Selective Inhibition of Inward Rectifier K⁺ Channels (Kir2.1 or Kir2.2) Abolishes Protection by Ischemic Preconditioning in Rabbit Ventricular Cardiomyocytes

Roberto J. Diaz,* Carsten Zobel,* Hee Cheol Cho, Michelle Batthish, Alina Hinek, Peter H. Backx, Gregory J. Wilson

**Abstract**—Volume regulatory Cl⁻ channels are key regulators of ischemic preconditioning (IPC). Because Cl⁻ efflux must be balanced by an efflux of cations to maintain cell membrane electroneutrality during volume regulation, we hypothesize that I_K1 channels may play a role in IPC. We subjected cultured cardiomyocytes to 60-minute simulated ischemia (SI) followed by 60-minute of simulated reperfusion (SR) and assessed percent cell death using trypan blue staining. Ischemic preconditioning (10-minute SI/10-minute SR) significantly (P<0.0001) reduced the percent cell death in nontransfected cardiomyocytes [IPC_CM 18.0±2.1% versus control (C_CM) 48.3±1.0%]. IPC protection was not altered by overexpression of the reporter gene (enhanced green fluorescent protein, EGFP). However, overexpression of dominant-negative Kir2.1 or Kir2.2 genes using adenoviruses (AdEGFPKir2.1DN or AdEGFPKir2.2DN) encoding the reporter gene EGFP prevented IPC protection [both IPC_CM+ AdEGFPKir2.1DN 45.8±2.3% (mean±SEM) and IPC_CM+ AdEGFPKir2.2DN 47.9±1.4% versus IPC_CM; P<0.0001] in cultured cardiomyocytes (n=8 hearts). Transfection of cardiomyocytes with AdEGFPKir2.1DN or AdEGFPKir2.2DN did not affect cell death in control (nonpreconditioned) cardiomyocytes (both C_CM+ AdEGFPKir2.1DN 45.8±0.7% and C_CM+ AdEGFPKir2.2DN 46.2±1.3% versus C_CM; not statistically significant). Similar effects were observed in both cultured (n=5 hearts) and freshly isolated (n=4 hearts) ventricular cardiomyocytes after I_K1 blockade with 20 μmol/L BaCl₂ plus 1 μmol/L nifedipine (to prevent Ba²⁺ uptake). Nifedipine alone neither protected against ischemic injury nor blocked IPC protection. Our findings establish that I_K1 channels play an important role in IPC protection. (Circ Res 2004;95:325-332.)

**Key Words:** ischemic preconditioning ■ cardiomyocytes ■ ischemia ■ potassium channels ■ gene transfer

Ischemic preconditioning (IPC) is a strong native protective mechanism that limits the amount of necrosis during a subsequent prolonged ischemia and reperfusion period. Although much is known about the cell membrane receptors and intracellular pathways involved in IPC, very little is known about the end effector(s) of IPC. Finding the end effector(s) of IPC is important to guide the development of new specific therapeutic approaches to protect the ischemic myocardium.

Our recent results in cardiomyocytes established that blockade of Cl⁻ channels inhibits cell volume regulation following hypoosmotic stress (regulatory volume decrease, RVD), which results in loss of protection against necrosis induced by both ischemic and pharmacological preconditioning. Because accumulation of metabolic by-products is responsible for increasing the intracellular osmotic load in ischemia and thereby increasing water uptake in cardiomyocytes, and because IPC only produces a small reduction in the estimated osmotic load in ischemic cardiomyocytes, we hypothesized that enhanced cell volume regulation as a result of Cl⁻ channel activation plays a significant role in the protection induced by IPC. Consistent with this hypothesis, IPC substantially reduces hypoosmotic-induced and ischemia-induced cell swelling in cardiomyocytes. In order for Cl⁻ ion movement to effectively regulate cellular volume, electroneutrality dictates that positively charged ions must accompany chloride ions. Although any cation could act as a counter-ion for Cl⁻, K⁺ is the most abundant intracellular cation. In addition, extracellular K⁺ increases by 2- to 3-fold during ischemia as a result of K⁺ efflux from cardiomyocytes. Passage of K⁺ out of the cardiomyocyte could occur through multiple K⁺ channels. Of these, voltage-gated channels such as for transient outward currents and delayed rectifier current are only open for relatively brief periods. Moreover, myocardium becomes electrically quiescent (ie,
approaching resting membrane potential) during severe and persistent ischemia, thus minimizing the activation of voltage-gated K⁺ currents. On the other hand, inward rectifier potassium (IRK) currents remain open during quiescence that increases during ischemia as a result of elevated extracellular K⁺ levels. Several IRK currents exist in heart: strongly rectifying currents (I_K1), G protein–regulated currents (I_KCN), ATP-sensitive currents (I_ATP), and Na⁺-sensitive currents (I_KNa) K⁺ channels. In the heart, I_KNa is primarily expressed in atrial and nodal cardiomyocytes, whereas I_Ks channels only open when [Na⁺] becomes a very high level (30 mmol/L). Thus, I_Ks and sarcolemmal I_ATP (sI_ATP) appear to be the most attractive candidates to allow K⁺ to move with Cl⁻ to regulate cardiomyocyte volume.

In the present study, we assessed whether I_Ks channels are involved in the protection induced by IPC against ischemia/reperfusion injury in fresh and cultured rabbit ventricular cardiomyocytes. We found that pharmacological inhibition of I_Ks abolished IPC cardioprotection and this finding does not appear to be related to nonspecific inhibition affecting sI_ATP. Moreover, knockdown of I_Ks channels by adenovirus transfection of either dominant-negative Kir2.1(C122S) gene (AdEGFPKir2.1DN) or the dominant-negative Kir2.2(C123S) gene (AdEGFPKir2.2DN) completely blocked the protection of IPC against cell death caused by the subsequent long period of simulated ischemia and reperfusion. To our knowledge, no previous published study has established a role for the I_Ks channel as a potential end effector in the cardioprotection of IPC.

### Materials and Methods

#### Isolation of Ventricular Cardiomyocytes

Calcium tolerant–isolated cardiomyocytes used in these studies were obtained from New Zealand white rabbits (weight = 3 to 3.5 kg) hearts by enzymatic dissociation using a method previously described (for details of cardiomyocyte isolation, see the expanded Materials and Methods in the online data supplement available at http://circres.ahajournals.org). Animal protocols conformed to the Guide for the Care and Use of Laboratory Animals published by NIH (publication No. 85-23, 1996). All animals were obtained from Reimens Fur Ranches Ltd. (St. Agatha, Ontario, Canada).

#### Recombinant Adenoviruses

To define the participation of the I_Ks channel in the protection induced by IPC, we used three different type-5 recombinant adenoviruses, AdEGFP, AdEGFPKir2.1(C122S) (AdEGFPKir2.1DN), and AdEGFPKir2.2(C123S) (AdEGFPKir2.2DN), which have been extensively studied in our laboratory. These recombinant adenoviruses encode a bidirectional construct, which includes a marker protein (enhanced green fluorescence protein, EGFP) used to confirm the transfer of each gene into cardiomyocytes. However, only the AdEGFPKir2.1DN and AdEGFPKir2.2DN viruses have a dominant-negative (DN) mutant, either the Kir2.1(C122S) or Kir2.2(C123S) gene, respectively, to knockdown the I_Ks channel (for details, see online data supplement).

#### Single Cell Electrophysiology Studies in Isolated and Cultured Cardiomyocytes

After ventricular cardiomyocytes were prepared as described, they were cultured on laminin-coated glass coverslips at a density of 4 to 5×10⁴ cells/35-mm dish in serum-free culture media 199 with Earle’s salts. After allowing 2 hours for cardiomyocyte attachment, the culture media was exchanged and the cells were either used directly for patch-clamp recordings (see later) or infected either with AdEGFP 25 TCID₅₀/cardiomyocyte, AdEGFPKir2.1DN 5 TCID₅₀/cardiomyocyte, or AdEGFPKir2.2DN 5 TCID₅₀/cardiomyocyte for patch-clamp recording after 48 hours in culture. Transfection efficiency using these viruses was ≥98% as assessed by EGFP phenotypic expression. After either 2 or 48 hours in culture, glass coverslips containing laminin-attached cardiomyocytes were transfected into a small recording chamber mounted on the stage of an inverted microscope (Olympus IX 70) and whole-cell patch-clamp recording of I_Ks current was performed as we have previously described (see online data supplement).

#### Ischemic Preconditioning Studies in Freshly Isolated Cardiomyocytes

After isolation (n = 4 hearts), cardiomyocytes were divided in several groups and subjected to the following protocol (also see the online Figure in the online data supplement). First, all cardiomyocytes were transferred into separate wells in a multi-well dish and then incubated at 37°C in a 100% O₂ atmosphere for 30 minutes (stabilization period). Then, preconditioned cardiomyocytes were subjected to 10 minutes of simulated ischemia (SI, pelleting cells under an oil layer in a 1.5-mL Eppendorf tube and followed by incubation in a 37°C water bath) followed by 20 minutes of simulated reperfusion (SR, resuspension of cell pellet in oxygenated S-MEM medium). Control (nonpreconditioned) and baseline (incubation in S-MEM medium at 37°C without SI or SR) cardiomyocytes were incubated in S-MEM medium for another 30 minutes to match each group protocol in time. Next, each group of cardiomyocytes was subjected to 45-minute SI/60-minute SR. I_Ks channels were blocked with 20 μmol/L Ba²⁺ (used as BaCl₂), given during the 45-minute SI period. To avoid toxic effects and any other nonspecific effect derived from Ba²⁺ uptake into cardiomyocytes via L-type calcium channels, we concurrently administered the L-type calcium channel blocker nifedipine (1 μmol/L). The Ba²⁺ concentration used was based on the concentration-response electrophysiology study performed in freshly isolated rabbit ventricular cardiomyocytes (Figure 1) with the Ba²⁺ concentration estimated at ~20 μmol/L chosen to produce an estimated 80% to 90% inhibition of I_Ks under the conditions of simulated ischemia (see online data supplement for details). Cell viability (as measured by percentage of dead cardiomyocytes) was determined after 45-minute SI and 60-minute SR by trypan blue staining as previously described. We counted at least 300 cardiomyocytes (live and dead) at each time point in each group.

#### Primary Culture of Cardiomyocytes

Ventricular cardiomyocytes were cultured using a method we have previously described (for details see online data supplement).

#### Ischemic Preconditioning Studies in Barium-Blocked I_Ks Channels in Cultured Cardiomyocytes

After 48 hours in culture, cardiomyocytes were subjected to the following experimental protocol (also see the online Figure in the online data supplement). After 30-minute stabilization in culture medium 199, cardiomyocytes were subjected to 60-minute simulated ischemia (SI) and 60-minute simulated reperfusion (SR). Prolonged ischemia was simulated by incubating cardiomyocytes in a severely hypoxic (~8% O₂), HEPES-based solution (SI solution) containing (in mmol/L) 139 NaCl, 12.0 KCl, 0.5 MgCl2, 0.9 CaCl₂, 5 HEPES, 10 2-deoxy-D-glucose, and 20 lactate, 0.1% BSA (pH 6.5, at 37°C), in a 100% N₂ atmosphere. Reperfusion was simulated by incubation of cardiomyocytes in oxygenated medium 199 (pH 7.4, at 37°C). KCl concentration was increased from physiological levels (4.7 mmol/L to 12 mmol/L to simulate the high K⁺ concentration environment in myocardium during ischemia; lactate was added to the SI solution to simulate the build up of metabolic end products and low pH during ischemia. Cardiomyocytes were preconditioned by incubating them in an IPC solution (a solution similar to the SI solution with the exception that it has no lactate added, pH 7.4, and contained 4.7 mmol/L of KCl) for 10 minutes followed by a 10-minute SR before the 60-minute SI. Control cardiomyocytes were
not preconditioned. $I_{K1}$ channels were blocked with Ba$^{2+}$ (20 μmol/L BaCl$_2$) administered during the 60-minute SI and in the presence of 1 μmol/L nifedipine. All cardiomyocytes (control, preconditioned, and baseline) received the same treatments (BaCl$_2$+nifedipine, nifedipine alone, or no drug). Baseline cardiomyocytes were incubated in medium 199 for the duration of protocols to determine the effect of drugs on cell viability under oxygenated conditions. Cardiomyocyte viability was determined by trypan blue staining before ischemia and end of 60-minute SI/60-minute SR, as described in the online data supplement.

### Ischemic Preconditioning Studies in $I_{K1}$ Channel Knockdown Cultured Cardiomyocytes

After 2 hours in culture, cardiomyocytes were infected with one of three recombinant adenoviruses: AdEGFP, AdEGFPK1r2.1DN, or AdEGFPK1r2.2DN. Culture dishes that did not meet the criteria for effective rate of cell transfection (≥98% of cardiomyocytes showing EGFP fluorescence) were not used for the study. Transfected cardiomyocytes were included in nine different groups: (1) EGFP-transfected control (C$_{CM}$/H9262/BaCl$_2$); (2) EGFP-transfected preconditioned (IPC$_{CM}$/H9262/BaCl$_2$); (3) EGFP-transfected baseline (Baseline$_{CM}$/H9262/BaCl$_2$); (4) AdEGFPK1r2.1DN-transfected control (C$_{CM}$/H11001/AdEGFPK1r2.1DN); (5) AdEGFPK1r2.1DN-transfected preconditioned (IPC$_{CM}$/AdEGFPK1r2.1DN); (6) AdEGFPK1r2.1DN-transfected baseline (Baseline$_{CM}$/AdEGFPK1r2.1DN); (7) AdEGFPK1r2.2DN-transfected control (C$_{CM}$/H11002/AdEGFPK1r2.2DN); (8) AdEGFPK1r2.2DN-transfected preconditioned (IPC$_{CM}$/AdEGFPK1r2.2DN); and (9) AdEGFPK1r2.2DN-transfected baseline (Baseline$_{CM}$/AdEGFPK1r2.2DN). Matched-control cardiomyocytes were used for nontransfected groups (preconditioned, control, and baseline). All cardiomyocytes were subjected to the same standard SI/SR and cell viability protocols described above for the barium studies in cultured cardiomyocytes. To establish an unbiased reproducibility of the results, we performed these studies in two stages: (1) nonblinded experiments to the observer (n=4 hearts) and (2) blinded experiments to the observer (n=4 hearts). Because the same outcome was observed in the two stages, we pooled these data.

### Effect of Sarcolemmal $I_{K_{ATP}}$ Channel Inhibition on Ischemic Preconditioning in Cardiomyocytes

To determine if $s_{K_{ATP}}$ channels may also have a role in IPC protection against ischemia/reperfusion injury in freshly isolated and 48-hour cultured cardiomyocytes, we inhibited $s_{K_{ATP}}$ channels with 30 μmol/L HMR1098 (a gift from Adventis Pharma Deutschland GmbH, Germany). All cardiomyocytes were subjected to the same control, preconditioning and baseline protocols described for the barium experiments. HMR1098 was added to the medium just before the 60-minute SI. Cardiomyocyte viability was determined as described in the barium studies.

### Statistical Analysis

All data are summarized and expressed as mean±SEM. All cardiomyocyte data were first tested for normality and homogeneity of variance. ANOVA analysis followed by Scheffe post hoc test was performed to determine significant differences (P<0.05) among groups and between two groups, respectively. Where the criteria for parametric analysis were not met, we performed a nonparametric analysis (Kruskal-Wallis followed by Mann-Whitney U) to determine whether a significant difference (P<0.05) existed between two groups.

### Results

#### Inhibition of $I_{K1}$ Conductance by Barium and Dominant-Negative Transfection With AdEGFPK1r2.1DN

In these studies, inward rectifier $K^+$ currents were inhibited in two ways. Inward rectifier $K^+$ currents ($I_{K1}$) measured at −110 mV were inhibited by 90% at steady state with BaCl$_2$ (10 μmol/L) as shown in Figure 1 (see “Discussion” and online data supplement for an estimate of $I_{K1}$ inhibition of 86% to 87% under simulated ischemia at 20 μmol/L Ba$^{2+}$ in the preconditioning protection studies). As expected, $I_{K1}$ current density decreased under culture conditions (Figure 1). Cardiomyocyte $I_{K1}$ conductance elicited at −110 mV was reduced by approximately 50% (control fresh cardiomyocytes −17.9±1.09 pA/pF versus control 48 hour-cultured cardiomyocytes −8.35±0.87 pA/pF; P<0.05) after 48 hours in culture. $I_{K1}$ currents were further reduced by transfection of cultured ventricular cardiomyocytes with recombinant adenovirus expressing Kir2.1DN or Kir2.2DN genes along with the EGFP. Adenovirus expressing EGFP alone (AdEGFP) were used as controls and AdEGFP had no effect on $I_{K1}$ current density after 48 hours in cultured cardiomyocytes compared with nontransfected control cardiomyocytes cultured for the same period of time (Figure 1). Transfection with either...
AdEGFPKIR2.1DN or AdEGFPKIR2.2DN resulted in a significant reduction (≈50%) of $I_{K1}$ current density (AdEGFPKIR2.1DN −3.62±0.31 or AdEGFPKIR2.2DN −4.49±0.21 versus AdEGFP −7.95±0.53; $P < 0.05$) (Figure 1). BaCl$_2$, administered at a concentration of 10 μmol/L to AdEGFP transfected cardiomyocytes, blocked $I_{K1}$ current density to about the same extent as in freshly isolated cardiomyocytes (data not shown). Neither Ba$^{2+}$ nor AdEGFPKIR2.1DN/AdEGFPKIR2.2DN transfection had any effect on average membrane potential (see online data supplement for details).

**Blockade of Ischemic Preconditioning Protection by Pharmacological Inhibition of $I_{K1}$ Channels in Ventricular Cardiomyocytes**

The effects of 20 μmol/L Ba$^{2+}$, sufficient to inhibit $I_{K1}$ current by about 86% to 87% (see Discussion and online data supplement) on protection against cell death by IPC in freshly isolated cardiomyocytes is shown in Figure 2A. As expected, there was no difference in the percentage of dead cardiomyocytes either after the initial stabilization period or immediately before the long SI among all the groups. Similarly, no difference among all the baseline groups (with and without barium) was found with regard to the percentage of dead cardiomyocytes at the end of the experimental period. As we have previously shown, ischemic preconditioning (IPC) significantly ($P < 0.0001$) reduced the percentage of dead cardiomyocytes after 45 minutes of SI and 60 minutes of SR, when compared with untreated control cardiomyocytes [IPC versus control (C)]. This amount of protection conferred by IPC in freshly isolated cardiomyocytes (46% reduction in mortality; Figure 2A) was similar to the amount of IPC protection observed in 48-hour cultured cardiomyocytes (49% reduction in mortality, Figure 2B) and with single-cycle IPC protection in vivo in rabbit hearts (42% reduction in infarct size) previously reported from our laboratory. Blockade of $I_{K1}$ channels with 20 μmol/L BaCl$_2$ (in the presence of 1 μmol/L nifedipine) significantly ($P < 0.0001$) reduced the protection induced by IPC (IPC+BaCl$_2$), while having no effect on treated control cardiomyocytes (C+BaCl$_2$). Nifedipine alone did not protect against ischemia or block IPC-induced protection (C+NF and IPC+NF, respectively). None of the treatments used in these protocols affected cell mortality in time-matched nonischemic baseline cardiomyocytes (data not shown). Similar results were obtained in 48-hour cultured cardiomyocytes (Figure 2B).
Blockade of Ischemic Preconditioning Protection by Targeted Knockdown of $I_{K1}$ Channel Subunits (Kir2.1 and Kir2.2) in Cultured Ventricular Cardiomyocytes

Because barium may not be completely specific for $I_{K1}$ channel blockade, we utilized a more definitive molecular approach to confirm the role of $I_{K1}$ channels in IPC. Cardiomyocytes were transfected with either a dominant-negative gene (AdEGFPKir2.1DN or AdEGFPKir2.2DN) to knockdown $I_{K1}$ channels or the AdEGFP gene as control. The percentage of dead cardiomyocytes did not differ among the different groups before the long SI/SR (Figure 3). In addition, the percentage of dead cardiomyocytes did not change over time (160 minutes) when cardiomyocytes were kept in oxygenated conditions at 37°C (data not shown). Knockdown of $I_{K1}$ channels by either AdEGFPKir2.1DN or AdEGFPKir2.2DN abolished IPC protection (Figure 3) when compared with cardiomyocytes transfected with the reporter gene (AdEGFP) alone or with nontransfected cardiomyocytes. Transfection of cardiomyocytes with AdEGFP neither changed cardiomyocyte mortality in controls (nonpreconditioned) nor affected IPC protection in cardiomyocytes.

Effect of Sarcolemmal $I_{KATP}$ Channel Blockade on Ischemic Preconditioning Cardioprotection

To determine if in our cardiomyocyte culture model $I_{KATP}$ channels were important for IPC, we tested whether blockade of these channels eliminated IPC protection in both freshly isolated cardiomyocytes and 48-hour cultured cardiomyocytes. Selective blockade of $I_{KATP}$ channels with 30 μmol/L HMR1098 had no effect on the protection by IPC, as shown in Figure 4A and 4B. HMR1098 alone did not affect cell viability in baseline cardiomyocytes in either cell model (data not shown).

Discussion

The present study defines a role for $I_{K1}$ channels in the protection produced by ischemic preconditioning (IPC). We show that inhibition of $I_{K1}$ channels with Ba$^{2+}$ or genetic knockdown of $I_{K1}$ channels using a dominant-negative approach abolishes IPC protection against ischemic cell death in cultured rabbit ventricular cardiomyocytes.

An initial concern in using Ba$^{2+}$ to inhibit $I_{K1}$ currents was the possible toxic effects of this agent on cardiomyocytes. Indeed, in pilot studies, we observed barium-associated increase of necrosis in cardiomyocytes under baseline conditions. This necrosis above baseline levels was prevented by treatment with nifedipine to block L-type calcium channels. Importantly, nifedipine did not interfere with the protective response to preconditioning nor did it produce a protective effect under control conditions.

Although Ba$^{2+}$ is a relatively nonspecific blocker of K$^+$ currents, it is unlikely that the inhibition of IPC protection by Ba$^{2+}$ application in our cardiomyocyte studies is a consequence of blockade of voltage-gated K$^+$ channels because the cardiomyocytes were electrically quiescent. On the other hand, Ba$^{2+}$ at levels used in our studies (20 μmol/L) can inhibit (40% to 50%) of $I_{KATP}$ current at hyperpolarized membrane potentials (−100 mV) although there is minimal inhibition at membrane potentials close to the resting poten-
two dominant-negative genes did not affect voltage-gated K⁺ currents in cultured cardiomyocytes, it would appear that these dominant-negative genes caused specific reductions in Iᵦᵢ current.

Because the amplitude of the Iᵦᵢ current is diminished in cardiomyocytes after 2 to 3 days in culture when compared with the Iᵦᵢ current in freshly isolated cardiomyocytes, one may question whether 48-hour cultured cardiomyocytes are appropriate for studying the role of Iᵦᵢ channel in IPC. In fact, despite a significant reduction of Iᵦᵢ current density to about half that in control cardiomyocytes overexpressing the EGFP gene after 48 hours in culture (Figure 1), IPC protection against ischemic cell death was as potent as observed in freshly isolated cardiomyocytes (Figures 2A and 4A). These results suggest that a critical level of Iᵦᵢ current is necessary for IPC to occur and that only reductions below this critical “threshold” level will lead to inhibition of IPC. Consistent with this suggestion, the absolute level of Iᵦᵢ current remaining in freshly isolated cardiomyocytes after inhibition with Ba²⁺ during our IPC studies is estimated to be similar to that present in cultured cardiomyocytes after dominant-negative knockdown of Iᵦᵢ. Specifically (also see online data supplement for further details), by accounting for the voltage-dependence of Iᵦᵢ block by Ba²⁺ as well as the changes in resting membrane potential of cardiomyocytes in our IPC studies (ie, −65 to −50 mV) due to K⁺ accumulation, Iᵦᵢ currents in our IPC studies with Ba²⁺ are estimated to be reduced by about 86% to 87% compared with a 75% to 80% reduction in Iᵦᵢ density recorded in cultured cardiomyocytes expressing dominant-negative Kir2.1/2 genes. It is possible that the extent of Iᵦᵢ inhibition necessary to block IPC protection may depend on the experimental conditions used (ie, fresh versus cultured cardiomyocytes), possibly as a result of a change in threshold of Iᵦᵢ current required to support IPC. Further studies will be necessary to fully unravel the dependence of IPC on the absolute level of Iᵦᵢ activity.

The results of the present study contrast with previous findings by Schultz et al who showed that a bolus intravenous administration of terikalant, an Iᵦᵢ channel blocker, 15 minutes before the long ischemia in vivo did not block ischemic preconditioning in anesthetized rats. This discrepancy could be due either to species differences (rat versus rabbit) in the role of Iᵦᵢ channels or, more likely, to inadequate Iᵦᵢ blockade by terikalant in that study. Indeed, terikalant is a relatively weak blocker of inward rectifier K⁺ channels in cardiomyocytes and Schultz et al could only block 39% of Iᵦᵢ current in guinea-pig ventricular cardiomyocytes with 30 μmol/L terikalant, which was the concentration selected to avoid simultaneous blockade of Iᵦᵢ and sᵦᵢ channel. As described earlier, our data suggest that this level of inhibition is insufficient to block IPC protection. Furthermore, terikalant also blocks Iᵦᵢ at micromolar concentrations, although this is unlikely to explain the inability of terikalant to block IPC-mediated cardioprotection.

The regulatory signaling underlying the protection produced by Iᵦᵢ currents against ischemic injury in our studies could involve several mechanisms. We have recently demonstrated that enhanced cell volume regulation is a key mechanism of IPC cardioprotection. Combining these results with
our previous observations that Cl\textsuperscript{−} channel inhibition blocks the protection against myocardial necrosis of both ischemia\textsuperscript{a} and pharmacological\textsuperscript{b} preconditioning suggests that outward Cl\textsuperscript{−} movement is involved in protecting cardiomyocytes from ischemic injury. Because ionic balance (electroneutrality) is required for volume regulation, our results suggest that I\textsubscript{K1} currents are required for IPC as a consequence of contribution to volume regulation. The direct contribution of I\textsubscript{K1} channels to cell volume regulation in cardiomyocytes remains to be investigated and is beyond the scope of the present study. Recently, Frie et al\textsuperscript{c} reported that preconditioning in single guinea pig cardiomyocytes produced a significant reduction in the average time for induction (ie, early activation) of outward K\textsuperscript{+} current in response to simulated ischemia. These results are consistent with I\textsubscript{K1} as a source for the outward K\textsuperscript{+} movement they have demonstrated.

To the extent to which enhanced cell volume regulation during ischemic stress is responsible for IPC protection against ischemia/reperfusion injury, transsarcolemmal K\textsuperscript{+} efflux in quiescent cultured cardiomyocytes could arise from background K\textsuperscript{+} currents other that I\textsubscript{K1}, most notably sI\textsubscript{KATP}. Addressing this issue, we found that sI\textsubscript{KATP} blockade with HMR1098 did not inhibit IPC in freshly isolated or 48-hour cultured quiescent cardiomyocytes. Under in vivo conditions, the role of sI\textsubscript{KATP} in IPC has been controversial. Consistent with the present study, blockade of sI\textsubscript{KATP} with HMR1883, of which HMR 1098 is the sodium salt, did not abolish the protective effect of IPC in rabbit hearts.\textsuperscript{27} Nevertheless, it has been shown that in Kir6.2 knockout mice lacking sI\textsubscript{KATP} channels, IPC failed to protect from myocardial necrosis\textsuperscript{28} and failed to improve posts ischemic functional performance.\textsuperscript{29,30} A more intense and rapid contracture has been reported to occur during the index ischemia in the Kir6.2 knockouts than in control hearts with subsequent functional recovery much worse than control, in the absence of preconditioning.\textsuperscript{31} It is clear that knockout of sI\textsubscript{KATP} channels results in a poor response of the myocardium to ischemic challenge under control conditions, likely shifting ischemic injury to higher levels. This makes it difficult to determine if sI\textsubscript{KATP} is playing a specific role in IPC protection. It remains possible that both sI\textsubscript{KATP} and I\textsubscript{K1}, in combination, may be contributing to IPC protection in vivo through enhanced cardiomyocyte cell volume regulation. Because our experimental results are limited to fresh and cultured cardiomyocyte models, some caution is warranted in extrapolation to the whole heart. Other K\textsuperscript{+} channels and transporters might also contribute to K\textsuperscript{+} efflux under ischemic conditions. Also, the precise quantitative connection among K\textsuperscript{+} efflux, I\textsubscript{K1} density and preconditioning requires further investigation.

Other than sI\textsubscript{KATP}, to date the putative ATP-sensitive mitochondrial K\textsuperscript{+} (mitoK\textsubscript{ATP}) channel has been the primary focus of interest and controversy in regard to ion channels in IPC. Whatever the possible role of mitoK\textsubscript{ATP} in IPC may be, it cannot, alone, account for the protection against necrosis afforded by IPC based on a recent review of the literature by Gross and Peart.\textsuperscript{32} Furthermore, there is no evidence that the I\textsubscript{K1} channel is expressed in mitochondria; therefore, involvement of mitochondria in the action of I\textsubscript{K1} in IPC, if any, would have to be indirect, conceivably through a signaling pathway terminating on the I\textsubscript{K1} channel.

In conclusion, our results indicate that I\textsubscript{K1} channels play an important role in the protection induced by ischemic preconditioning and support the I\textsubscript{K1} channel as a strong candidate for the role as one end effector in ischemic preconditioning of the myocardium.

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Data Supplement

I. Isolation of Adult Rabbit Ventricular Cardiomyocytes by Enzymatic Digestion

Cardiomyocytes were isolated by enzymatic dissociation as we have previously described. Briefly, donor rabbits were first anesthetized with a mixed solution of pentobarbital (60 mg/Kg) and heparin (200 IU/kg). The heart was excised and immediately perfused on a non-recirculating Langendorff apparatus at 75 mmHg of perfusion pressure with oxygenated (95% O₂-5% CO₂) Joklik-modified minimal essential medium solution (S-MEM, Gibco Inc.), at 37°C, pH 7.4 and supplemented with (mmol/L) CaCl₂ 1.2, MgSO₄ 0.2, creatine 20, taurine 60, NaHCO₃ 24, and HEPES 10 (~300 mOsm). Hearts were perfused with calcium-containing S-MEM solution (2-3 min) before perfusing with nominally calcium-free S-MEM solution supplemented with EGTA (0.016 mol/L) and 0.1% bovine serum albumin (BSA, Pentex fraction V, Sigma Chemical Co. Inc.) for 6-8 min. Subsequently, all hearts were perfused in recirculating mode, with the same calcium-free S-MEM solution (coronary flow rate=10-15 ml/min) supplemented with collagenase type II (200 IU/ml, Worthington Inc.), specific activity range=225-275 IU/mg, for 10-20 min. Isolated cardiomyocytes were filtered through a nylon mesh, centrifuged (45 g for 3-min x 2) and resuspended in calcium-free S-MEM solution containing 2% BSA. The calcium concentration was then increased in steps over a 30-min period, to a final concentration of 1.07 mmol/L. The cell suspension was centrifuged again for 2 min at 45 g, the supernatant discarded, and the cell pellet resuspended in 15 ml of calcium-containing (1.2 mmol/L) S-MEM solution supplemented with 0.1% BSA. Using this procedure, we generally obtained 8-9 million cells/g of tissue with a yield of 70-80% rod-shaped cardiomyocytes.
II. Expanded single cell electrophysiology method used in isolated and cultured cardiomyocytes.

After ventricular cardiomyocytes were prepared as described above, they were cultured on laminin-coated glass coverslips at a density of 4-5 x 10^4 cells/35-mm dish in serum-free culture media with Earle’s salts. After allowing 2 hours for cardiomyocyte attachment, the culture media was exchanged and the cells were either used directly for patch-clamp recordings (see below) or infected either with AdEGFP 25 TCID_{50}/cardiomyocyte, AdEGFPKir2.1DN 5 TCID_{50}/cardiomyocyte or AdEGFPKir2.2DN 5 TCID_{50}/cardiomyocyte for patch-clamp recording after 48 hours in culture. Transfection efficiency using these amounts of virus was ≥98% as assessed by EGFP phenotypic expression. After either 2 or 48 hours in culture, glass coverslips containing laminin-attached cardiomyocytes were transferred into a small recording chamber mounted on the stage of an inverted microscope (Olympus IX 70) and whole-cell patch-clamp recording of I_{K1} current were performed as we have previously described. Briefly, the chamber was perfused with physiological salt solution containing (mmol/L): 140 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 10 Glucose, 0.5 CdCl₂ (at room temperature ~22-23°C). Whole-cell patch-clamp recordings were performed (Axopatch 200A, Axon Instruments). Recording pipettes were prepared from polished thin-walled borosilicate glass (1.5 mm diameter, World Precision Instruments) using a Flaming-Brown micropipette puller (Sutter Instruments) and had a resistance of 2-4 MΩ. The pipette solution contained (mmol/L) 90 K-Aspartate, 20 KCl, 1 MgCl₂, 10 HEPES, 10 EGTA, 5 Na₂-ATP, pH 7.3 with 30 mmol/L KOH. Cardiomyocytes suitable for patch-clamping were identified based on their morphological appearance (rod-shaped, absence
of blebs) and EGFP fluorescence. In agreement with Rust et al\(^3\), infected cardiomyocytes did not display morphological alterations when compared to time matched non-infected cardiomyocytes. Cardiomyocyte capacitance was estimated by integrating capacity currents, followed by compensation for pipette series resistance. To measure \(I_{K1}\) current, voltage steps from -130 to -10 mV, in 10 mV increments, were applied for 500 ms with an interval of 1 second from a holding potential of -40 mV. The data were acquired with pClamp 6 software (Axon Instruments) and analyzed with pClamp 8. The data were not corrected for the liquid junction potential, which was 7.2 mV as determined by the method of Barry and Lynch.\(^4\)

### III. Simulated Ischemia and Reperfusion for Cell Necrosis Studies in Freshly Isolated Cardiomyocytes

Ischemia was simulated in cardiomyocytes using a method previously by Vander Heide\(^5\) and Armstrong\(^6\), and extensively used in our laboratory.\(^1,7\) Briefly, 1.5 ml of the cardiomyocyte suspension was placed in a 1.5 ml Eppendorff tube and centrifuged (45 g x 2-min) to form a 8-10 mm thick cell pellet. The supernatant was discarded, except for a volume equivalent to about 1/3 of the cell pellet thickness. A 3-4 mm thick mineral oil layer was then placed on top of the cell pellet and supernatant to prevent oxygen uptake and incubated at 37°C in a water bath. This method of simulated ischemia was intended to reproduce the major metabolic consequences of ischemia by packing cardiomyocytes in a cell pellet by centrifugation (45 g for 2-3 min) to limit the access to oxygen of cardiomyocytes so that oxidative phosphorylation is inhibited and anaerobic glycolysis is promoted with the consequent of breakdown of metabolic-by-products leading to
accumulation of lactate, reduction of pH and squaring (cell change in the length:width ratio from 3:1 to 1:1) of cardiomyocytes as ATP stores are depleted and the production of high energy phosphates via anaerobic glycolysis is limited. Under these conditions, with extracellular volume less than 50% of total volume, extracellular K+ concentration can be expected to be similar to that in whole hearts during ischemia, with concentrations rising into the 10 to 20 mmol/L range.

Reperfusion was simulated, in freshly isolated cardiomyocytes, using a method we have previously characterized. Briefly, a 100-150 µl sample of the ischemic cell pellet was taken with a pipette and resuspended in 1.0 ml of oxygenated calcium-containing buffer supplemented with 0.1% BSA and incubated on a multidish cell culture plate at 37°C with agitation in an O₂ atmosphere.

IV. Primary Culture of Adult Rabbit Ventricular Cardiomyocytes

Isolated rabbit ventricular myocytes were placed in culture using a modified version of a method we have previously described. Cardiomyocytes were placed in 35-mm laminin-coated petri dishes, with a density of 2.5 x 10⁵ cells/dish, each containing 2 ml of culture medium 199 (Gibco, Burlington, ON, Canada) with Earle’s salts supplemented with 10% fetal bovine serum, 10 µM cytosine β-D-arabinofuranoside, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 0.08 mg/ml gentamicin. Cardiomyocytes were then incubated at 37°C in a humidified 5% CO₂-95% air mix for 48 hours. Culture medium was replaced by fresh medium every 24 hours of incubation.
V. Comparison of Fresh vs Cultured Cardiomyocyte Models Regarding Level of Injury Produced by Simulated Ischemia and Preconditioning Protection.

There are limitations in isolated cardiomyocyte models (freshly isolated cardiomyocytes versus primary cultured cardiomyocytes) that may influence the amount of injury one may induce in cardiomyocytes. Because of these differences, we adjusted the simulated ischemia protocol in each cardiomyocyte model to obtain a level of injury (measured as an increase in the percentage of dead cardiomyocytes) similar to the level of ischemic injury (measured as an increase in the percentage of necrosis in the region at risk) observed in myocardium after a prolonged period of regional ischemia followed by reperfusion in whole heart. In whole heart studies, using an index ischemia of 30-min duration at 37°C, we have observed a control level of injury of about 50-55% of necrosis (in the region at risk) with IPC producing an average of 42% reduction in the percentage of myocardial necrosis. In freshly isolated cardiomyocytes we had to adjust the index ischemia to 45-min duration to obtain a similar level of injury (50-55% of cardiomyocytes dead), with IPC producing an average of 46% reduction in the percentage of dead cardiomyocytes. In cultured cardiomyocytes, we had to extend the duration of the index ischemia to 60 min, lower extracellular pH to 6.5, increase KCl concentration to 12 mmol/L, replace glucose with a metabolically inactive substrate, and make the medium severely hypoxic to obtain equivalent amount of injury (50-55% of dead cardiomyocyte), with IPC producing an average of 49% reduction in the percentage of dead cardiomyocytes. Cardiomyocyte models of simulated ischemia similar to ours have been used by many other investigators to study ischemic injury.
VI. Assessment of Cardiomyocyte Viability by Trypan Blue Staining

in either Freshly Isolated or Cultured Cardiomyocytes.

In freshly isolated cardiomyocytes, cell viability was assessed by trypan blue exclusion. Trypan blue staining was used to distinguish viable (cells are not stained because their membrane is intact and thus they exclude trypan blue) from dead cells (cells which cannot exclude trypan blue and are stained blue colour). In freshly isolated cardiomyocytes, cell viability was assessed by pelleting the cardiomyocytes in an eppendorff tube at the end of simulated reperfusion and obtaining a 25 µl of the cell pellet which was then mixed with a 25 µl of hypotonic (85 mOsm) trypan blue solution as we have previously described (Diaz et al 1999). Then, a sample from this mixture was mounted on a hemocytometer for cell counting using a microscope. Thus, we studied as many groups as was technically feasible to evaluate by an observer at the same time frame. The strategy of sample staggering was also used to have all groups evaluated in one experiment using the same batch of isolated cardiomyocytes. We usually counted a total of at least 300 cells, from 4-5 microscopic fields, for each group sample. For studies performed in 48-hour cultured cardiomyocytes, small cell aliquotes (50-100 µl of total cell isolate pellet) from the freshly isolated cardiomyocytes were resuspended in 2 ml culture medium 199 supplemented as we have described in the method section of the manuscript and placed in as many laminin-coated 35-mm Petri dishes as the total number of groups being evaluated. Thus, at one time, in each experiment from one cell isolation from a heart, we assessed simultaneously all the different cell groups. Cell viability was assessed in this cultured cardiomyocyte model by exposing cardiomyocytes to trypan blue staining solution at the end of simulated reperfusion. We counted at least 300...
cardiomyocytes, from 5-6 microscopic fields, using an inverted microscope. In both cell studies (fresh and cultured cardiomyocytes), the % of dead cell was calculated in each group, from cardiomyocytes isolated or cultured from each heart (n=4-8), using the following formula: number of dead cells/total number of cells. From these data we then calculated the mean percentage of dead cardiomyocyte in each group. Each heart provided sufficient myocytes to seed as many cell culture plates as needed to performed an experiment for every specific group simultaneously (e.g., control (C), ischemically preconditioned (IPC), C+Ba\textsuperscript{2+}, IPC+Ba\textsuperscript{2+}, C+nifedipine, IPC+nifedipine, and all baseline controls).

VII. Adenoviral-Mediated Transfections of Cultured Cardiomyocytes

After isolation, cardiomyocytes were placed in culture for 48 hours as described in the culture protocol above. Following attachment to the laminin after 2 hours in culture, cardiomyocytes were infected with a recombinant replication deficient adenovirus type 5 (Ad5) lacking the E1 and E3 regions and encoding either the enhanced green fluorescent protein gene (EGFP) alone or in combination with the dominant negative mutant gene Kir2.1(C122S), designated as Kir2.1DN, or Kir2.2(C123S), designated as Kir2.2DN, as we have previously described.\textsuperscript{2} Each 35-mm dish containing cultured cardiomyocytes, was infected with 3-4 µl of a viral stock solution, AdEGFP (1:5 dilution), AdEGFPKir2.2DN (1:3 dilution) and AdEGFPKir2.2 (1:3 dilution) after 2 hours in culture. Culture media was then exchanged after 24 and 48 hours in cultured. The AdEGFP virus, an adenovirus encoding the EGFP reporter gene, was purchased from Quantum Biotechnologies Inc., Montreal, Canada. All viruses used were plaque-purified
and titers were determined using the 50% tissue culture infectious dose method (TCID\textsubscript{50}): AdEGFP \(3.6 \times 10^9\) TCID\textsubscript{50} ml\(^{-1}\), AdEGFPKir2.1DN \(1.4 \times 10^{10}\) TCID\textsubscript{50} ml\(^{-1}\), and AdEGFPKir2.2DN \(2.8 \times 10^9\) TCID\textsubscript{50} ml\(^{-1}\). Transfection was confirmed by the demonstration of green fluorescence on cultured cardiomyocytes when observed under an UV light and a GFP filter in a deconvolution LEICA microscope. Transfection efficiency was approximately 98% using this transfection method. Transfections were performed in the presence of 5% fetal bovine serum and antibiotics.

VIII. Estimating the Percentage Inhibition of I\textsubscript{K1} During the Preconditioning Protection Experiments.

Block of I\textsubscript{K1} is both time- and voltage-dependent (as can be seen from our electrophysiological studies illustrated in Figure 1). Thus, the amount of current block in our IPC studies is rather complex and depends on several factors as described below. It is the steady-state I\textsubscript{K1} block in quiescent cells that is the most relevant in establishing the link between I\textsubscript{K1} and IPC protection. Obviously, it is difficult to exactly determine the steady-state I\textsubscript{K1} block by Ba\textsuperscript{2+} at 20 µmol/L concentration under our conditions for the IPC experiments, especially under the conditions of simulated ischemia using freshly isolated cardiomyocytes. However, we can make reasonable estimates of the range of I\textsubscript{K1} current block. Estimating the range of Ba\textsuperscript{2+} block on I\textsubscript{K} current can be readily done by using our electrophysiological measurements of Ba\textsuperscript{2+} block at various voltages and Ba\textsuperscript{2+} concentrations along with reasonable estimates of the resting membrane potential in our IPC studies. In our previous study published in the Journal of Physiology\textsuperscript{2} we measured Ba\textsuperscript{2+} block of I\textsubscript{K} current at several membrane potentials in both freshly isolated
cardiomyocytes and in 48-hour cultured cardiomyocytes (with and without \( I_{K1} \) reduction with both Kir2.1DN and Kir2.2DN gene transfection). As shown in our previous publication in the *Journal of Physiology* (Figure 8), there is no detectable difference in the \( Ba^{2+} \) blocking properties of \( I_{K1} \) under these different experimental conditions, supporting our suggestion that the molecular composition of \( I_{K1} \) does not change during time in culture or as a consequence of \( I_{K1} \) knockdown using our dominant-negative genes. Therefore, we used our results obtained in cultured cardiomyocytes at 48 hours to provide estimates for steady-state block in our IPC studies, although precisely the same estimates would be obtained if we used the data from the freshly isolated cardiomyocytes. After 48 hours in culture, the IC50s for \( BaCl_2 \)-block of \( I_{K1} \) current in our rabbit cardiomyocytes at \(-110 \text{ mV}, \ -100 \text{ mV} \) and \(-90 \text{ mV} \) were \( 1.71\pm0.16 \mu\text{mol/L} \), \( 1.94\pm0.2 \mu\text{mol/L} \) and \( 2.54\pm0.28 \mu\text{mol/L} \), respectively (from our *J Physiology* paper and unpublished observations of C. Zobel and P. Backx). Theoretically, if \( Ba^{2+} \) occurs within the selectivity filter and if the measured IC\(_{50}\) reflects \( Ba^{2+} \) binding to the channel pore, then the IC\(_{50}\) is expected from many previous biophysical studies in ion channels to depend exponentially on the electric field. By plotting the log (IC\(_{50}\)) as a function of voltage, we estimated the binding constant at 0mV (i.e. IC\(_{50}(0)\), the y intercept) and the “fractional electrical distance” (i.e. \( \delta \) which represents the depth of binding of \( Ba^{2+} \) in the channel pore). From these plots, we found that the IC\(_{50}(0)\) was \( 3.15 \mu\text{mol/L} \) and the \( \delta \) was 0.56. Assuming that the external \( K^+ \) levels range between 10 and 20 mmol/L in our IPC studies on freshly isolated cardiomyocytes, then the resting membrane potential is between \(-65 \text{ mV} \) and \(-49 \text{ mV} \), assuming that the internal \( K^+ \) level is 140 mmol/L. These data indicate an IC50 for \( Ba^{2+} \) somewhere between 2.7-2.9 µmol/L (for extracellular \( K^+ \) concentration of 10 mmol/L.
and 20 mmol/L, respectively) in our IPC studies. From this we predicted that (using the
formula \( \%\text{block} = \frac{1}{1 + (K_a/[Ba^{2+}])} \) a steady-state \( I_{K_1} \) blockade of 86-87% at 20
\( \mu \text{mol/L Ba}^{2+} \). Based on the above calculations, our cardiomyocyte necrosis data (for 20
\( \mu \text{mol/L Ba}^{2+} \)) showed that the inhibition of IPC protection occurs when the current
density reduced to below 20% of the level observed in freshly isolated cardiomyocytes.
This (absolute) level of \( I_{K_1} \) current in the presence of 20 \( \mu \text{mol/L Ba}^{2+} \) in our IPC
experiments is actually remarkably similar to the \( I_{K_1} \) current measured in our cultured
cardiomyocytes after dominant negative knockout of \( I_{K_1} \) using either the Kir2.1DN or
Kir2.2DN constructs, which also block preconditioning. Indeed, in our cultured
cardiomyocytes \( I_{K_1} \) density is reduced by about half relative to freshly isolated
cardiomyocytes while dominant negative suppression resulted in a further reduction of
about 50% (56% for Kir2.1DN and 47% for Kir2.2DN). Briefly, culturing for 48h
resulted in a reduction of average current density to -8.35±0.87 pA/pF (p<0.05, reduction
of current ~55% compared to fresh cardiomyocytes). Infection with AdEGFP alone had
no impact on \( I_{K_1} \) current densities. Overexpression of Kir2.1DN reduced current density
to -3.62±0.31 pA/pF (p<0.05, relative reduction ~56% compared to cultured control
cells) while overexpression of Kir2.2DN diminished current density to -4.49±0.21 pA/pF
(p<0.05, relative reduction ~47% compared to cultured control cells). Thus, the amount
of current reduction was similar between Kir2.1DN and Kir2.2DN. Following dominant-
negative knockdown of \( I_{K_1} \), the \( I_{K_1} \) current is reduced to about 20 to 25% of the level
observed in the freshly isolated cardiomyocytes for Kir2.1DN and Kir2.2DN, respectively.
IX. **Effect of Ba$^{2+}$, AdEGFPKir2.1DN and AdEGFPKir2.2DN on Average Membrane Potentials in Cultured Cardiomyocytes.**

With 4 mmol/L external K$^+$, the average membrane potential (not corrected for liquid junction potentials) obtained from cardiomyocytes cultured for 48 hours under our various experimental conditions are as follows: for AdEGFP -75.07±0.97 mV (mean±SEM) (n=17), for AdEGFPKir2.1DN 73.87±1.87 mV (n=10) and for AdEGFPKir2.2DN -74.25±2.04 mV (n=9). There was no significant difference among these groups. The absence of alterations in membrane potential despite significant reductions of I$_{K1}$ density (~55% relative to control current density after 48 hours culture) is not unexpected since I$_{K1}$ currents are the predominant currents setting the resting membrane potential. Indeed, once corrected for the liquid junction potential which is +7.2mV) the resting membrane potential in our cultured cardiomyocytes are very close to the predicted Nernst potential for K$^+$ ion distribution across the cell membrane.

XI. **Experimental Protocols for Cell Necrosis Studies in Freshly Isolated and 48-hour Cultured Cardiomyocytes (see Figure below).**
Experimental protocol for freshly isolated ventricular cardiomyocytes (A) and 48-hour cultured cardiomyocytes. A- Freshly isolated cardiomyocytes were first stabilized for 30 min and then either ischemically preconditioned (IPC) with 10-min simulated ischemia (SI, pelleting under an oil layer, 37°C) followed by 10 min of simulated reperfusion (SR, re-suspension in oxygenated S-MEM medium), or not preconditioned (control, C). Next, cardiomyocytes were subjected to 45-min SI/60-min SR. Time-matched oxygenated cardiomyocytes (Baseline) were also studied. B- 48-hour cultured cardiomyocytes were initially stabilized for 30 min and then either ischemically preconditioned (IPC\textsubscript{CM}) with 10-min simulated ischemia (SI, severe hypoxia plus metabolic inhibition) followed by 10-min simulated reperfusion (SR, re-oxygenation in culture medium 199) or not preconditioned (control, C\textsubscript{CM}). Next, cardiomyocytes were subjected to 60-min SI/60-min SR.
SR. Time-matched oxygenated cardiomyocytes (Baseline\textsubscript{CM}) were also studied.

Cardiomyocytes viability was assessed by trypan blue staining at different time points (*).

References


