosphate-3-kinase inhibition (PI3K) hereby blocking protein kinase B (Akt) phosphorylation. Akt and Erk phosphorylation are both components of the RISK pathway of cell survival. The SAFE pathway was inhibited using AG490 ((E)-2-Cyano-3-(3,4-dihydrophenyl)-N-(phenylmethyl)-2-propenamide) as an inhibitor of Janus kinase 2 (JAK2)/STAT3 phosphorylation. Dimethyl Sulfoxide (DMSO) was used as a solvent. In the HL-1 cell experiments, the water-soluble XE991 was used. All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) except for the water-soluble XE991 (purchased from Tocris Bioscience.)

Western Blots

HL-1 cells were harvested by scraping 60 min after the onset of simulated reperfusion using M-PER mammalian protein extraction reagent (Thermo Scientific, Waltham, MA, USA #78,501) containing phosphatase inhibitor cocktail 2 and 3 (Sigma Aldrich #P5726, # P0044) and complete mini tablet (Roche Diagnostics GmbH #11,836,153,001). The cell lysates were kept at –20°C. Plasmalemma was degraded by submerging the cell lysates in liquid nitrogen followed by heating to 65°C. This cycle was repeated 3 times interspersed by vortexing and followed by centrifugation at 10,000 g for 10 min. Total protein content in the supernatant was measured using a Pierce 660 nm protein assay (Thermo Scientific #22,660). Each sample was diluted to an equal protein concentration of 30 μ g protein per 100 μ L dilution. Samples were heated to 65°C for 15 min to denature proteins. Equal amounts of samples (4.5 μ g protein) were loaded per well on an Any kD™ Criterion™ TGX Stain-Free™ Protein Gel (Bio-Rad #567-8125) followed by gel electrophoresis for

40 min at 250 V. Protein size was measured by comparison to the loaded protein ladder (Spectra™ Multicolor Broad Range Protein Ladders, Thermo Scientific #26,623). Gel activation and total protein measurement were performed using the ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA) and the gel was blotted onto a nitrocellulose membrane using a trans blot turbo transfer system. The nitrocellulose membrane was subsequently blocked in 5% skimmed milk powder in TBS-T for 1 hr before washing in TBS-T. The membrane was cut transversely according to relevant markers and incubated with primary antibodies against phosphorylated and non-phosphorylated Akt, Erk and STAT3 (antibodies are specified below) at 4°C overnight.

The following day the membrane was washed in TBS-T and incubated with a secondary antibody (polyclonal HRP-488 conjugated goat anti-rabbit immunoglobulin, DAKO #P0448) for 1 hr at room temperature. The membrane was washed in TBS-T and TBS before activation of the nitrocellulose membrane using Amersham ECL™ Prime Western Blotting detection reagent (GE Healthcare #2232). The membrane was analyzed using the ChemiDoc™ MP Imaging System. Western blots were normalized to total protein measured by the Stain-Free technology.²⁶

The primary antibodies used in this study were all purchased from Cell Signaling Technology (Danvers, MA, USA). Akt (pan) (C67E7) Rabbit mAb #4691, Phospho-Akt (Ser473) (D9E) XP Rabbit mAb #4060, p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb #4695, Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP Rabbit mAb #4370, STAT3 (79D7) Rabbit mAb #4904, Phospho-STAT3 (Tyr705) (D3A7) XP Rabbit mAb #9145.

Reverse Transcription PCR for Kv7 Subtypes

RT-PCR was performed to identify specific Kv7 channel subtypes. Brain and heart tissue from rat and mouse were, together with HL-1 cells, examined by reverse transcription PCR (RT-PCR). Brain tissue (incl. frontal, parietal and cerebellum) was included as all 5 Kv7 subtypes are present in the brain.²⁷ All tissues were snap-frozen in liquid nitrogen and kept at -80°C until analysis. Total RNA was purified from brain tissue using a Qiagen RNeasy minikit (Qiagen #74,104) and from heart tissue and HL-1 cells using TRIzol Reagent (Ambient #15,596,018). Conversion to cDNA was performed with a RevertAid First Strand cDNA Synthesis kit (Fischer Scientific #K1622) using 1

µg total RNA from tissue samples and cultured cells, respectively. For all Kv7 subtypes, RT-PCR was performed using 1 µL of cDNA as template, a GoTaq G2 DNA polymerase (Promega #M7841) and the following PCR program: 94°C, 3 mins for 1 cycle, 94°C for 30 s, the primer-specific annealing temperature (see [table s1](#)) for 30 s, and 72°C for 20 s for 35 cycles, and 72°C for 7 mins for 1 cycle. Beta-2 microglobulin (*B2m*) was used as a reference gene for successful cDNA synthesis. For the *B2m*-specific RT-PCR, 1 µL cDNA was used as a template for all tissue and cell samples using the following PCR program: 94°C, 3 mins for 1 cycle, 94°C for 30 s, 60°C for 30 s, and 72°C for 25 s for 35 cycles, and 72°C for 7 mins for 1 cycle. All primers and the annealing temperatures used for RT-PCR are listed in [table s1](#).

Quantitative Polymerase Chain Reaction

Total RNA was purified from HL1-cells as well as rat and mouse heart and brain tissue as described for RT-PCR (see above). cDNA was synthesized from 1 µg of purified total RNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). For qPCR, samples (2 µL cDNA) were subjected to PCR amplification using the Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Fisher Scientific #K0222) on an Aria Mx3000P qPCR System (Agilent Technologies, Santa Clara, CA). Standard curves were generated by diluting cDNA from rat brain or mouse brain successively and applying it into separate wells. The qPCR protocol consisted of 40 cycles of denaturation (30 s at 95°C), annealing and synthesis (60 s at 59°C). The primers used were identical to the primers used for RT-PCR for the individual target genes (see [table s1](#)). Rat and mouse *B2m* were used as reference genes.

Calculations and Statistical Analyses

All data are expressed as median (range). Due to relatively small sample size, we used Kruskal-Wallis non-parametric test with Dunn's post-hoc test for multiple comparisons. GraphPad PRISM 7.05 (GraphPad Software, La Jolla, California, USA) was used for statistical analysis.

Results

Cardioprotective Effects of Kv7.1-5 Inhibition in Rat Hearts

XE991 (Kv7.1-5 inhibition) reduced median IS compared to vehicle (57.3 (range: 46.0-83.6)) when administered

pre-ischemically (36.0 (11.5–81.5), $p=0.02$), post-ischemically (35.2 (23.7–70.9), $p=0.05$) and both pre- and post-ischemically (30.7 (10.1–47.2), $p=0.009$) (Figure 2A). Cardioprotection by XE991 was supported by increased recovery in LVDP after 20 mins of reperfusion in hearts treated with XE991 ($p=0.006$) (Figure 2B).

Cardioprotective Effects of Kv7.1 Channel Inhibition in Rat Hearts

Chromanol 293B (10 μM) (Kv7.1 inhibition) did not reduce IS (69.6 (41.5–82.1)) compared to vehicle (57.3 (46.0–83.6), $p=0.5$) (Figure 2A). The post-ischemic recovery in LVDP was similar in the Chromanol 293B and vehicle groups (Figure 2B).

Cytoprotective Effect in HL-1 Cells

XE991 (100 μM) administration prior to ischemia did not significantly change PI/Hoechst ratio (1.12 (range: 1.09–1.14) compared to control (0.99 (0.98–1.03), $p=0.33$). PI/Hoechst in cells exposed to XE991 (100 μM) administration during simulated ischemia was 0.80 (0.69–0.84, $p=0.06$) and XE991 (100 μM) administration throughout the IR-protocol reduced PI/Hoechst ratio to 0.58 (0.56–0.60, $p=0.006$). XE991 (1 μM) and XE991 (10 μM) did not significantly reduce PI/Hoechst (0.98 (0.97–1.06);

$p=0.91$ and 0.84 (0.81–0.89); $p=0.13$). No reduction in PI/Hoechst was observed by post-ischemic XE991 administration (0.97 (0.88–1.04); $p=0.68$) (Figure 3A).

KV7.1 inhibition did not reduce PI/Hoechst using 10 μM Chromanol 293B (0.88 (0.85–1.25), $p>0.99$), 100 μM Chromanol 293B (1.13 (1.10–1.19), $p>0.99$), 1 μM HMR 1556 (1.08 (0.90–1.27), $p>0.99$), 10 μM HMR 1556 (0.99 (0.86–1.20), $p>0.99$) or 100 μM HMR 1556 (0.88 (0.78–1.11), $p>0.99$) compared to vehicle (1.00 (0.99–1.01)) (Figure 3B).

Protein Kinases

XE991 did not significantly increase pAkt/Akt (2.17 (range: 1.44–2.88), $p = 0.36$) but pErk/Erk was significantly increased (2.16 (2.11–2.20), $p<0.001$) compared to vehicle (0.78 (0.36–2.19) and 1.05 (0.84–1.12), respectively). Insulin was used as a positive control for pAkt/Akt upregulation and increased pAkt/Akt (7.48 (6.79–7.94), $p=0.002$) (Figure 4A and B). pSTAT3/STAT3 was not increased by XE991 (0.49 (0.41–0.57), $p=0.03$) but rather slightly suppressed compared to vehicle (1.00 (0.77–1.15)) (Figure 4C).

Inhibition of RISK and SAFE Pathway in XE991-Treated HL-1 Cells

As we observed changes in phosphokinases with XE991 treatment, we examined whether inhibition of these

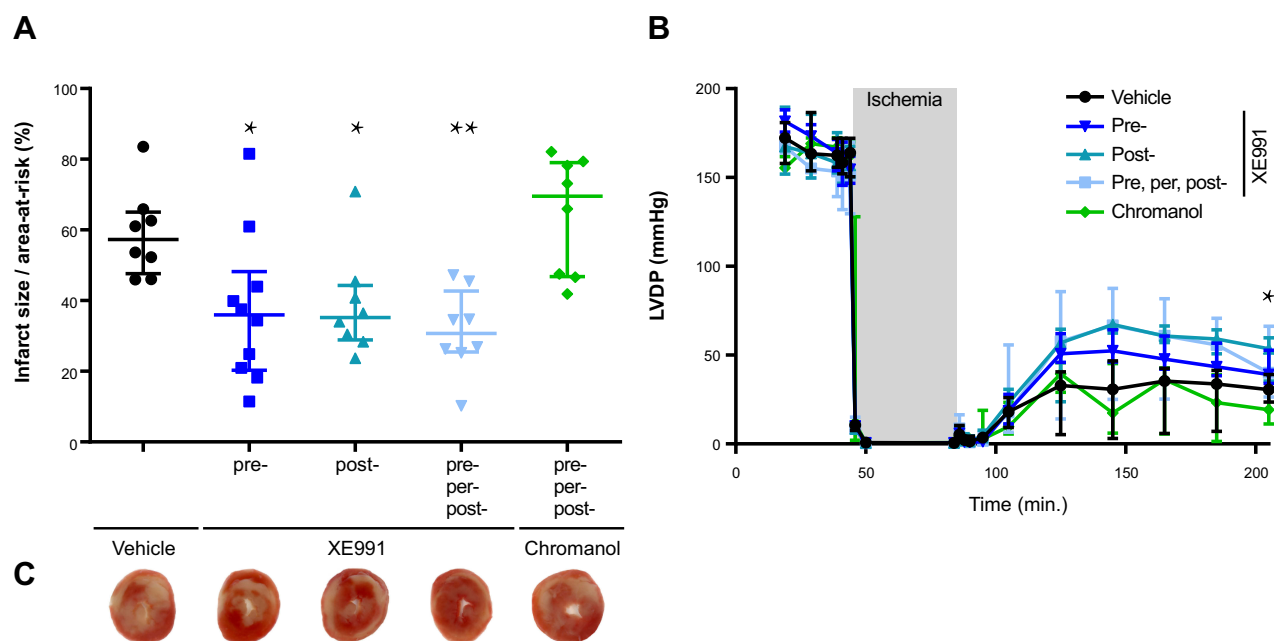


Figure 2 Myocardial infarct sizes as a percentage of area-at-risk in rat hearts (A) and LVDP (left ventricular-developed pressure) (B). Representative triphenyl tetrazolium chloride (TTC) stained sections of the rat heart for evaluation of infarct size (C). Rats are perfused with KH buffer containing vehicle, XE991 prior to ischemia (pre-), XE991 during reperfusion (post-), XE991 throughout the perfusion protocol (pre-, per-, post-) or chromanol 293B throughout the perfusion protocol (chromanol; pre-, per-, post-). Vehicle; DMSO 1 mL/L. XE991; KV7.1–5 potassium channel blocker. Chromanol 293B; Specific Kv7.1 inhibitor. Data are median (IQR). * $P<0.05$, ** $P<0.01$.

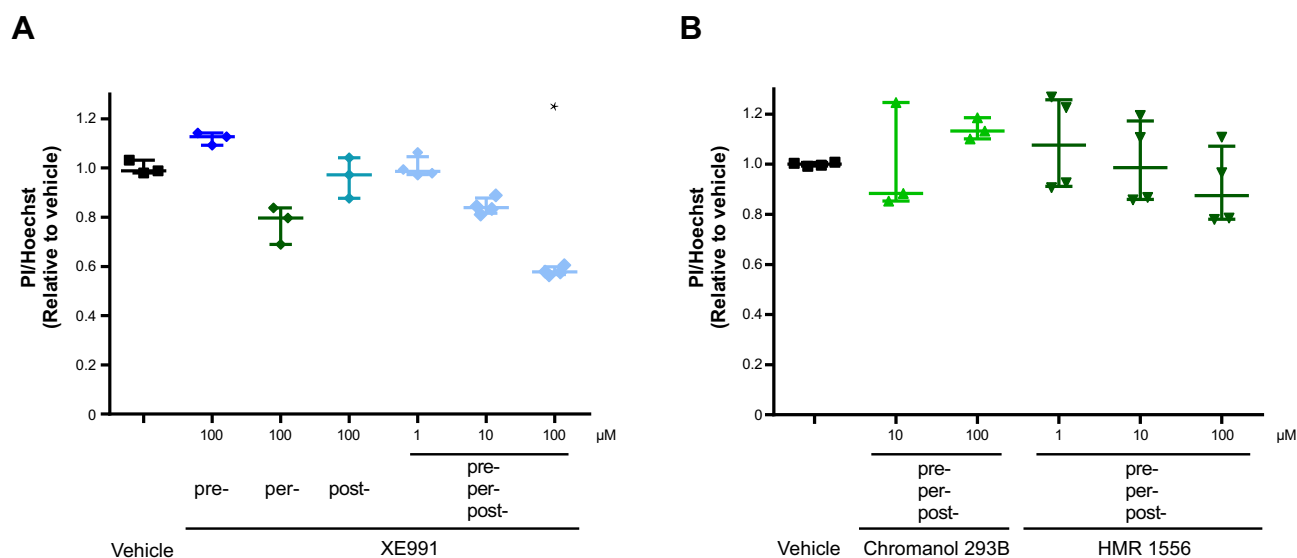


Figure 3 The left panel shows the protective effects of different XE991 administration schedules on HL-1 cell survival following simulated ischemia/reperfusion assessed by PI/Hoechst staining (A). XE991 was administered prior to ischemia (pre-), during ischemia (per-) or during reperfusion (post-) (100 μM XE991) or throughout the perfusion protocol (pre-, per-, post) (1+10+100 μM XE991). The right panel shows the effect of chromanol 293B (10+100 μM) and HMR 1556 (1+10+100 μM) administration throughout the perfusion protocol (pre-, per-, post-) compared to vehicle (B). XE991; Kv7.1–5 blocker. Chromanol 293B and HMR 1556; Kv7.1 blocker. Data are median (IQR). *P<0.05. All data are normalized to vehicle.

survival kinases affected HL-1 cell protection by pre, per and post-ischemic XE991 inhibition (Figure 1B). We also inhibited pSTAT3/STAT3 although no increase in pSTAT3/STAT3 was observed. The blockers of Akt (Wortmannin), Erk1/2 (PD98059) and STAT3 (AG490) did not affect PI/Hoechst per se ($p>0.99$ for all). None of the blockers affected the cytoprotective effect of XE991 (Figure 4G–I). Successful inhibition was confirmed by Western blotting (Figure 4D–F and Fig. s1).

RT-PCR and qPCR for Kv7 Subtypes in Rat Heart and HL-1 Cells

All five Kv7 subtypes were expressed in left ventricular myocardial tissue from rat hearts with Kv7.1, Kv7.3 and Kv7.4 having the most prominent expression (Figure 5A and B). In HL-1 cells and mouse hearts, Kv7.1 and Kv7.4 showed the highest expression (Figure 5A and C). Brain tissue from mouse and rats, used as a positive control, showed expression of all five Kv7 channels.

Discussion

The present study confirms the cardioprotective effect of Kv7.1–5 inhibition by XE991 and extends our previous findings by demonstrating that known cardioprotective pro-survival kinases are involved in the cardioprotective mechanisms only to a minor extent. In the cell experiment, XE991 administration is cytoprotective only when administered

throughout the simulated ischemia/reperfusion protocol but borderline protection was also observed with XE991 administration only during simulated ischemia. The same temporal effect of XE991 was not observed in the isolated heart perfusion experiments. Predominantly Kv7.1 and Kv7.4 are present in HL1 cells although small amounts of Kv7.2, Kv7.3 and Kv7.5 were also detectable. Kv7.1 inhibition did not offer protection suggesting Kv7.4 channels as the most likely channel to mediate cardioprotection by XE991.

Efforts to reduce reperfusion injury frequently target pro-survival signaling pathways as demonstrated in the mechanism of ischemic conditioning. The RISK and SAFE pathways are prevailing signaling pathways in cardioprotection.²⁸ Phosphorylation of Akt, Erk and STAT3 are central elements in pro-survival signaling through RISK and SAFE.^{7,8} We observed minor alterations of these phosphokinases during reperfusion in cells treated with XE991. Blocker experiments targeting these phosphokinases did not modify the cardioprotective capacity of XE991. Other studies demonstrating cardioprotection achieved by recruitment of the RISK pathways typically report a larger upregulation of pAkt/Akt and pErk/Erk^{29,30} than we observed by XE991. Insulin, included as a positive control of pAkt/Akt-upregulation and known to provide simultaneous cardioprotection,³¹ resulted in a pronounced phosphorylation of Akt. The cardioprotective effects by XE991 remained the same in cells co-exposed to wortmannin and PD98059, which successfully inhibited RISK

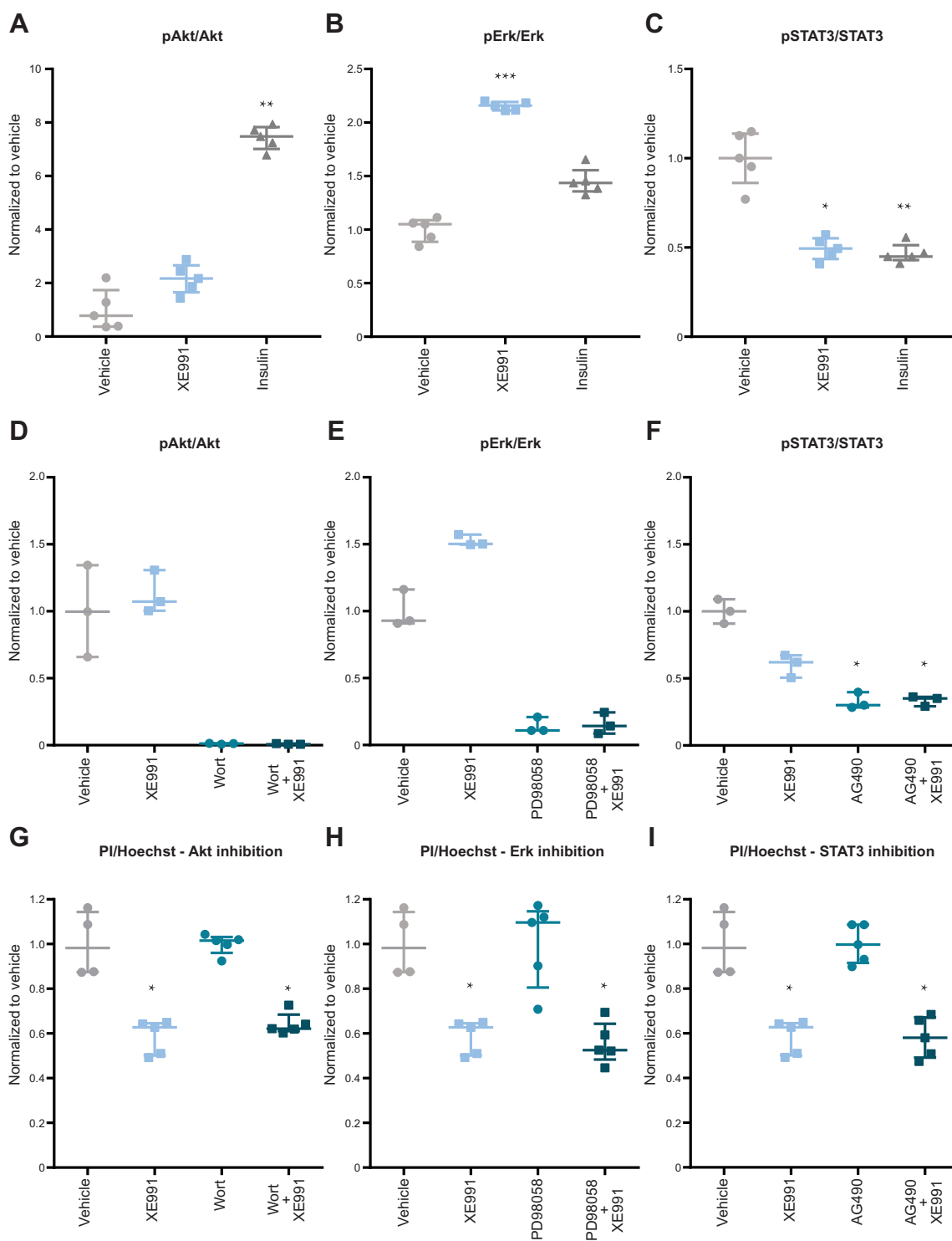


Figure 4 HL-I cells exposed to anoxia/reoxygenation and harvested for Western blot analysis after 1 hr of reoxygenation (**A–F**). The effect of XE991 on p-Akt/Akt (**A**), p-Erk/Erk (**B**) (RISK pathway) and p-STAT3/STAT3 (**C**) (SAFE pathway) is illustrated in the upper panels and [Fig. s1](#). Insulin was used as a positive control of phosphorylation of Akt. We inhibited the increase in p-Akt/Akt by co-administration of wortmannin (wort) (**D**). PD98058 (**E**) inhibited the upregulation of p-Erk/Erk and AG490 reduced P-STAT3/STAT3 activation (**F**). The lower panels show the effect of p-Akt/Akt (**G**), p-Erk/Erk inhibition (**H**) and p-STAT3/STAT3 (**I**) on the protective effect of XE991 on HL-I cell injury following 1 hr of stabilization, 5.5 hrs of anoxia and 2 hrs of reoxygenation assessed by PI/Hoechst staining. Vehicle; DMSO 1 mL/L. XE991; KV7.1–5 blocker. Data are median (IQR) and normalized to vehicle. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

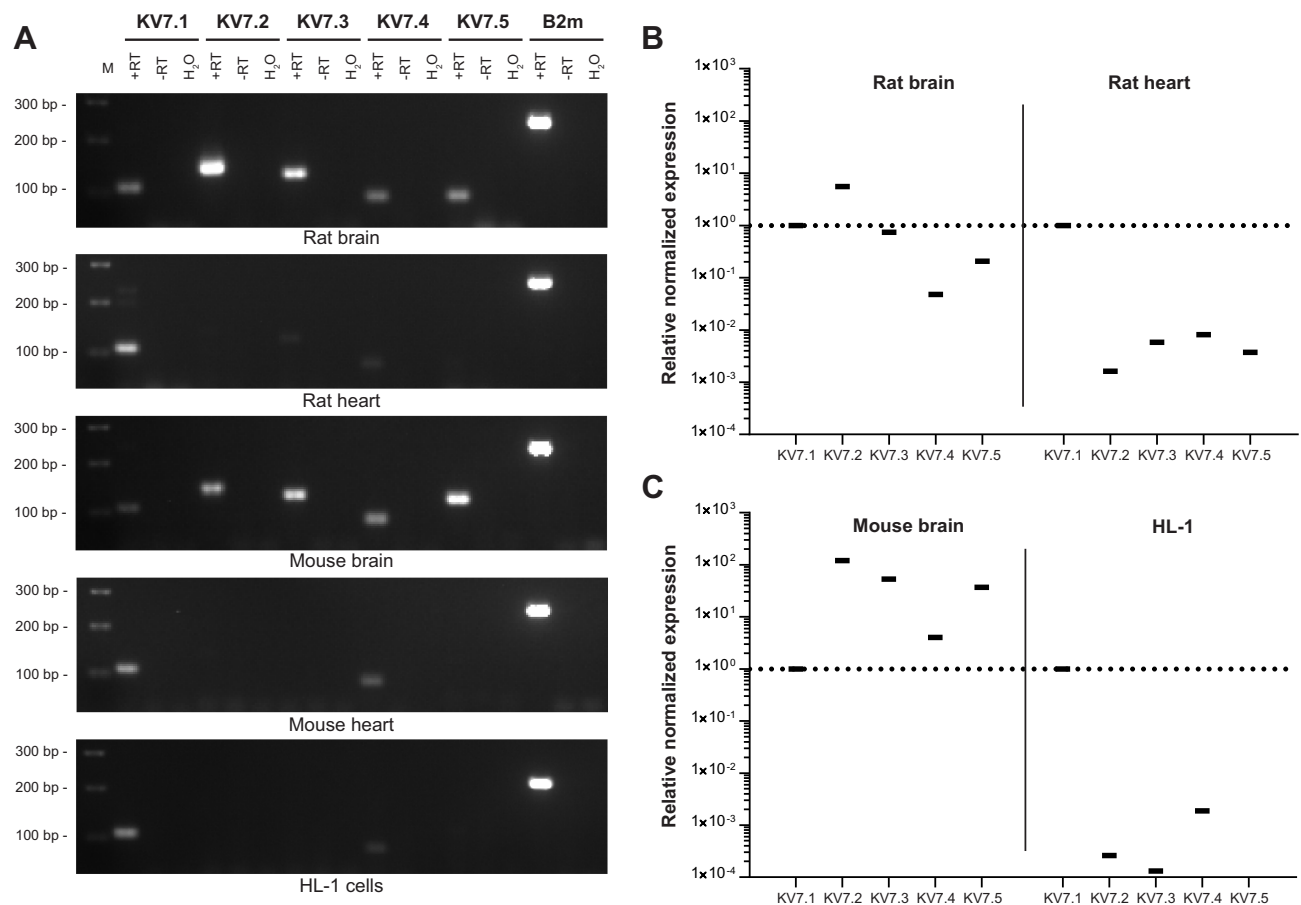


Figure 5 Kv7.1–5 mRNA expression in rat and mouse heart tissue and HL-1 cells illustrated with RT-PCR (A) and qPCR (B and C). Rat and mouse B2m were used as reference genes for normalization as appropriate and qPCR data are illustrated relative to Kv7.1 in the respective tissues. Brain tissue was used as a positive control of channel expression. All 5 Kv7 subtypes were present in the rat heart with Kv7.1, Kv7.3 and Kv7.4 having the most prominent expression. In mouse heart and HL-1 cells, Kv7.1 and Kv7.4 showed the highest expression (A+C) and only very small amount of Kv7.2, Kv7.3 and Kv7.5 was observed (Kv7.5 less than 1×10^{-4} not shown in figure). Conversion of total RNA to cDNA was performed with reverse transcriptase (+RT) and samples without reverse transcriptase (–RT) was used for quality control. Expected band sizes were identical for rat and mouse for Kv7.1 (105 bp), Kv7.2 (138 bp), Kv7.3 (126 bp), and Kv7.4 (85 bp). For Kv7.5, the expected band sizes were 122 bp for mouse and 87 bp for rat, respectively. Amplification of beta-2 microglobulin (B2m) was performed as a control for successful cDNA synthesis (band size 240 bp). Primer sequences are displayed in [table s1](#).

Abbreviation: M, molecular marker.

pathway activation. Upregulation of pSTAT3/STAT3 is associated with cardioprotective signaling³² but we observed a downregulation by XE991 treatment and inhibition of STAT3 phosphorylation by AG490 did not influence the protective effects of XE991. We therefore conclude that cardioprotection by XE991 channel inhibition is largely independent of these major pro-survival kinases.

We have previously demonstrated additive protective effects of combining XE991 treatment with IPC in isolated rat hearts¹⁴ which suggest independent mechanisms of protection by IPC and XE991. Protection by IPC rely on the upregulation of the prosurvival kinases mentioned above. The results of the present study further support that the mechanisms of protection by XE991 are independent of the multiple protective pathways activated by IPC.

Clinical translation of ischemic preconditioning in cardioprotection has proven difficult despite encouraging results in a very large number of experimental studies. It is suggested that targeting only one cardioprotective pathway in the complex process of reperfusion injury may be insufficient in a complex clinical setting encompassing comorbidity, medications, etc., and that combinations of therapies may be needed to facilitate translation of cardioprotection to patient benefit.^{33–35} The additive effect of XE991 to IPC and the alternate mechanisms of action suggest that Kv7 inhibition may constitute an attractive, supplementary treatment modality to ischemic preconditioning or other pharmacological conditioning modalities relying on RISK and SAFE pathway activation.

XE991 inhibits all known Kv7 channels, including the abundantly present Kv7.1 sub-channel involved in cardiac

repolarization. Selective inhibitors of sub-channels Kv7.2–5 are not readily available, but Chromanol 293B and HMR1556 inhibit Kv7.1. Inhibition of Kv7.1 with Chromanol 293B did not protect the isolated rat hearts, nor did inhibition with Chromanol 293B and HMR1556 increase HL-1 cell survival. Our data suggest that protection is mediated through other Kv7 channel members (Kv7.2–5). The results of our RT-PCR and qPCR analysis revealed highest mRNA expression for Kv7.1, Kv7.3 and Kv7.4 in the rat heart and only weak expression of Kv7.2 and Kv7.5. In HL-1 cells we predominantly observed mRNA expression of Kv7.1 and Kv7.4 although limited expression of Kv7.2, Kv7.3 and Kv7.5 was also present. Altogether, these results suggest that the protection by Kv7 channel inhibition is most likely mediated by the Kv7.4 subchannel. Involvement of Kv7.4 in cardioprotection has also previously been suggested by Testai et al,¹⁵ as discussed below and inhibition of another voltage-gated potassium channel involved in cardiac repolarization, KCNE2, was recently reported to attenuate cardiac ischemia/reperfusion injury as well.³⁶ Inhibition of Kv7 channels by XE991 is associated with prolongation of QTc³⁷ which is associated with malignant cardiac arrhythmias.^{38,39} Cardiac ischemia is also associated with malignant arrhythmias and prolongation of QTc.⁴⁰ We are therefore concerned that unselective Kv7 channel inhibition, encompassing Kv7.1 inhibition, may cause adverse effects such as long QT syndrome (LQTS) and lethal cardiac arrhythmias in clinical use, but selective inhibition of Kv7.4 may represent an attractive pharmacological target.

In agreement with our results, Testai and colleagues have suggested Kv7.4 channels to be involved in cardiac ischemia and reperfusion injury.¹⁵ However, in contrast to our findings, they demonstrated that Kv7.2–5 opening by retigabine reduced IR-injury in Langendorff perfused rat hearts. This discrepancy may be explained by the timing of Kv7 channel modulation. While the authors administered retigabine (Kv7.2–5 opener) prior to ischemia and not during ischemia and/or reperfusion, we administered XE991 (Kv7.1–5 inhibitor) throughout the perfusion protocol in our contemporary study.¹⁴ Hence, timing of Kv7 channel inhibition may explain that both Kv7 opening and Kv7 inhibition have cardioprotective capacity. Our HL-1 cell experiments support this notion, as we did not observe protection by XE991 administered prior to ischemia, but rather a tendency toward an adverse effect. Nor was XE991 protective when administered during reperfusion only, but protection was observed when XE991 was administered during ischemia or throughout the perfusion

protocol. In isolated rat hearts, however, we also observed protection by both pre-ischemic and post-ischemic administration of XE991 and speculate that the protection in isolated rat hearts by pre-ischemic administration of XE991 was actually mediated by per-ischemic effects, as we did not washout XE991 prior to ischemia.

We observed protection by XE991 when administered from the onset of reperfusion in Langendorff hearts but not in HL-1 cells. The lack of post-ischemic protection of HL-1 cells by XE991 may be attributable to the inevitable delay in XE991 administration when substituting the ischemia buffer with the XE991-containing reperfusion buffer thus allowing for full exposure to reoxygenation of the single-layered cardiomyocytes before XE991 was administered.

Overall, our results indicate that the Kv7 channels should be inhibited during ischemia or at the latest at the onset of reperfusion. If Kv7 inhibitors could be dispensed at the onset of reperfusion to reduce IR injury in the ischemic heart undergoing revascularization, it could offer an attractive supplementary treatment modality, potentially together with other conditioning strategies targeting RISK and SAFE pathway activation such as ischemic conditioning. Further investigation for such a combined approach is needed.

Our study is limited by the lack of a selective Kv7.4 inhibitor to confirm the importance of this specific subchannel in cardioprotection and future studies should investigate specific Kv7.4 inhibition or target Kv7.4 by RNA interference. XE991 is considered to specifically inhibit Kv7 channels, however off-target effect should not be discarded.⁴¹ We did not include a washout period between XE991 administration and ischemia in our Langendorff experiments, and consequently, we are not able to properly distinguish the effects of XE991 prior to ischemia with the effects during ischemia in the isolated rat hearts. The rat heart samples used to examine the presence of Kv7 subunits contain coronary vascular tissue, in which Kv7 channels are also present,^{27,42,43} this may have influenced the RT-PCR and qPCR analysis and explain the weak expression of Kv7.2 and Kv7.5 bands in the rat heart that is barely present in HL-1 cells. Very small amounts of Kv7.2, Kv7.3 and Kv7.5 is expressed in HL-1 cells but a role of these channels in cardiac ischemia/reperfusion should not be completely discarded. Finally, the HL-1 cell line is derived from atrial cardiomyocytes and its behavior compared with ventricular cardiomyocytes arranged in sarcomeres should be interpreted with caution.

Conclusion

XE991 yields cardioprotection presumably by modulating the voltage-gated Kv7.4 channels. The protective mechanism is RISK and SAFE pathway independent and Kv7 channel inhibition may be used in a multifaceted pharmacological cardioprotective approach when administered during ischemia and early reperfusion.

Abbreviations

AAR, area-at-risk; IR, ischemia and reperfusion; IPC, ischemic preconditioning; KH, Krebs–Henseleit; LVDP, left ventricular developed pressure; PI/Hoechst, propidium iodide and Hoechst staining; RT-PCR, reverse transcriptase; TTC, 2,3,5-triphenyltetrazoliumchloride.

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Disclosure

The authors report no conflicts of interest in this work.

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