Activation of Sonic hedgehog signaling in ventricular cardiomyocytes exerts cardioprotection against ischemia reperfusion injuries

Ludovit Paulis[1], Jeremy Fauconnier[1], Olivier Cazorla[1], Jérome Thireau[1], Rafaela Soleti[2], Bastien Vidal[1], Aude Ouillé[1], Marion Bartholome[1], Patrice Bideaux[1], François Roubille[1], Jean-Yves Le Guennec[1], Ramaronson Andriantsitohaina[2], Carmen Martínez[2], Alain Lacampagne[1,*]

1, INSERM U1046, Université Montpellier1, Université Montpellier2, Montpellier, France.

2, INSERM U1063, Stress oxydant et pathologies métaboliques, LUNAM Université, Angers, France.

Supplementary material

Ethics Statement

All experiments were conducted in accordance with relevant guidelines and regulations, conformed to European Parliament Directive 2010/63/EU and the Guide for the Care and Use of Laboratory Animals of the NIH (No. 85-23, revised 1996). The protocols were approved by the local ethics committee rules (CEEA-LR-12079).

Isolated cells preparation

Ventricular cardiomyocytes were isolated from 200–250 g male adult Wistar rats as previously described[1]. The rats were anesthetized by i.p. injection of pentobarbital

sodium (100 mg/kg) with heparin (100 U). The heart was rapidly excised, rinsed in ice-cold Hanks–HEPES buffer (in mM NaCl 117, KCl 5.7, $NaHCO_3$ 4.4, KH_2PO_4 1.5, $MgCl_2$ 1.7, HEPES 21, glucose 11.7, taurine 20, pH at 7.15) mounted on a Langendorf perfusion system and perfused (3 ml/min, at 37°C) first with Hanks–HEPES buffer for 5 min to be cleared from blood and then with buffer supplemented with 1.2 mg/mL collagenase type 4 (Worthington, Lakewood, NJ, USA) for 13–18 min. The heart was placed into Hanks–HEPES buffer supplemented with 1 mg/mL 2,3-Butanedione monoxime (BDM) and 1 mg/mL BSA. The atria were removed and the ventricles were gently dissociated with scissors and pipettes in the same medium. The suspension was filtered through a nylon mesh (200 μm) and 4x incubated for decantation of the cells for 10 min. After each decantation of the supernatant, myocytes were resuspended in a Hanks–HEPES buffer containing 0 mM Ca^{2+} (+ 1 mg/mL BDM and 1 mg/mL BSA), 2 times in Hanks–HEPES buffer containing 0.3 mM Ca^{2+} (+ 0.6 mg/mL BDM and 1 mg/ml BSA) and finally Hanks–HEPES buffer containing 1 mM Ca^{2+} (+ 1 mg/mL BSA only).

Cardiac Histology

After 24 hours of reperfusion, rats were euthanized and Evans Blue dye was injected into the heart. Hearts were excised and left ventricles were cut into transverse slices. These slices were incubated with 2,3,5 triphenyltetrazolium chloride (TTC) solution for 30 min at 37°C and then observed by light microscope (x10).

Inhibitors and chemicals

Control cells and cells loaded with MPs were also incubated under the same conditions as described above with different inhibitors: cyclopamine (Ccl, 30 μM, SHH pathway

inhibitor), LY294002 (LY, 25 μM, PI3-K inhibitor), N^{ω}-nitro-L-arginine (L-NNA, 100 μM, NOS inhibitor, 1H-[1.2.4]oxadiazolo[4.3-a]quinoxalin-1-one (ODQ, 10 μM, soluble guanylate cyclase, sGC, inhibitor), glibenclamide (Glib, 1 μM, K_{ATP} channel inhibitor). Unless stated, all chemicals were obtained from Sigma (St. Louis, MO, USA).

Nitric oxide measurement

Cardiomyocytes were loaded with the NO sensitive fluorescent dye: DAF-FM (20 μM, 4-amino-5-methylamino-2,7-difluorofluorescein diacetate, for 30 min, Invitrogen). Cells were then resuspended in Tyrode´s solution (1.8 mM Ca^{2+}) at 22°C. DAF fluorescence confocal images were acquired in the x-y mode (Zeiss LSM 510, 25x, NA=0.8 water immersion, Le Pecq France), with 488 nm excitation with argon ion laser and 510-530 nm emission filter. The fluorescence values were corrected for the background fluorescence from a region of the image without cells ($F-F_0$) and fluorescence/background ratio (($F- F_0$)/ F_0) was calculated. All images were processed and analyzed using Image J v 1.36b software (National Institute of Health, USA).

Cellular electrophysiology

Whole-cell patch-clamp experiments were performed at room temperature (22-24°C) with an Axopatch 200B (Axon instrument, Burlingham, CA, USA). Patch pipettes had resistance of 2 MΩ. Currents were normalized to the cell membrane capacitance (pA/pF). Series resistances were compensated before recordings.

To record APs, pipettes solution contained (in mM): KCl (130), HEPES (25), ATP(Mg) (3), GTP(Na) (0.4), EGTA (0.5); pH 7.2 (KOH). Bathing solution contained (in mM): NaCl (135), MgCl2 (1), KCl (4), Glucose (11), HEPES (2), CaCl2 (1.8); pH 7.4 (NaOH).

APs were elicited by 0.2 ms current injection of supra-threshold intensity. During experiments, cells were stimulated by trains of 30 stimuli at 0.5 Hz.

To record I_{CaL}, bath solution contained (in mM): $CaCl_2$ (1.8), TEACl (140), $MgCl_2$ (2), glucose (10), HEPES (10), pH 7.4 (TEAOH). Pipette solution contained (in mM): CsCl (140), HEPES (10), EGTA (10), NaGTP (0.4), MgATP (3), pH 7.2 (CsOH). I_{CaL} was elicited by test depolarization (150 ms) from -80 to -10 mV at 0.1 Hz. Current-voltage relationship was determined using 10 mV voltage step from -80 to +50 mV. Its amplitude was estimated as the difference between peak I_{CaL} and current level at the end of the pulse.

K^+ currents were recorded with the solution used for APs, but with 10 μM tetrodotoxin (TTX) and 2 mM cobalt (Co^{2+}) added in the external medium to block I_{Na} and I_{CaL}, respectively. They were elicited from a HP of -80 mV by test depolarization (1s) ranging from -120 mV and 0 mV at 0.1 Hz. Voltage steps were performed 2 times on each cells before and after Glibenclamide application and used to determine the glibenclamide sensitive components of the current.

Ca^{2+} transient and cell shortening

Isolated cardiomyocytes were loaded with Indo-1 AM (10 μM, Invitrogen, Molecular Probes, Eugene, OR, USA) at room temperature for 30 min and then washed out with free HEPES-buffered solution (117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 mM $MgCl_2$, 21 mM HEPES, 11 mM glucose, 20 mM taurine, pH 7.2) containing 1.8 mM Ca^{2+}. Unloaded cell shortening and calcium concentration [Ca^{2+}] (indo-1 dye) were studied using field stimulation (1 Hz, 22 °C, 1.8 mM external Ca^{2+}).

Sarcomere length (SL) and fluorescence (405 and 480 nm) were simultaneously recorded (IonOptix Myocyte Calcium and Contractility Recording System, Dublin, Ireland) [2].

Mechanical recording and sarcomere length measurement

The Ca^{2+}-activated force of single permeabilized myocytes was measured as described previously [2]. Intact isolated myocytes were permeabilized in a relaxing solution (pCa 9; pCa= -log [Ca^{2+}], see below) containing 0.3% vol/vol Triton X-100 and protease inhibitors (phenylmethylsulfonylfluoride (PMSF), leupeptin, and transepoxysuccinyl-leucine-guanidobutylamide (E-64)) at room temperature for 6 min. Cells were then rinsed twice with the same solution without Triton X-100 and maintained at 4 °C.

Permeabilized myocytes were attached to a piezoresistive strain gauge (AE801 sensor, Memscap; 500 Hz unloaded resonant frequency, compliance of the strain gauge 0.03 μm/μN) and to a stepper motor-driven micromanipulator (MP-285, Sutter Instrument Company) with thin stainless steel needles and optical glue (NOA 63, Norland Products) that is polymerized by 2 min UV illumination. SL was determined online during the experiment by applying a fast Fourier transform algorithm on the video images of the cell, using IonOptix acquisition software. Force was normalized to the cross-sectional area measured from the imaged cross-section. Slack SL was measured before attachment to serve as the origin. pCa–force relationships were established at 2.3 μm SL at 22 °C. The active tension at each pCa was represented by the difference between total tension and relaxed tension. Cells that did not maintain 80% of the first maximal tension or a visible striation pattern were discarded. Although cells were kept isometric during contraction, sarcomeres typically changed length, especially when activation was maximal. Only cells that were well attached and with a minimal SL change (<0.1 μm)

were kept. Active tensions at submaximal activations were normalized to maximal isometric tension (obtained at pCa 5). Force measurements were low-pass filtered at 100 Hz and recorded on a thermal paper recorder (Dash IV, Astro- Med). A single cell was positioned at the tip of a conical micro capillary that received the outlet of 10 micro capillaries connected to 5-mL syringes containing experimental solutions with a range of Ca^{2+} concentration from pCa 9 to pCa 4.5 (flow rate 200 μL/min). For each cell, the relation between force and pCa was fitted to the equation force = $[Ca^{2+}]^{nH}$ / (K + $[Ca^{2+}]^{nH}$) where nH is the Hill coefficient and pCa50 is the pCa for half-maximal activation, corresponding to –(log K)/nH.

Ca^{2+} activating solutions were prepared daily by mixing relaxing (pCa 9.0) and maximal activating (pCa 4.5) stock solutions. The relaxing and activating solutions contained 12 mM phosphocreatine, 30 mM imidazole, 1 mM free Mg^{2+}, 10 mM EGTA, 3.3 mM Na_2ATP, and 0.3 mM DTT with either pCa 9.0 (relaxing solution) or pCa 4.5 (maximal activating solution), pH 7.1 adjusted with KOH. Ionic strength was adjusted to 180 mM with K-acetate.

Microparticles preparation

Microparticles *(*MPs) were obtained from the human lymphoid CEM T cell line (ATCC, Manassas, VA, USA). Cells were seeded at 10^6 cells/ml, cultured in serum-free X-VIVO 15 medium (Lonza, Walkersville, MD, USA) and then treated subsequently with phytohemagglutinin (5 µg/ml) for 72 h, with phorbol-12-myristate-13 (20 ng/ml) and actinomycin D (0.5 µg/ml) for 24 h (MPs^{SHH+}) or only with actinomycin D (0.5 µg/ml) for 24 h (MPs^{SHH-}). A supernatant was obtained in two centrifugation steps at 750 *g* for 15 min and at 1500 *g* for 5 min to remove cells and large debris. Remaining MP-containing

supernatant was subjected at 14,000 *g* for 45 min to pellet MPs. MP pellet was subjected at two series of centrifugations at 14,000 *g* for 45 min. Finally, MP pellet was recovered in 400 μL sterile NaCl (0.9% w/v) [3]. MP content was adjusted to protein content determined against bovine serum albumin (BSA) standards to contain 10 μg proteins per ml.

Incubation with SHH protein or MPs

Cells were counted and seeded to 150.10^3 cells/mL and subsequently incubated with 0.4 µg/mL recombinant human carrier bound SHH (C24II) protein (N-SHH, R&D Systems, Minneapolis, MN, USA) or 10 µg/mL MPs for 4 hours at 22°C while being gently shaken. The time for incubation was determined according to preliminary experiments in which incubation for 0 h, 2 h, 4 h and 6 h was tested and the effects on calcium transient and shortening were most pronounced after 4 h.

Western blot

Proteins were extracted from cardiomyocytes-pellets homogenized in lysis buffer (10 mM Tris-malate, 20 mM β-glycerolphosphate, 50 mM NaF, 10 mM benzamidine, 0.2 mM Na_3VO_4, 40 μM leupeptin, 500 µM PMSF, 10 μM E64, 2 mM EDTA, 5 mM DTT, 1% Triton X100, pH 6.8, at 4°C). The homogenates were centrifuged at 10,000 *g* for 5 min at 4°C. Total protein concentration was determined by RC DC kit (Bio-Rad, Hercules, CA, USA). The protein extracts were solubilized in Laemmli-urea buffer (10 mM Tris-malate, 20 mM β-glycerolphosphate, 50 mM NaF, 10 mM benzamidine, 0.2 mMNa_3VO_4, 40 µM leupeptin, 500 µM PMSF, 10 µM E64, 1 mMEDTA, 1 mMEGTA, 8 M urea, 2 M thiourea, 3% SDS, 5 mMDTT, 1% Triton X100, pH 6.8, at 4°C) and boiled

for 5 min at 95°C. Protein samples (50 µg) were separated using SDS-PAGE (10%) electrophoresis and transferred to polyvinylidene membrane by semidry Western blot protocol and blocked with 3% BSA for 1 hour at room temperature. Membranes were stained with goat antibody against Smo (ab58591) (1:400, Abcam, Cambridge Science Park, Cambridge, UK) and Ptc (sc-6147) (1:1000, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) overnight at 4°C and peroxidase-conjugated secondary anti-rabbit (1:3,000) / anti-goat (1:1,0000) IgG antibodies (Pierce, Rockford, IL, USA). The bands were identified using chemiluminescence (Super Signal West Pico, Pierce), densitometrically evaluated and expressed in arbitrary units (A.U.).

RNA extraction and real-time RT-PCR

RNA was extracted from cardiomyocytes pellets using TRIzol reagent according to manufacturer's protocol (Euromedex, Souffelweyersheim, France), treated with DNase I (Invitrogen) at 37°C for 30 min. cDNA was synthesized using superscript II reverse transcriptase (Invitrogen) at 42°C for 50 min. Quantitative PCR was performed using a LightCycler rapid thermal cycler (Roche, Meylan, France). Twenty μL reaction mixture contained 10 µL of Absolute QPCR SYBR Green Capillary mix (thermo strat DNA polymerase, reaction buffer, deoxyrybonucleoside triphosphate mix, 3 mM$MgCl_2$, SYBR green I dye; Thermo Fischer Scientific, llkirch, France) supplemented with 0.5 µM primer mix and 5 µL of cDNA. The data were normalized to GAPDH. The amplification program included the initial denaturation step at 95°C for 15 min, 40 cycles of denaturation at 95°C for 1 sec, annealing at 60°C for 10 sec and extension at 72°C for 20 sec. Melting curves were used to determine the specificity of PCR products.

Numerical simulation

The numerical simulation was performed using the an endocardial ventricular cell model described by [4]. This model is available online from the CellML repository (http://models.cellml.org/exposure/ea62c9c8a502afe364350d353ebf4dd5/pandit_clark_giles_demir_2001_mouse_ventricle.cellml/view) and was developed with Cellular Open Resource [5]. The current activated by SHH was simulated by the following equation derived from an instantaneous inward rectifying current equation having a negative slope:

$$i_{shh} = \frac{g_{shh} \cdot (V - E_K)}{1 + e^{\frac{F \cdot (V - E_K)}{R \cdot T}}} + \left(1 - \frac{1}{1 + e^{\frac{F \cdot (V - E_K)}{R \cdot T}}}\right) \cdot 0.1$$

Where E_K is the equilibrium potential for K^+, G_{SHH} is the I_{SHH} conductance, R is the gas constant, T is the temperature and F the Faraday constant.

References

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SUPPLEMENTAL FIGURES AND LEGENDS:

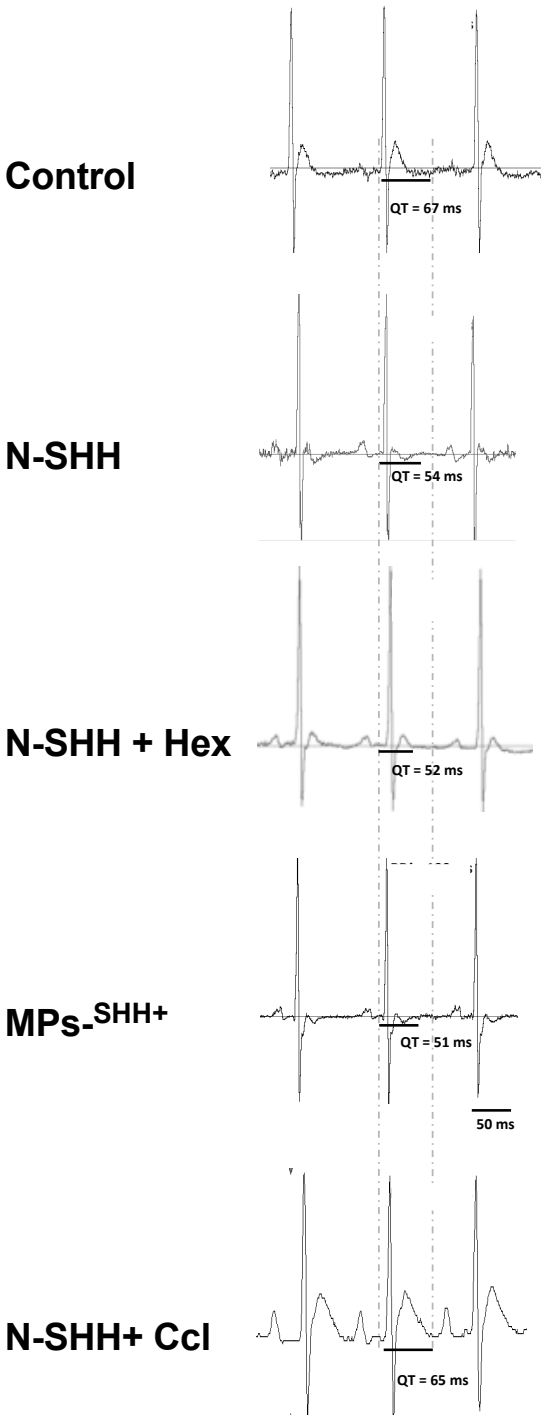

Figure S1 Representative ECGs**.** ECGs were recorded in control animal treated with N-SHH, MPs^{SHH+}, N-SHH + cyclopamine and N-SHH + hexamethonium.

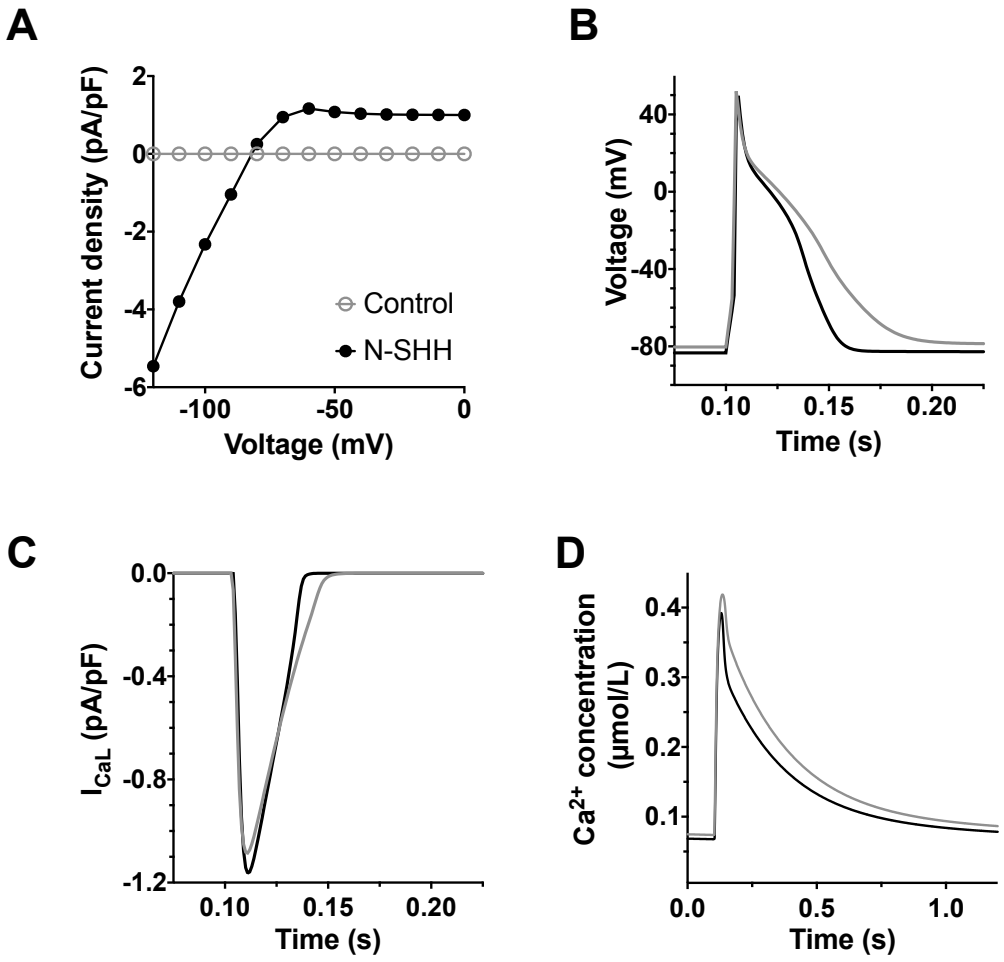

Figure S2 Numerical simulation of action potential, I_{CaL} and Ca^{2+} transient after adding an additional inward rectifying K^+ current. An equation matching for the inward rectifying current elicited by SHH was empirically built and added to the model (online model from the CellML). (A) Current-voltage relationship of the current simulated. (B) Time-course of the action potential (AP) following an electrical stimulation. (C) Time course I_{CaL} during the action potential in the presence (black line) and in the absence (dash line) of this potassium current. (D) Calcium transient during the action potential in the presence (black line) and in the absence (dash line) of this potassium current.

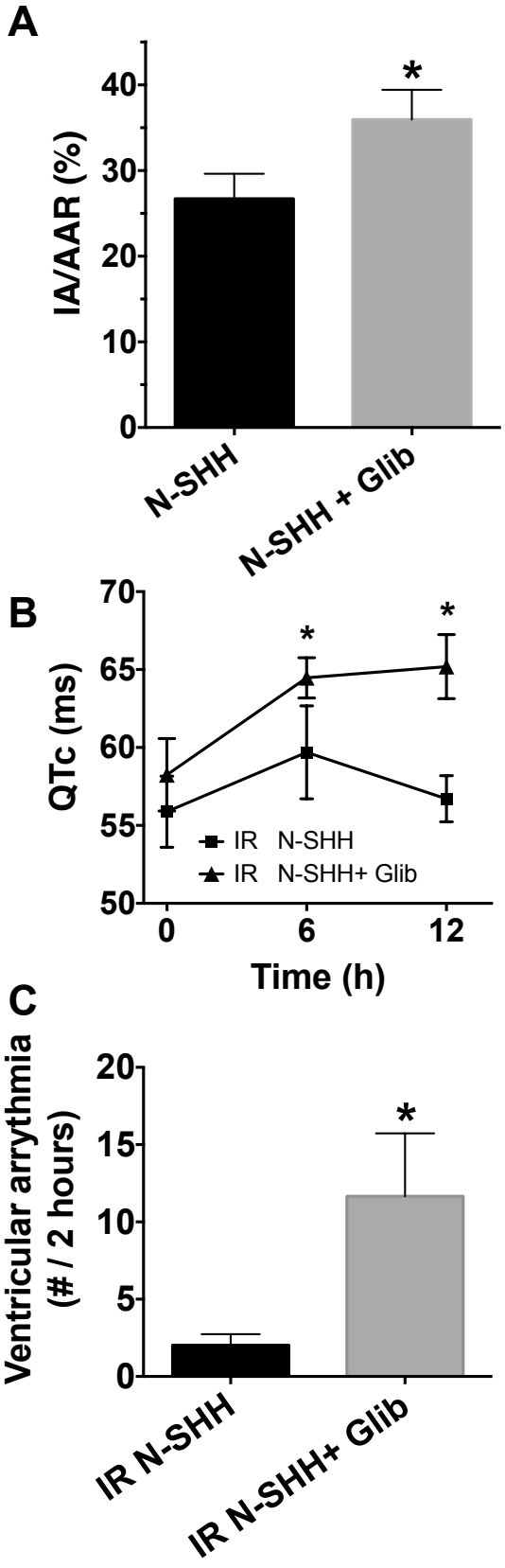
A
IA/AAR (%)
40
30
20
10
0
*
N-SHH
N-SHH + Glib
B
QTc (ms)
70
65
60
55
50
*
*
IR N-SHH
IR N-SHH+ Glib
0
6
12
Time (h)
C
Ventricular arrythmia
(# / 2 hours)
20
15
10
5
0
*
IR N-SHH
IR N-SHH+ Glib

Figure S3 Cardioprotective effect of SHH is blocked by glibenclamide. (A) Reperfusion injuries were quantified by normalizing the infarct area (IA) to the area at risk (AAR). Treatment with N-SHH reduced infarct size after 24 h of reperfusion (same data as in figure 6) and this cardioprotective effect was prevented by glibenclamide. (B) Summarizes the time course of QTc over a period of 12 h of reperfusion following 30min ischemia, in animal treated (i.p. injected 15 min prior reperfusion) either with the vehicle, recombinant N-SHH, or N-SHH + glibenclamide (Glib). (C) Summarizes the number of arrhythmias recorded over a period of 2 hours following reperfusion showing that he protective effect of SHH was abolished by glibencalmide (N-SHH n=8, N-SHH+Ccl n=6; * $p<0.05$).