Role of sarcolemmal ATP-sensitive potassium channel in oxidative stress-induced apoptosis: mitochondrial connection

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Marinovic J, Ljubkovic M, Stadnicky A, Bosnjak ZJ, Bienengraeber M. Role of sarcolemmal ATP-sensitive potassium channel in oxidative stress-induced apoptosis: mitochondrial connection. Am J Physiol Heart Circ Physiol 294: H1317–H1325, 2008. First published January 11, 2008; doi:10.1152/ajpheart.00840.2007.—From time of their discovery, sarcolemmal ATP-sensitive K⁺ (sarcKATP) channels were thought to have an important protective role in the heart during stress whereby channel opening protects the heart from stress-induced Ca²⁺ overload and resulting damage. In contrast, some recent studies indicate that sarcKATP channel opening can lead to cardiac protection. Also, the role of the sarcKATP channel in apoptotic cell death is unclear. In the present study, the effects of channel inhibition on apoptosis and the specific interaction between the sarcKATP channel and mitochondria were investigated. Apoptotic cell death of cultured HL-1 and neonatal cardiomyocytes following exposure to oxidative stress was significantly increased in the presence of sarcKATP channel inhibitor HMR-1098 as evidenced by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling and caspase-3,7 assays. This was paralleled by an increased release of cytochrome c from mitochondria to cytosol, suggesting activation of the mitochondrial death pathway. sarcKATP channel inhibition during stress had no effect on Bcl-2, Bad, and phospho-Bad, indicating that the increase in apoptosis cannot be attributed to these modulators of the apoptotic pathway. However, monitoring of mitochondrial Ca²⁺ with rhod-2 fluorescent indicator revealed that mitochondrial Ca²⁺ accumulation during stress is potentiated in the presence of HMR-1098. In conclusion, this study provides novel evidence that opening of sarcKATP channels, through a specific Ca²⁺–related interaction with mitochondria, plays an important role in preventing cardiomyocyte apoptosis and mitochondrial damage during stress.

METHODS

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin.

HL-1 cells. HL-1 cells [atria-derived mouse cardiac myocyte cell line (9)] were a gift from Dr. William C. Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA). They were cultured in Claycomb medium (SAFC Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (FBS), 4 mM glucose, 10 μM norepinephrine, 100 U/ml penicillin, and 100 μg/ml streptomycin on gelatin/fibroin-coated flasks and maintained in a humidified 5% CO₂ incubator at 37°C. The HL-1 cells were used for experimentation after reaching ~80% confluence.

Cultured neonatal cardiomyocytes. To confirm our data obtained with the cardiac HL-1 cell line, we also used primary cardiomyocytes. One-day-old Sprague-Dawley neonatal rats (~20 rats per isolation) were used to culture primary cardiomyocytes.

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were decapitated, the hearts were excised, and ventricular myocardium was minced in ADS buffer (in mM: 116 NaCl, 20 HEPES, 1 NaH2PO4, 5.5 glucose, 5.4 KCl, and 0.8 MgSO4 with 3 mg/ml phenol red, pH 7.35) with 0.15 mg/ml collagenase ( Worthington CLS II, Lakewood, NJ) and 0.52 mg/ml pancreatin (Life Technologies, Grand Island, NY) and incubated in a shaker at 37°C for 20 min at 100 rpm. Tissue pieces were allowed to settle, and the supernatant was collected, suspended in 1 ml of newborn calf serum ( GibC0, Carlsbad, CA), and centrifuged at 1,000 × g for 6 min. The cell pellet was resuspended in 1 ml of newborn calf serum and stored at 37°C. The procedure was repeated until all tissue was digested. The cells were then resuspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 17% fetal bovine serum, 5% FBS, 50 μm penicillin, 50 μg/ml streptomycin, and 20 mm HEPES at pH 7.2 and preplated for 2 h on cell culture dishes to separate ventricular myocytes from the faster-attaching nonmyocytes. The ventricular myocytes in the supernatant were collected and plated on gelatin-coated dishes and chamber slides. The neonatal cardiomyocytes were used for experiments after demonstrating confluence and rhythmic contractions (after 72 h).

Electrophysiology. For electrophysiological recordings of sarKATP channel activity, HL-1 and neonatal cardiomyocytes were lifted from culture dishes with 0.05% trypsin-0.53 mM EDTA, washed, and suspended in a Tyrode solution containing (in mM) 132 NaCl, 10 glucose, 5 KCl, 1 CaCl2, and 1.2 MgCl2 at pH 7.4. Single-channel activity was monitored from excised inside-out membrane patches at room temperature, as reported previously (38). Brieﬂy, the ﬁre-polished borosilicate glass patch pipettes (Garner, Claremont, CA) had resistance of 7–10 MΩ when ﬁlled with pipette solution. Single-channel currents were ampliﬁed with an EPC-7 ampliﬁer, ﬁltered at 0.5 kHz (low-pass Bessel 8-pole ﬁlter), digitized (Digidata 1322A), and analyzed with pCLAMP9 (Axon Instruments, Foster City, CA) and Origin7 (OriginLab, Northampton, MA). The channel open probability (Po) was determined as the fraction of time spent in the open state for recordings of 60-s duration. Recording solutions contained symmetrical concentrations of K+. The pipette solution consisted of (in mM) 145 KCl, 0.5 CaCl2, 0.5 MgCl2, and 10 HEPES at pH 7.2, and the bath/internal solution consisted of 145 KCl, 0.5 MgCl2, 2 EGTA, and 10 HEPES with 50 μM or 0 μM KATP at pH 7.2. The sarKATP channel inhibitor HMR-1098 (50 μM, Aventis, Frankfurt, Germany) was applied in the internal solution.

Oxidative stress. HL-1 and neonatal cardiomyocytes were exposed to oxidative stress by replacement of regular DMEM from culture dishes with serum- and glucose-free DMEM containing H2O2 (200 μM) and FeSO4 (50 μM). The combination of H2O2 and FeSO4 produces hydroxyl radicals via the Fenton reaction (20). After 20 min the H2O2-containing medium was replaced with DMEM containing 50 μM MnTRO (Invitrogen, Carlsbad, CA), and Origin7 (OriginLab, Northampton, MA). The channel open probability (Po) was determined as the fraction of time spent in the open state for recordings of 60-s duration. Recording solutions contained symmetrical concentrations of K+. The pipette solution consisted of (in mM) 145 KCl, 0.5 CaCl2, 0.5 MgCl2, and 10 HEPES at pH 7.2, and the bath/internal solution consisted of 145 KCl, 0.5 MgCl2, 2 EGTA, and 10 HEPES with 50 μM or 0 μM KATP at pH 7.2. The sarKATP channel inhibitor HMR-1098 (50 μM, Aventis, Frankfurt, Germany) was applied in the internal solution.

Assessment of apoptosis. HL-1 and neonatal cardiomyocytes (3 × 105 cells/well at time of experiment) were grown on laminin-coated four-well LabTech II chamber slides (Nalgene Nunc, Naperville, IL) and exposed to oxidative stress as described above. Fifteen hours after oxidative stress, the cells were ﬁxed in 1% paraformaldehyde, and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) was performed according to manufacturer’s instructions with a commercial kit (ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit, Chemicon International, Temecula, CA). Apoptotic nuclei stained with fluorescein isothiocyanate (TUNEL assay) were visualized by confocal microscopy. TO-PRO-3 was used to stain all nuclei. TUNEL-positive cells were counted, and the number of apoptotic cells was expressed as a percentage of the total number of cells (indicated by TO-PRO staining). For assessment of caspase-3 activation, both cell types were stained 3 h after stress with a CaspTag caspase-3,7 in situ assay kit (Chemicon International, Temecula, CA) and sulforhodamine-labeled caspase-3-positive cells were imaged by confocal microscopy. In HL-1 cells, the activation of caspase-3 was expressed as the number of cells with active caspase-3 relative to the total number of cells, as indicated by TO-PRO-3 staining. In neonatal cardiomyocytes, the intensity of red sulforhodamine ﬂuorescence, which is proportional to the activation of caspase-3,7, was expressed relative to the ﬂuorescence intensity in the Control group (not exposed to oxidative stress), which was set at 100%. From each well, images from 10 random microscopic ﬁelds were taken at ×20 magniﬁcation.

Measurements of cytochrome c release. Three hours after oxidative stress HL-1 and neonatal cardiomyocytes were lifted from incubation plates (7 × 106 cells/100-mm dish). To separate cytosolic and mitochondrial fractions, protein was extracted and differentially centrifuged by previously described methods (41). To test for cross-contamination between the cytosolic and mitochondrial fractions, activities of lactate dehydrogenase and citrate synthase in both fractions were determined (41). Lactate dehydrogenase activities for HL-1 cells (with neonatal cardiomyocytes in parenthesis) were 0.245 ± 0.034 (0.275 ± 0.064) and 0.005 ± 0.002 (0.012 ± 0.002) U/mg of protein in the cytosolic and mitochondrial fractions, respectively. The activities of citrate synthase were 0.818 ± 0.077 (1.386 ± 0.089) and 0.008 ± 0.003 (0.012 ± 0.004) U/mg of protein in the mitochondrial and cytosolic fractions, respectively. Therefore, cross-contamination between the cytosolic and mitochondrial fractions was considered negligible. Cytosolic and mitochondrial protein (25 μg) were loaded on a polyacrylamide gel (Bio-Rad Readygel, Tris-HCl 4–20%) and separated by electrophoresis (100 mV for 1 h). After transfer, the blots were blocked in Tris-buffered saline containing 5% nonfat dry milk and incubated with mouse cytochrome c immunoglobulin G1 antibody (BD Biosciences, San Jose, CA) at a dilution of 1:500. After being washed in Tris-buffered saline containing 0.05% Tween 20, the blot was incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated secondary anti-mouse antibody (Santa Cruz Biotechnol, Santa Cruz, CA). Bands were visualized by chemiluminescence (Pierce Biotechnology, Rockford, IL) on radiographic ﬁlm and analyzed by densitometry with the UN-SCAN-IT program (Silk Scientiﬁc, Orem, UT).

Monitoring of mitochondrial Ca2+ during oxidative stress. HL-1 and neonatal cardiomyocytes grown on laminin-coated coverslips were incubated for 1 h at 37°C in culture medium containing 1 μM rhod-2 AM (Invitrogen, Carlsbad, CA), a Ca2+-sensitive ﬂuorescent indicator that is taken up preferentially by mitochondria. After loading, the cells were washed three times and left for an additional 30 min in culture medium to allow complete deesteriﬁcation of the dye. Mitochondrial localization of rhod-2 was conﬁrmed in each experiment by MitoTracker Green (Invitrogen). Coverslips were then transferred to a recording chamber on the stage of the confocal microscope and perfused with modiﬁed Tyrode solution containing (in mM) 132 NaCl, 10 HEPES, 5 glucose, 5 KCl, 1 CaCl2, and 1.2 MgCl2 (pH 7.4). Oxidative stress was induced by switching to a glucose-free Tyrode
solution with added H$_2$O$_2$ (200 μM) and FeSO$_4$ (50 μM). In the experiments with sarcK$_{ATP}$ channel inhibition, HMR-1098 (50 μM) was included in the perfusion solution. Rhod-2 fluorescence was detected at 590 nm every 60 s. Time lapse of mitochondrial Ca$^{2+}$ was included in the perfusion solution. Rhod-2 fluorescence was evaluated by calculating relative rhod-2 fluorescence ($100 \times \text{F/F}_0$ where F is measured rhod-2 fluorescence and F$_0$ is that at beginning of experiment).

Assessment of Bcl-2 proapoptotic and antiapoptotic factors. After oxidative stress HL-1 cells were lifted from incubation plates, lysed, and homogenized. After electrophoresis (25 μg protein/lane), transfer to the nitrocellulose membrane, and membrane blocking, the protein from cell homogenates was probed with rabbit polyclonal anti-Bcl-2 (Chemicon International, Temecula, CA) and rabbit polyclonal anti-Bad and mouse monoclonal anti-phospho (p)-Bad (both from Cell Signaling Technology, Beverly, MA) primary antibodies and the blotting procedure was finished as described above.

Statistical analysis. Results are expressed as means ± SE. Data were analyzed with Origin7 software. Statistical analysis was performed by one-way analysis of variance with Bonferroni’s post hoc test; n indicates the number of independent experiments in each group. Differences were considered significant when the P value was <0.05.

RESULTS

HMR-1098 inhibits sarcolemmal K$_{ATP}$ channels in HL-1 and neonatal cardiomyocytes. To confirm the presence of functional sarcK$_{ATP}$ channels in HL-1 and neonatal cardiomyocytes and their sensitivity to HMR-1098, the single-channel activity was monitored from inside-out membrane patches in the presence and absence of a specific sarcK$_{ATP}$ channel inhibitor, HMR-1098. As shown in Fig. 1, the currents recorded at −80 mV in the presence of 50 μM intracellular ATP were inhibited by HMR-1098 in both HL-1 and neonatal cardiomyocytes. The channel opening was also blocked by 1–2 mM intracellular ATP with and without sarcK$_{ATP}$ channel inhibitor HMR-1098. As shown in Fig. 1, the currents recorded at −80 mV in the presence of 50 μM intracellular ATP were inhibited by HMR-1098 in both HL-1 and neonatal cardiomyocytes. The channel opening was also blocked by 1–2 mM intracellular ATP (not shown), and recorded unitary current amplitude and unitary conductance were typical for the cardiac sarcK$_{ATP}$ channel.

Inhibition of sarcolemmal K$_{ATP}$ channel aggravates apoptotic cardiomyocyte death. To test whether inhibition of the sarcK$_{ATP}$ channel affects apoptotic cell death, HL-1 and neonatal cardiomyocytes were exposed to oxidative stress in the presence and absence of HMR-1098 (Fig. 2A). In HL-1 cells, exposure to stress increased the percentage of TUNEL-positive cells compared with control (19.9 ± 1.7% in Stress group vs. 7.6 ± 1.0% in Control group, Fig. 2B). The percentage of apoptotic HL-1 cells was further increased in the presence of HMR-1098 (31.5 ± 2.8% in Stress+HMR group, P < 0.05 compared with Stress group; n = 5), while HMR-1098 pretreatment had no effect on stress-induced damage (20.9 ± 3.3% in Stress+pHMR group). In the absence of stress, HMR-1098 did not affect apoptotic cell death (9.3 ± 1.0% in HMR group). Application of the mitochondrial Ca$^{2+}$ uniporter inhibitor ruthenium red resulted in attenuation of stress-induced apoptosis in the presence of HMR-1098 (15.2 ± 3.9% in Stress+RR group and 18.7 ± 4.1 in Stress+HMR+RR group, respectively, P < 0.05 vs. Stress+HMR; n = 4). Similar results were found in neonatal cardiomyocytes, with 22.0 ± 2.9% of TUNEL-positive cells in the Control group, 58.7 ± 9% in the Stress group, 83.5 ± 8.4% in the Stress+HMR group (P < 0.05 vs. Stress, n = 5), and 15.5 ± 1.9% in the HMR group. These findings were confirmed by the in situ caspase-3,7 assay (Fig. 3A). As shown in Fig. 3B, activation of executioner caspases-3 and -7 was detected in a greater percentage of HL-1 cells when HMR-1098 was included during exposure to stress (6.3 ± 1.4% in Stress+HMR group vs. 3.0 ± 0.6% in Stress group, P < 0.05; n = 5). Again, HMR-1098 alone did not affect the baseline levels of caspase-3,7 activation (0.4 ± 0.02% and 0.4 ± 0.1% in Control and HMR groups, respectively). Also, in neonatal cardiomyocytes oxidative stress in the presence of HMR-1098 caused greater activation of executioner caspases-3 and -7 compared with the cells exposed to...
stress alone (306 ± 18% in Stress group vs. 403 ± 23% in Stress+HMR group, P < 0.05; n = 5).

Sarcolemmal K$_{ATP}$ channel inhibition potentiates involvement of mitochondrial death pathway. To investigate whether inhibition of the sarcoK$_{ATP}$ channel modulates the mitochondrial death pathway, cytochrome c release from mitochondria to the cytosol was measured in both HL-1 and neonatal cardiomyocytes (Fig. 4, A and B, respectively). Oxidative stress increased the cytochrome c release from mitochondria to cytosol relative to control. This increase was significantly augmented by administration of HMR-1098, suggesting that inhibition of the sarcoK$_{ATP}$ channel during stress compromises mitochondrial integrity.

Augmentation of mitochondrial Ca$^{2+}$ loading during stress by sarcolemmal K$_{ATP}$ channel blockade. To test possible mechanism by which the sarcoK$_{ATP}$ channel might affect mitochondrial homeostasis, changes in mitochondrial Ca$^{2+}$ content were monitored in both HL-1 and neonatal cardiomyocytes during exposure to stress in the presence and absence of HMR-1098. As shown in Fig. 5, exposure to oxidative stress of HL-1 cells induced a gradual increase of rhod-2 fluorescence that at 50 min reached 110.1 ± 5.7% of baseline. Rhod-2 fluorescence was increased significantly more when cardiomyocytes were treated with HMR-1098 during stress (139.9 ± 10.0% of baseline, P < 0.05; n = 6), indicating greater mitochondrial Ca$^{2+}$ loading. In neonatal cardiomyocytes oxidative stress-induced rise in rhod-2 fluorescence peaked at 118.1 ± 3.3% and 137.7 ± 2.9% in Stress and Stress+HMR groups, respectively (P < 0.05; n = 5).

Inhibition of sarcolemmal K$_{ATP}$ channel has no effect on Bcl-2 and Bad. To investigate whether inhibition of sarcoK$_{ATP}$ channel modulates some of the anti- and proapoptotic regulators, the levels of Bcl-2, Bad, and p-Bad were assessed. As demonstrated in Fig. 6A, exposure of HL-1 cells to stress with or without HMR-1098 had no effect on expression of Bcl-2. As seen in Fig. 6B, oxidative stress increased level of total Bad and p-Bad but did not affect the p-Bad-to-Bad ratio compared with control. The addition of HMR-1098 during stress did not induce further changes, indicating that inhibition of the sarcoK$_{ATP}$ channel does not affect the amount of proapoptotic dephosphorylated Bad.

DISCUSSION

In the present study we demonstrated that inhibition of the sarcoK$_{ATP}$ channel augments oxidative stress-induced apoptosis by affecting the mitochondrial death pathway. More specifically, a link between the sarcoK$_{ATP}$ channel and mitochondrial Ca$^{2+}$ homeostasis during stress was identified.

The protective role of sarcoK$_{ATP}$ channels was hypothesized from the time of their discovery because of their ability to sense intracellular metabolic conditions (32). This metabolic gating of the sarcoK$_{ATP}$ channel is a result of the channel’s molecular composition. The channel consists of an ATP-
inhibited Kir6.2 subunit that forms the pore and a nucleotide-binding SUR2A modulatory subunit (19). The channel opening under conditions of increased cardiac workload or ischemic challenge has a hyperpolarizing effect on the cell membrane, thus attenuating the cytosolic Ca\(^{2+}\) overload and cellular damage (3, 44).

Although previous studies have investigated the physiological role of the sarcK\(_{\text{ATP}}\) channel and its contribution to the phenomenon of preconditioning, the involvement of the sarcK\(_{\text{ATP}}\) channel in modulation of apoptotic cell death in the heart is unclear. In a study by Ichinose et al. (18) that investigated diazoxide-induced preconditioning in neonatal rat cardiomyocytes, HMR-1098 failed to abolish preconditioning-induced protection from oxidative stress-induced apoptosis and did not result in an increased rate of apoptosis. Conversely, in a similar experimental setting we found that inhibition of the sarcK\(_{\text{ATP}}\) channel aggravates apoptotic cell death after stress, as evidenced by an increase in the number of TUNEL-positive cells as well as the increase in activation of executioner caspases-3 and -7. The disparity in the obtained results may be explained by a difference in timing of application of HMR-1098, since Ichinose et al. used the inhibitor only during the application of diazoxide, but not during the exposure to oxidative stress. Our previous study (28) demonstrated that the timing of administration of HMR-1098 is crucial, such that HMR-1098 has no effect on cell death if it is not applied during exposure to oxidative stress but rather only before the stress. In the present study, sarcK\(_{\text{ATP}}\) channel inhibition did not affect apoptosis of cardiomyocytes in the absence of stress (HMR group) or when it was applied only before, but not during, stress (Stress group vs. Stress + HMR group). These results are in agreement with the widely accepted “dormant” nature of the sarcK\(_{\text{ATP}}\) channel under conditions of metabolic abundance. Indeed, various studies using Kir6.2 knockout mice found that the phenotypic differences between the knockout and wild-type mice become apparent only when the mice are exposed to a certain type of stress (exercise, hypertension, ischemia-reperfusion) (25).

Stressors that disturb cardiomyocyte homeostasis such as hypoxia, oxidative stress, and ischemia-reperfusion activate the mitochondrial death pathway. These noxious signals are transmitted to mitochondria either by directly affecting mitochondrial function or indirectly via proapoptotic members of the Bcl-2 family. Therefore, mitochondria play a central role in integrating cellular disturbances and initiating the mitochondrial death process (10).

Assessment of Bcl-2 level revealed that inhibition of the sarcK\(_{\text{ATP}}\) channel does not affect its expression. Furthermore, administration of HMR-1098 alone also had no effect on expression of proapoptotic Bad. Since dephosphorylation of...
Bad results in Bad activation and is stimulated by the rise in intracellular Ca\(^{2+}\), we also assessed whether inhibition of the sarcK\(_{\text{ATP}}\) channel affects the amount of p-Bad. Measurements of p-Bad and assessment of the p-Bad-to-Bad ratio revealed no significant difference between groups exposed to stress in the presence and absence of HMR-1098. This suggests that the increase in apoptosis by sarcK\(_{\text{ATP}}\) channel inhibition during stress is unlikely to be due to alterations in protein levels of the Bcl-2 family.

Mitochondria, besides being an essential ATP-producing organelle, are important regulators of cellular ionic homeostasis and play a major role in transmission and amplification of cellular death signals. During metabolic stress, mitochondria can act as buffers that attenuate the cytosolic Ca\(^{2+}\) overload. However, if excessive, the mitochondrial Ca\(^{2+}\) accumulation may lead to initiation of cell death (7). Mitochondrial Ca\(^{2+}\) overload is a strong trigger of mitochondrial permeability transition pore opening, release of cytochrome c to the cytosol, and activation of the apoptotic machinery (17, 21). Since sarcK\(_{\text{ATP}}\) channel profoundly affects the cytosolic Ca\(^{2+}\) loading during metabolic challenge (3, 44), we further hypothesized that the effects of sarcK\(_{\text{ATP}}\) channel inhibition on apoptotic cell death are closely related to mitochondrial Ca\(^{2+}\).

Our measurements of mitochondrial Ca\(^{2+}\) during exposure of cardiomyocytes to oxidative stress revealed that mitochondrial Ca\(^{2+}\) loading is significantly increased in the presence of sarcK\(_{\text{ATP}}\) channel inhibitor. Since it is widely accepted that the downstream effects of the sarcK\(_{\text{ATP}}\) channel opening are membrane hyperpolarization and resulting attenuation of intracellular entry of Ca\(^{2+}\) during stress (3, 13), we conclude that the observed effects of HMR-1098 can be explained by an aggravated stress-induced cytosolic Ca\(^{2+}\) overload in the presence of the inhibitor. The cytosolic Ca\(^{2+}\) is taken up by the mitochondria (37, 43), resulting in the rise of mitochondrial Ca\(^{2+}\) and

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**Fig. 4.** Inhibition of sarcK\(_{\text{ATP}}\) channel increases cytochrome c release from mitochondria to cytosol following stress. A, top: representative Western blot shows cytosolic and mitochondrial fractions from HL-1 cells from all experimental groups probed with anti-cytochrome c antibody. Bottom: summary graph showing that exposure to oxidative stress increased the release of cytochrome c from the mitochondria to the cytosol, which was further augmented by HMR-1098. HMR-1098 had no effect on cytochrome c release in absence of stress (n = 5; *P < 0.05). B: representative Western blot (top) and summary graph (bottom) obtained from neonatal cardiomyocytes (n = 5; *P < 0.05).
subsequent mitochondrial damage. In fact, the finding that ruthenium red, an inhibitor of mitochondrial Ca\(^{2+}\)/H\(_{11001}\) uniporter, attenuates the HMR-1098-induced potentiation of apoptosis supports this conclusion.

Mitochondrial Ca\(^{2+}\) overload is a known trigger for cytochrome c release by affecting cytochrome c-cardiolipin interaction in the mitochondrial inner membrane (35). Our measurements revealed that the apoptosis-triggering cytochrome c release was potentiated in the presence of sarcKATP channel inhibitor (n = 5; *P < 0.05, Stress vs. Stress + HMR).

Fig. 5. SarcKATP channel inhibition increases mitochondrial Ca\(^{2+}\) (mitoCa\(^{2+}\)) loading during stress. A: representative confocal microscopy images of rhod-2 fluorescence at baseline and after exposure to oxidative stress in HL-1 cells. Top: cells exposed to stress in absence of channel inhibitor. Bottom: in the presence of HMR-1098. B: time courses of relative rhod-2 fluorescence (expressed relative to baseline) during exposure to stress in HL-1 (left) and neonatal (right) cardiomyocytes. Oxidative stress caused a gradual increase in rhod-2 fluorescence that was potentiated in the presence of sarcKATP channel inhibitor (n = 5).

Fig. 6. Inhibition of sarcKATP channel has no effect on Bcl-2 and Bad. A: representative Western blot images showing whole HL-1 cell lysates from all experimental groups probed with anti-Bcl-2 antibody and reprobed with anti-troponin I (loading control). Data are summarized at bottom, indicating no difference between experimental groups (n = 5). B: representative Western blot images showing whole cell lysates probed with anti-Bad and anti-phospho (p)-Bad antibodies and summary graph of p-Bad-to-Bad ratios (n = 5, bottom). Although oxidative stress in the presence and absence of HMR-1098 increased expression of total Bad and p-Bad in the whole cell lysate compared with control, it did not affect the p-Bad-to-Bad ratios, and no further changes were observed in the presence of HMR-1098.
release to the cytosol after exposure to stress is greater in the presence of sarcKATP channel inhibitor, further corroborating the finding of functional interaction between sarcKATP channel and mitochondria. This study therefore offers new evidence for a connection between the activity of the sarcKATP channel and the downstream function of mitochondria. The reverse, where the sarcKATP channel activity is modulated by the mitochondria, was demonstrated in earlier studies. Since mitochondria produce most of the intracellular ATP and metabolize ADP, both major regulators of the sarcKATP channel activity, it is apparent that mitochondrial function will inevitably affect the sarcKATP channel (2). This metabolic communication between sarcKATP channel and different intracellular compartments including mitochondria was shown to be facilitated by the existence of an intracellular phosphotransfer network that overcomes diffusion barriers within the cell (11). Therefore, a constant two-way interaction between the sarcKATP channel and mitochondria helps to maintain cellular homeostasis during stress.

Interestingly, the ability of the sarcKATP channel to affect mitochondrial function was investigated in a recent study by Pasdois et al. (34). A transient inhibition of sarcKATP before cardiac ischemia-reperfusion was found to induce preconditioning-like effects and protect the heart by affecting the mitochondria. These protective effects of HMR-1098 were suggested to be the result of a transient increase in intracellular Ca2+ during application of sarcKATP channel inhibitor. Since a transient moderate increase in cytosolic Ca2+ before ischemia-reperfusion is known to induce cardiac preconditioning (31), these findings are not counterintuitive. Therefore, it is possible that the sarcKATP channel closing before cardiac stress induces a moderate Ca2+ loading and Ca2+-induced preconditioning, while closing of the channel during stress results in excessive Ca2+ loading leading to cell death. In both cases, mitochondria are affected through increases in cytosolic Ca2+. However, in our experimental setting, pretreatment with HMR-1098 followed by its washout before exposure to oxidative stress had no effect on cell damage. Differences in obtained results from our study and that of Pasdois et al. might possibly be explained by differences in the measured end point (apoptosis vs. infarct size), the experimental model (Langendorff-perfused heart vs. cell culture), or the type of stress. Furthermore, in a similar experimental setting (isolated heart exposed to global ischemia-reperfusion), HMR-1098 pretreatment also had no effect on infarct size and contractile performance after ischemia-reperfusion (40). The discrepancy in results from these studies using a very similar experimental approach is hard to explain and requires further investigation. However, it is obvious that, in order to observe the effect of sarcKATP channel inhibitor, the sarcKATP channel needs to be open. Since under normal stress-free conditions within the heart the sarcKATP channels are expected to be closed, the question is why would the sarcKATP channels open in preischemia conditions as suggested by data from the study of Pasdois et al.? In our study we used the sarcKATP channel inhibitor HMR-1098, which was previously demonstrated to inhibit the sarcKATP channel currents in HL-1 cells (14), an effect confirmed in the present study in both HL-1 and neonatal cardiomyocytes. One advantage of using HMR-1098 compared with other KATP channel inhibitors is its selectivity for the sarcKATP channels without affecting the KATP channels in the mitochondria (36). Earlier studies using putative mitochondrial KATP (mitoKATP) channel openers and inhibitors (diazoxide and 5-HD) already characterized the mitoKATP channel as being protective against apoptotic cell death in cardiac myocytes (1). The protective effects of mitoKATP channel opening, with resulting mitochondrial K+ influx, include prevention against excessive mitochondrial Ca2+ loading through mitochondrial membrane potential depolarization and consequently reduced driving force for the mitochondrial Ca2+ entry (27, 29). Therefore, the most likely mechanism of protection by activation of both sarcKATP and mitoKATP channels is related to attenuation of mitochondrial Ca2+ overload and its fatal downstream outcome. Although HMR-1098 has been used in a great number of studies as a specific sarcKATP channel blocker, potential other, yet unrecognized, targets might contribute to the observed effects of the present and previous studies. However, that is an inherent drawback of all pharmacological agents. On the other hand, the observed effects of the sarcKATP blocker in the present study can have implications for the clinical setting in which sulfonylureas are commonly clinically used for glycaemia regulation in patients suffering from Type 2 diabetes. Since a number of Type 2 diabetic patients taking sulfonylureas also suffer from ischemic heart disease, the safety of these drugs in this patient population has been questioned, but without a definite answer (6, 12, 15). The present study, although performed with an in vitro model, provides insight into other intracellular effects of sulfonylureas in the heart.

From the results of this study we conclude that opening of the sarcKATP channels plays an important role in prevention of cardiomyocyte apoptosis during metabolic stress. This effect is due to the specific interaction between the sarcKATP channel and mitochondria, namely through sarcKATP channel-induced modulation of mitochondrial Ca2+ homeostasis and cytochrome c release.

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