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Preconditioning by isoflurane elicits mitochondrial protective mechanisms independent of sarcolemmal K_{ATP} channel in mouse cardiomyocytes

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Abstract

Cardiac mitochondria and the sarcolemmal (sarc)K_{ATP} channels contribute to cardioprotective signaling of anesthetic-induced preconditioning (APC). Changes in mitochondrial bioenergetics influence the sarcK_{ATP} channel function, but whether this channel has impacts on mitochondria is uncertain. We used the mouse model with deleted pore-forming Kir6.2 subunit of sarcK_{ATP} channel (Kir6.2 KO) to investigate whether the functional sarcK_{ATP} channels are necessary for isoflurane activation of mitochondrial protective mechanisms. Ventricular cardiomyocytes were isolated from C57Bl6 wild type (WT) and Kir6.2 KO mouse hearts. Flavoprotein autofluorescence, mitochondrial ROS production and mitochondrial membrane potential were monitored by laser-scanning confocal microscopy in intact cardiomyocytes. Cell survival was assessed using H_2O_2-induced stress. Isoflurane (0.5 mM) increased flavoprotein fluorescence to 180±14% and 190±15% and ROS production to 118±2% and 124±6% of baseline in WT and Kir6.2 KO myocytes, respectively. TMRE fluorescence decreased to 84±6% in WT and to 86±4% in Kir6.2 KO myocytes. This effect was abolished by 5HD. Pretreatment with isoflurane decreased the stress-induced cell death from 31±1% to 21±1% in WT and from 44±2% to 35±2% in Kir6.2 KO myocytes. In conclusion, Kir6.2 deletion increases sensitivity of intact cardiomyocytes t o oxidative stress, but does not alter the isoflurane-elicited protective mitochondrial mechanisms, suggesting independent roles for cardiac mitochondria and sarcK_{ATP} channels in APC by isoflurane.

Keywords

Kir6.2 KO mouse; ventricular cardiomyocytes; cardiac mitochondria; cardiac K_{ATP} channels; isoflurane

Introduction

Anesthetic-induced preconditioning (APC) is an infarct size limiting strategy. A complex network of signaling pathways that are activated in APC[1] ultimately converge on two key

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effectors of protection, the mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channels and the sarcolemmal ATP-sensitive K^+ (sarcK_{ATP}) channels.[2, 3] However, it is unclear whether these two effectors of APC operate in a mutually dependent manner or constitute independent cytoprotective mechanisms. The latter would suggest separate, possibly parallel mechanisms of protection that facilitate the survival of cardiomyocytes even when some effectors of protection are dysfunctional.

The mitoK_{ATP} channels play an important role both as triggers and end-effectors of the volatile anesthetic-induced APC.[2] Opening of this channel limits mitochondrial Ca^{2+} loading via a mild depolarization of the mitochondrial membrane potential that decreases driving force for Ca^{2+} influx. The channel participates also in the cellular signal transduction by inducing a small burst of the mitochondrial reactive oxygen species (ROS) that function as signaling molecules in APC. Previous studies have suggested that communication between mitochondria and the sarcK_{ATP} channels is important for cardiomyocyte survival, and demonstrated that uncoupled mitochondria may activate the sarcK_{ATP} channels in the myocardial cells.[4]

It is generally accepted that opening of sarcK_{ATP} channels is cardioprotective by inducing hyperpolarization of the cell membrane and therefore limiting cytosolic Ca^{2+} influx and mitochondrial Ca^{2+} overload, two important noxious stimuli.[5,6] But, it is less clear whether opening of the sarcK_{ATP} channel could be protective by other, yet unknown mechanisms, such as facilitating the mitoK_{ATP} channel opening.

Studies aimed to delineate the role of mitoK_{ATP} and sarcK_{ATP} channels in protection by APC are hampered by limited selectivity of their pharmacological modulators.[7-11] One important reason is that molecular structure of the mitoK_{ATP} channel is still undetermined, even though the molecular structure of the cardiac sarcK_{ATP} channel has been well characterized. The cardiac sarcK_{ATP} channel is formed by association of two subunits, the pore-forming inwardly rectifying Kir6.2 channel, encoded by KCNJ11 gene, and the regulatory sulphonylurea receptor SUR2A, an ATP-binding cassette (ABC) transporter that is encoded by ABCC9 gene.[12, 13] The mouse model with knockout of KCNJ11 coding for the inward rectifier Kir6.2 channel (Kir6.2 KO)[14] is a genetic model that enables us to more directly assess the role of sarcK_{ATP} channels in the anesthetic-induced and mitochondria-mediated APC.

The purpose of the present study was to investigate whether the functional sarcK_{ATP} channel is required for isoflurane-elicited activation of specific mitochondrial protective mechanisms that ultimately lead to protection of intact ventricular cardiomyocytes.

Methods

The study was conducted in accordance with guidelines set forth by the Medical College of Wisconsin, the Animal Welfare Act Regulation and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). All experimental protocols of this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical College of Wisconsin, Milwaukee, WI.

Animals

Experiments were performed using 10 to 14 week-old male mice, weighing 25-35g. Wild-type (WT) C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, MI). The Kir6.2 KO mice were a kind gift from the laboratory of Dr. Susumu Seino (Kobe University, Kobe, Japan) via Dr. John A Auchampach (Medical College of Wisconsin, Milwaukee, WI) and Dr. Richard J Gumina (Ohio State University, Columbus, OH)

laboratories. Mice were housed in groups of 4 per cage in the temperature-controlled room with a 12:12 hour dark-light cycle, in Biomedical Resource Center of the Medical College of Wisconsin. Animals were fed standard chow with free access to tap water.

Isolation of mouse ventricular cardiomyocytes

Mouse ventricular cardiomyocytes were isolated as reported previously.[15] Each mouse was injected (i.p.) with 100 IU heparin and anesthetized with 3 mg Inactin (sodium thiobutabarbital, Sigma-Aldrich, St Louis, MO). Following thoracotomy, the heart was rapidly excised and arrested in the ice-cold Ca^{2+-}free Tyrode solution containing (in mM): NaCl 135, KCl 4.7, $MgCl_2$ 1.2, HEPES 10, glucose 5 and taurine 5, at pH 7.4. The aorta was cannulated under dissecting microscope. The heart was mounted on temperature-controlled (37°C) Langendorff apparatus and perfused at a constant flow of 3.0 ml/min with Ca^{2+} free Tyrode solution for 4 min, and then with 0.128 mg/ml of Liberase-TM blendzyme (Roche, Indianapolis, IN) in the Ca^{2+-}free Tyrode solution for 8-13 min. Following digestion, the left ventricle was excised and minced in 5 ml of Tyrode solution containing 20% fetal calf serum. Tissue suspension was gently agitated to release single cardiomyocytes. The cells were sedimented for 20 min at room temperature, resuspended in Tyrode solution containing 5% fetal calf serum and allowed to settle for another 20 min during which the extracellular Ca^{2+} was stepwise increased to 1 mM. Myocytes were stored in the Tyrode solution at room temperature. The rod-shaped cells with distinct cross-striations and intact surface membrane were used for experiments within 5 h after isolation.

Electrophysiolog

The presence of functional cardiac sarcKATP channels in WT myocytes, and their absence in Kir6.2 KO myocytes was confirmed electrophysiologically. Activity of single $sarcK_{ATP}$ channels was monitored in the inside-out patch clamp configuration[16] at the membrane potential of +40 mV. The extracellular/pipette solution contained (in mM) 145 KCl, 0.5 $CaCl_2$, 0.5 $MgCl_2$, and 10 HEPES at pH 7.4. The intracellular/bath solution contained (in mM) 145 KCl, 0.5 $MgCl_2$, 2 EGTA, 10 HEPES and 0.005 K_2ATP at pH 7.2. The channel opener pinacidil (100 μM, Sigma-Aldrich, St Louis, MO), and the channel blockers glibenclamide (1 μM, Sigma-Aldrich, St Louis, MO) and HMR-1098 (30 μM, Sanofi-Aventis Pharma, Frankfurt, Germany) were applied in the bath solution. The heat-polished borosilicate glass patch pipettes (Garner, Claremont, CA) had resistance of 4-7 MΩ when filled with the pipette solution. Recordings were performed at room temperature using EPC-7 amplifier (List, Darmstadt-Eberstadt, Germany) with Digidata 1322A interface (Axon Instruments/Molecular Devices, Union City, CA) and pClamp10 software. Current signal was low-pass filtered at 500 Hz through an eight-pole Bessel filter and sampled at 1 kHz. Single channel data were analyzed with pCLAMP10 (Axon Instruments/Molecular Devices, Union City, CA) and Origin7 (OriginLab, Northampton, MA) software. The all-points amplitude histograms were constructed from 60 second recordings. The channel open probability (Po) was determined from the ratio of the area under the peaks in amplitude histograms fitted by a multi-Gaussian distribution, using the equation Po = [1 –(Pc)1/N] where Pc is the channel closed probability and (N) is the number of channels in the patch.

Laser scanning confocal microscopy

Experiments were conducted on isolated intact cardiomyocytes in the recording chamber mounted on the stage of a laser-scanning confocal microscope (Eclipse TE2000-U, Nikon, Tokyo, Japan) with the x60/1.4 oil-immersion objective (Nikon, Tokyo, Japan). Data were stored on hard disk of PC computer and analyzed off-line using MetaMorph6.2 software (Universal Imaging, West Chester, PA) and NIH ImageJ software. Results are presented as percent change in fluorescence intensity relative to baseline (F_0) recorded before the cell exposure to isoflurane ($F/F_0 \times 100$).

Analysis of mitochondrial redox state

Isolated cardiomyocytes were placed in the recording chamber and superfused with Tyrode solution. Autofluorescence of endogenous flavoproteins (FP) was excited at 488 nm by an argon laser and the emitted light was collected at 500-530 nm by a photomultiplier and was digitized. To assess the mitochondrial matrix redox state of intact myocytes, FP fluorescence was recorded for 25 min at room temperature as reported previously.[17, 18] The FP oxidation, resulting in enhanced autofluorescence signal, is compensatory to mitochondrial uncoupling.[3] For statistical analysis, the averaged baseline fluorescence of time points recorded before anesthetic exposure was compared to the averaged fluorescence of time points recorded during exposure to the anesthetic.

Analysis of ROS production

The mitochondrial ROS production was measured using the ROS-sensitive indicator 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester, CM-H2DCFDA (Molecular Probes, Eugene, OR) as described previously.[17, 19] CM-H2DCFDA is a membrane-permeable indicator that diffuses into the cell where endogenous esterases cleave acetate groups yielding the membrane impermeable, nonfluorescent CM-H2DCF. In turn, oxidation of deestrified CM-H2DCF by ROS yields a fluorescent CM-DCF. Isolated cardiomyocytes were loaded with 2 μM CM-H2DCFDA for 20 min, followed by a 10-min washout. The cells were superfused with Tyrode solution at room temperature and fluorescence of individual myocytes was recorded for 28 min. CM-DCF fluorescence was excited at 488 nm by an argon laser and emission was collected at 500-550 nm. The neutral density filters (ND32) minimized the dye bleaching. The baseline fluorescence was compared with the averaged fluorescence of time points recorded during the application of isoflurane.

Measurement of mitochondrial membrane potential (ΔΨm)

Isolated myocytes were incubated for 10 min with the mitochondrial membrane potential-sensitive fluorescent dye tetramethylrhodamine ethyl ester (TMRE; 100 nM; Invitrogen, Carlsbad, CA) in the absence and the presence of isoflurane. The TMRE fluorescence was monitored at the excitation and emission wavelengths of λex/λem=543/570-610 nm using a green HeNe laser.[19] The experimental protocols are shown in Fig. 1A.

Assessment of cardiomyocyte response to stress

Myocyte survival was assessed in vitro using taurine-free Tyrode solution, since taurine may have an effect on the key mediators of cardioprotection.[20] Approximately 1 ml of cell suspension was placed in a chamber on the stage of an inverted Olympus MT2 microscope (Olympus, Tokyo, Japan), and the cells were allowed to settle for 10 min. Oxidative stress was induced by exposing myocytes to 2.0 mM H_2O_2 (Calbiochem, LA Jolla, CA) for 30 min at room temperature. In experimental groups that underwent APC, the myocytes were exposed to 0.5 mM isoflurane for 10 min, followed by a 10 min anesthetic washout prior to application of H_2O_2. In each experiment approximately 100 myocytes were counted by one blinded and one not blinded investigator. The living cell count was based on cell morphological criteria (intact surface membrane, distinct cross-striation, rod shape) and exclusion of Trypan blue.[2] Percent cell death was calculated from the living cell count before and after oxidative stress. A labeled grid on the glass floor of the chamber ensured counting the same cardiomyocytes before and after the stress. The experimental protocols are shown in Fig. 1B.

Drugs

The volatile anesthetic isoflurane (Abbott Laboratories, North Chicago, IL) was dispersed in experimental solutions by sonication and delivered to the recording chamber from an airtight glass syringes. At the end of each experiment, samples of the anesthetic-containing solution were taken from the outflow of the recording chamber, and anesthetic concentrations were analyzed by a gas chromatography method using Shimadzu gas chromatograph (Shimadzu, Kyoto, Japan). The mean isoflurane concentration was 0.5±0.04 mM, equivalent to 1 MAC (minimum alveolar concentration) in mice. To investigate the effects of mitoK_{ATP} channels on mitochondrial membrane potential, the mitoK_{ATP} channel blocker 5-hydroxydecanoate (5HD, 500 μM; Sigma-Aldrich, St. Louis, MO) was used in the confocal microscopy and cell viability experiments. The sarcK_{ATP} channel blockers HMR-1098 (30 μM, Sanofi-Aventis Pharma, Frankfurt, Germany) and glibenclamide (1 μM, Sigma-Aldrich, St Louis, MO) were used in electrophysiological experiments.

Statistical analysis

Data are presented as means±SEM, (n) represents the number of cell isolations from individual hearts, i.e. the number of mice. Confocal microscopy data were analyzed with Origin7 software (OriginLab, Northampton, MA). Statistical significance was determined using analysis of variance for multiple comparisons and Student's t test where appropriate. Patch clamp data were analyzed with pCLAMP10 (Molecular Devices, Union City, CA) and Origin7 (OriginLab, Northampton, MA) software. Statistical significance of results was determined using analysis of variance with Scheffe post hoc test. Differences with at the two-tailed $P<0.05$ were considered significant.

Results

Activity of single sarcK_{ATP} channels in mouse ventricular cardiomyocytes

To verify the presence of functional sarcK_{ATP} channels in WT mouse cardiomyocytes and their absence in Kir6.2 KO myocytes, single K_{ATP} channel activity was monitored from inside-out membrane patches at +40 mV membrane potential in the presence of 5 μM ATP or in the absence of ATP. Figure 2A shows that in patches from WT myocytes the channel activity was high, with Po of 0.214±0.035 (n=8). Po was inhibited by 97% (Po 0.004±0.002, n=8) upon application of 30 μM HMR-1098 (Fig. 2B) or 1 μM glibenclamide, confirming the identity of the channel. By contrast, all patches excised from Kir6.2 KO myocytes were silent (Fig. 2C and 2D). No channel opening was evoked in the absence or presence of low ATP, or on application of the channel opener pinacidil (100 μM). When applied directly to the inside-out membrane patches from WT myocytes isoflurane (0.5 mM) increased channel Po by 33% (Fig. 3A and 3B), from mean control of 0.111±0.022 to 0.157±0.0.031 (n=8), and increased the number of open channels (Fig. 3C, histograms).

Effect of isoflurane on FP fluorescence in WT and Kir6.2 KO cardiomyocytes

In the present study, FP autofluorescence was used to assess the mitochondrial uncoupling. Figure 4 shows that isoflurane significantly enhanced FP fluorescence to 180±14% and 190±15% of baseline (100%) in cardiomyocytes from WT (n=6) and Kir6.2 KO (n=5) hearts, respectively. Similar responses in both groups suggested the absence of functional sarcK_{ATP} channels does not alter isoflurane-induced enhancement of FP oxidation and mitochondrial uncoupling.

Effect of isoflurane on mitochondrial ROS production in WT and Kir6.2 KO cardiomyocytes

Fig. 5 shows the time course of changes in mitochondrial ROS generation quantified by CM-DCF fluorescence intensity before and during acute application of isoflurane. During 18

min exposure to isoflurane, the CM-DCF fluorescence significantly increased to 118±2% and 124±6% of baseline (100%) in cardiomyocytes from WT (n=6) and Kir6.2 KO (n=5) hearts, respectively. Acute isoflurane increased the mitochondrial ROS production in both WT and KO myocytes, suggesting mitochondrial ROS generation by isoflurane is preserved in the absence of functional sarcK_{ATP} channels.

Mitochondrial membrane depolarization by isoflurane in WT and Kir6.2 KO cardiomyocytes

Volatile anesthetics cause a partial depolarization of the mitochondrial membrane potential which contributes to protection afforded by APC and may in part be mediated by mitoK_{ATP} channel opening.[3, 17] We tested the effects of isoflurane on mitochondrial membrane potential in cardiomyocytes from WT (n=6) and Kir6.2 KO (n=5) hearts using TMRE. Fig. 6A shows that acute isoflurane decreased TMRE fluorescence to 84±6% of control (100%) in WT cardiomyocytes, suggesting partial mitochondrial depolarization. This effect was abolished by the mitoK_{ATP} channel blocker 5HD. A similar effect was observed also in Kir6.2 KO myocytes, where isoflurane decreased TMRE fluorescence to 86±4% of control, and 5HD abolished this effect (Fig. 6B), suggesting the function of mitoK_{ATP} channel is preserved in Kir6.2 KO myocytes in the absence of functional sarcK_{ATP} channel.

APC with isoflurane alters cardiomyocyte response to stress

Oxidative stress, due in part to enhanced generation of H_2O_2 during reperfusion period causes cardiomyocyte death.[21] We investigated whether APC with isoflurane may protect WT and Kir6.2KO cardiomyocytes from cell death by H_2O_2. Fig. 7 shows that in the time control group, where myocytes were superfused only with taurine-free Tyrode solution, the cell death among myocytes from WT (n=9) and Kir6.2 KO (n=6) hearts was relatively low (4±1% and 4±2%, respectively). H_2O_2 increased cell death to 31±1% in WT myocytes (Fig. 7A) and myocyte pretreatment with isoflurane (APC) attenuated this effect to 21±1%. Bracketing APC with 5HD abolished isoflurane protection and cell death increased to 35±3%. By contrast, H_2O_2 increased Kir6.2 KO myocyte death to 44±2% and APC with isoflurane attenuated this effect to 35±2%. Interestingly, blocking the mitoK_{ATP} channels with 5HD had no significant effect on survival of Kir6.2 KO myocytes (Fig. 7B, APC vs. APC+5HD). The cell death among Kir6.2 KO myocytes exposed to H_2O_2 in the absence or presence of APC was significantly higher than among WT myocytes (Fig. 7 A and B). Thus APC with isoflurane can elicit protection of both WT and Kir6.2 KO myocytes, in the presence and absence of functional sarcK_{ATP} channel. However, susceptibility to stress-induced cell injury is significantly greater in Kir6.2 KO, which confirms the importance of sarcK_{ATP} channels for myocyte protection from stress.

Discussion

The present study demonstrates that in isolated intact mouse cardiomyocytes a volatile anesthetic isoflurane elicits competent mitochondrial protective events characteristic for APC signaling.[17] These include: (i) oxidation of mitochondrial flavoproteins, an indicator of mitochondrial uncoupling; (ii) generation of small bursts of ROS that function as signaling molecules in APC; and (iii) partial depolarization of mitochondrial membrane potential via opening of mitoK_{ATP} channel. Although isoflurane elicited these protective events in both WT and Kir6.2-KO cardiomyocytes, the latter appear more sensitive to oxidative stress than WT myocytes with the functional sarcK_{ATP} channels.

The effect of isoflurane on mitochondrial redox state was evaluated based on changes in native FP fluorescence. Increase in flavoprotein oxidation, due to accelerated electron flow through the respiratory chain, is a compensatory response to mitochondrial membrane

depolarization and uncoupling, which occur in acutely isolated cardiomyocytes[18, 22] and cultured cells [23] during exposure to anesthetics. The mitoK$_{ATP}$ channels, previously identified as effectors of protection by preconditioning, appear to mediate APC-induced mitochondrial depolarization and compensatory flavoprotein oxidation.[23,24] Isoflurane-induced increase in FP fluorescence in rat cardiomyocytes is sensitive to mitoK$_{ATP}$ channel blockade by 5HD and could be mimicked by the mitoK$_{ATP}$ channel opener diazoxide[3] Our study shows that isoflurane increases FP fluorescence similar in mouse WT and Kir6.2 KO cardiomyocytes. This suggests that isoflurane activation of the signaling cascade that leads to mitoK$_{ATP}$ channel opening does not rely on the sarcK$_{ATP}$ channel opening and therefore may constitute an independent pathway. Our data support findings by Suzuki et al.[25] showing that oxidation of flavoproteins by diazoxide is preserved in Kir6.2 KO cardiomyocytes, and confirm the presence of functional mitoK$_{ATP}$ channels. It also suggests that Kir6.2 is not a subunit of the mitoK$_{ATP}$ channel.

A moderate increase in ROS generation appears critical for initiating the APC signaling cascade.[26, 27] Our study demonstrated that isoflurane elicits a burst of ROS in mouse WT and Kir6.2 KO cardiomyocytes. A similar effect of isoflurane was reported in rat cardiomyocytes.[19] The mechanism by which isoflurane induces a burst of ROS involves opening of mitoK$_{ATP}$ channels[28] and/or partial inhibition of the electron transport chain.[19] Thus, mitochondria appear to be the primary source of ROS generated in cardiomyocytes during exposure to isoflurane.[19] These findings correlate with FP fluorescence data, showing both WT and Kir6.2 KO myocytes exhibit a similar mitochondrial response to isoflurane. Furthermore, ROS may directly activate the sarcK$_{ATP}$ channel[29, 30] possibly by modifying its sensitivity to ATP.[31] In addition, ROS generated in response to volatile anesthetics can activate PKC,[32] which in turn may enhance opening of sarcK$_{ATP}$ channel.[33, 34] Taken together these findings suggest the opening of sarcK$_{ATP}$ channel is downstream of a signaling burst of ROS, and likely downstream or parallel to mitoK$_{ATP}$ channel opening.

Modulation of the mitochondrial membrane potential by volatile anesthetics plays an important role in the mechanism of cardioprotection and may involve the mitoK$_{ATP}$ channel opening.[18, 23, 24] Indeed, isoflurane elicits partial depolarization of cardiac mitochondria in both WT and KO myocytes. These findings corroborate the results from our previous studies in rats and the human embryonic stem cell-derived cardiomyocytes[3, 35] underscoring similarities in the mechanisms of APC signaling among different species. Application of 5HD together with isoflurane abolished its effect on mitochondrial membrane potential in WT and Kir6.2 KO myocytes supporting a notion that this effect occurs via activation of the mitoK$_{ATP}$ channel. This suggests that Kir6.2 KO myocytes exhibit normal mitochondrial responses to isoflurane, including opening of the mitoK$_{ATP}$ channels.

Our cell survival experiments showed that compared to WT the Kir6.2 KO myocytes are more sensitive to stress-induced cell death. This is in line with findings by Suzuki et al.[25]who showed that basal ischemic damage is greater in Kir6.2 KO than WT hearts, as judged by greater magnitude of ischemic contracture and poorer recovery of ventricular function after reperfusion. Thus, the functional sarcK$_{ATP}$ channel is important endogenous effector of protection activated in response to noxious stimuli. Genetic disruption of sarcK$_{ATP}$ channel may enhance susceptibility toward stress in KO mice due to increased intracellular Ca^{2+} loading[36] or disruption of metabolic networks.[37]

Interestingly, APC with isoflurane attenuated cell death of both WT and Kir6.2 KO myocytes, suggesting the APC protective signaling pathways may function in parallel. Intact mitochondrial pathways (mitochondrial redox state, ROS burst and mitoK$_{ATP}$ channel opening) in Kir6.2 KO myocytes could likely be the parallel mediators of protection. That

would imply the existence of multiple but independent pathways that can afford protection even if other pathways are dysfunctional, suggesting an evolutionary mechanism of a vital importance that ensures survival of cardiomyocytes, the terminally differentiated cells, that cannot be regenerated following detrimental ischemic events.

Previous studies demonstrated that diazoxide-induced preconditioning is abolished in the hearts from Kir6.2 KO mice.[38] Hu et al.[39] reported that Kir6.2 knockout impairs the left ventricular response to stress: systolic overload following chronic transverse aortic constriction. Furthermore, the hearts of transgenic mice expressing a mutant Kir6.2 channel with reduced ATP sensitivity exhibited incompetent protection by ischemic preconditioning and poor recovery after metabolic inhibition.[40]

When investigating the cellular mechanisms of APC with isoflurane we tested the possibility of a cross-talk between two molecular signaling pathways that contribute to preconditioning, the sarcK_{ATP} channels and mitochondria. After initial reports by Gross and Auchampach[41] and Auchampach et al.[42] numerous studies focused on the role of sarcK_{ATP} channel in cardioprotection. Opening of these channels decreases action potential duration and cytosolic Ca^{2+} influx, thus protecting the heart from ischemic damage.[43, 44] The significance of mitochondrial pathways, including the opening of mitoK_{ATP} channels also has been documented. Communication between mitochondria and sarcK_{ATP} channel is important for myocyte survival.[4] However, the interdependence of these two end-effectors of protection is not completely understood, except for indications that they may be activated at different stages of APC.[2, 45, 46] The present study suggests for the first time that the sarcK_{ATP} channels and mitochondria might be independent, but complimentary effectors of protection by APC.

In conclusion, knockout of Kir6.2 subunit, the pore of cardiac sarcK_{ATP} channel does not perturb the protective mitochondrial responses elicited by isoflurane: uncoupling, mitochondrial ROS burst and mitochondrial membrane depolarization, and does not abolish cellular protection by APC in mouse myocytes. This suggests that mitochondria and sarcK_{ATP} channels may function independently, in parallel cellular protective pathways that complement each other. Increased susceptibility of Kir6.2 KO cardiomyocytes to H_2O_2-induced cell death supports the importance of sarcK_{ATP} channels for protection from stress.

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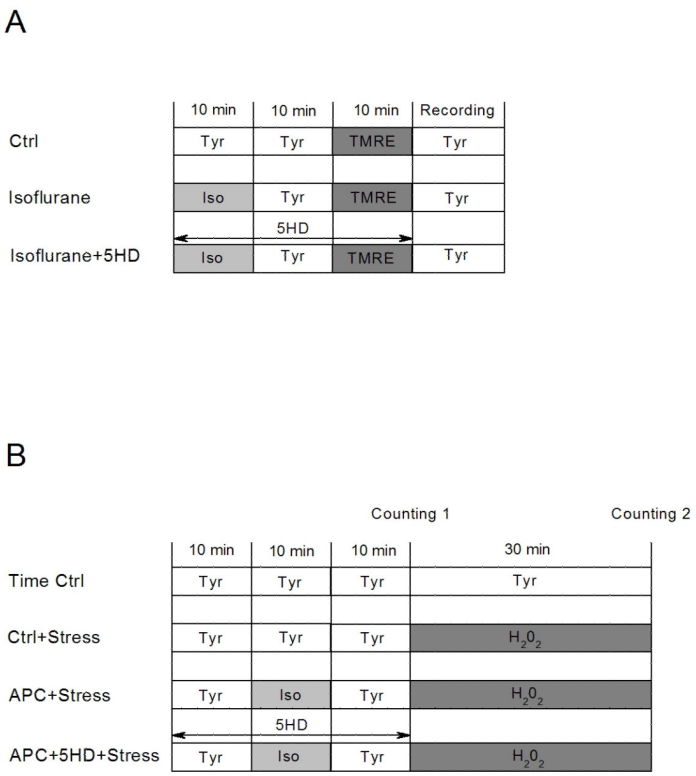

Figure 1.

Study protocols. (A) Protocol for mitochondrial membrane potential measurement. (B) Protocols for cell survival experiments. Detailed descriptions are provided in Methods. Ctrl, control; Tyr, Tyrode solution; TMRE, tetramethylrhodamine ethyl ester; Iso, isoflurane; 5HD, 5-hydroxydecanoate; APC, anesthetic preconditioning; Stress, oxidative stress with H_2O_2.

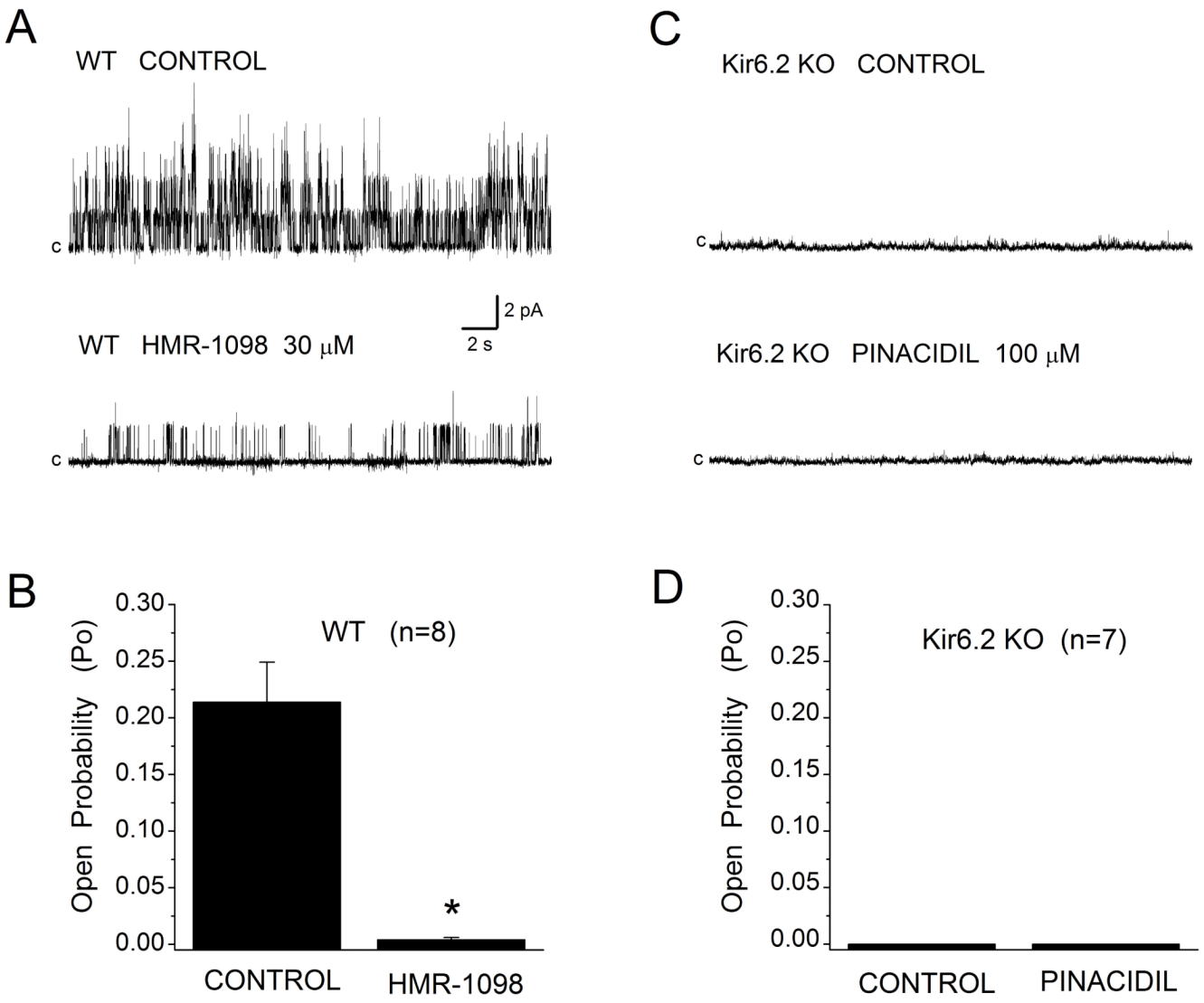

Figure 2.

The sarcK_{ATP} channel in mouse ventricular cardiomyocytes. (A) Single channel activity was monitored in the inside-out patch configuration at Em +40 mV in the absence (Control) or presence of sarcK channel blocker HMR-1098. (B) HMR-1098 (30 μM) inhibited channel activity by 97%. Data are mean±SEM, n=8. (C, D) No channel activity was detected in membrane patches excised from Kir6.2 KO myocytes when monitored in control conditions or during application of channel opener pinacidil (100 μM), n=7.

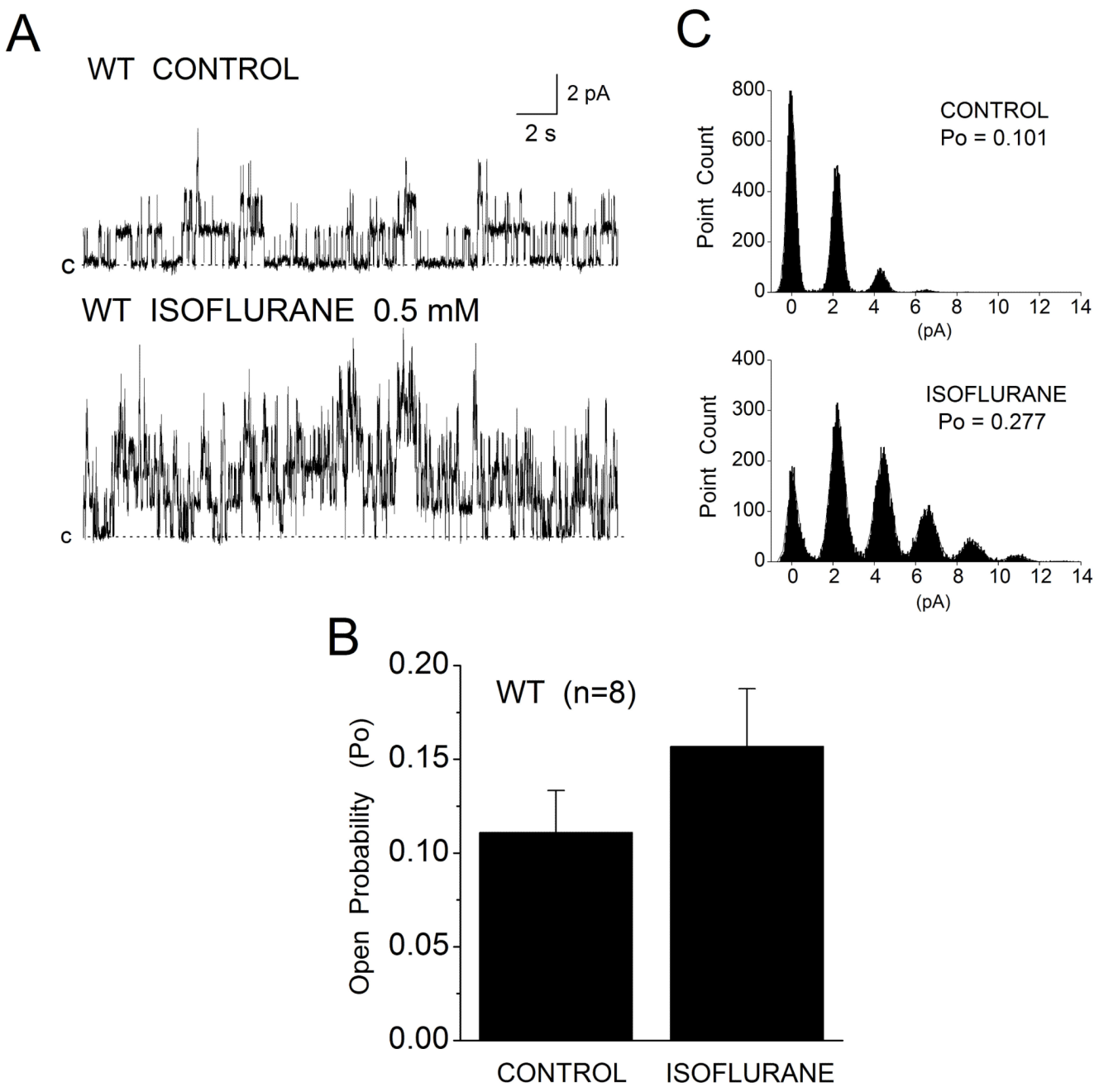

Figure 3.

Acute isoflurane enhances sarcK_{ATP} channel opening in inside-out patches from mouse WT myocytes. (A) Example recordings of single sarcK_{ATP} channel activity monitored from the same patch in the absence (CONTROL) and the presence of 0.5 mM isoflurane (ISOFLURANE). Isoflurane increased the probability of channel opening (Po) by 33% (B) and increased the number of open channels (C). Data are mean±SEM, n=8.

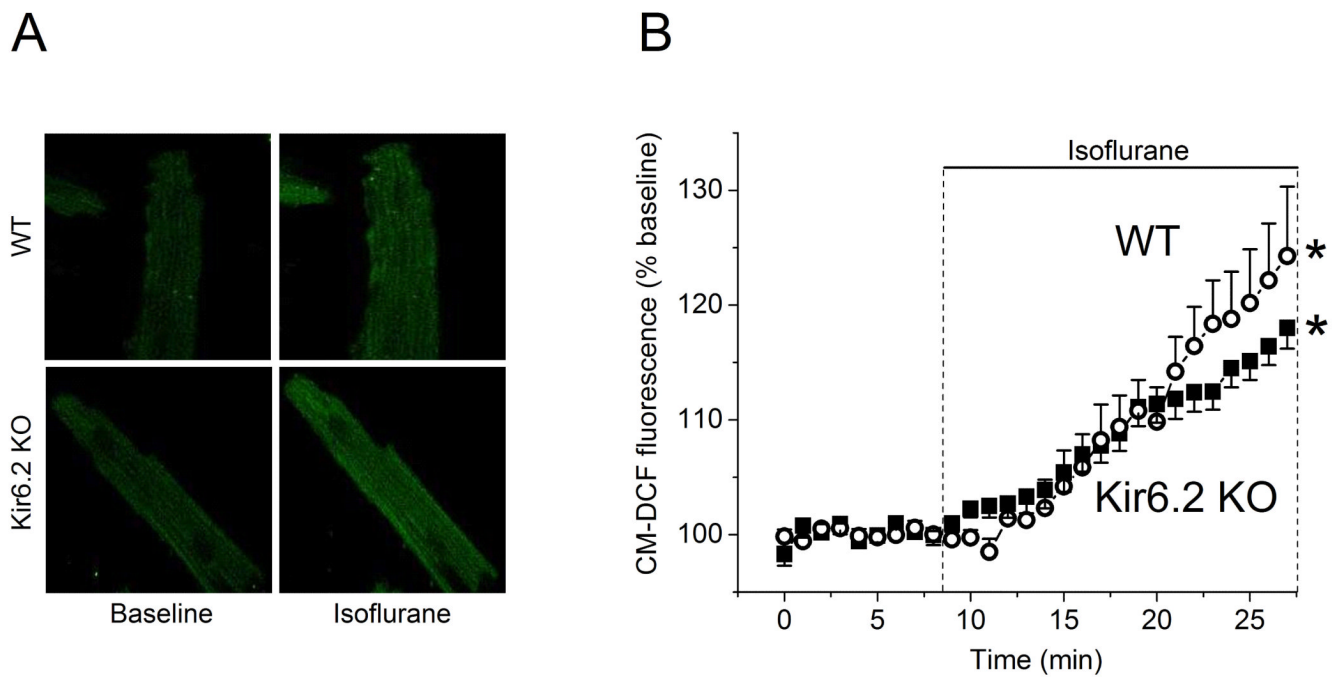

Figure 4.

Acute isoflurane alters the mitochondrial redox state of intact mouse ventricular cardiomyocytes. Autofluorescence of mitochondrial flavoproteins (FP) was monitored as the indicator of mitochondrial redox state. (A) Example images of FP fluorescence captured in WT and Kir6.2 KO cardiomyocytes before (Baseline), during (Isoflurane) and after (Washout) exposure to 0.5 mM isoflurane. (B) Time course of changes in FP fluorescence during application of isoflurane. Data are expressed as change from the baseline (100%), data points are mean±SEM. Isoflurane increased oxidation of mitochondrial FP in cardiomyocytes from WT (closed symbols, n=6) and Kir6.2 KO (open symbols, n=5) hearts. *P< 0.05 vs. baseline. Washout between 14 and 25 min.

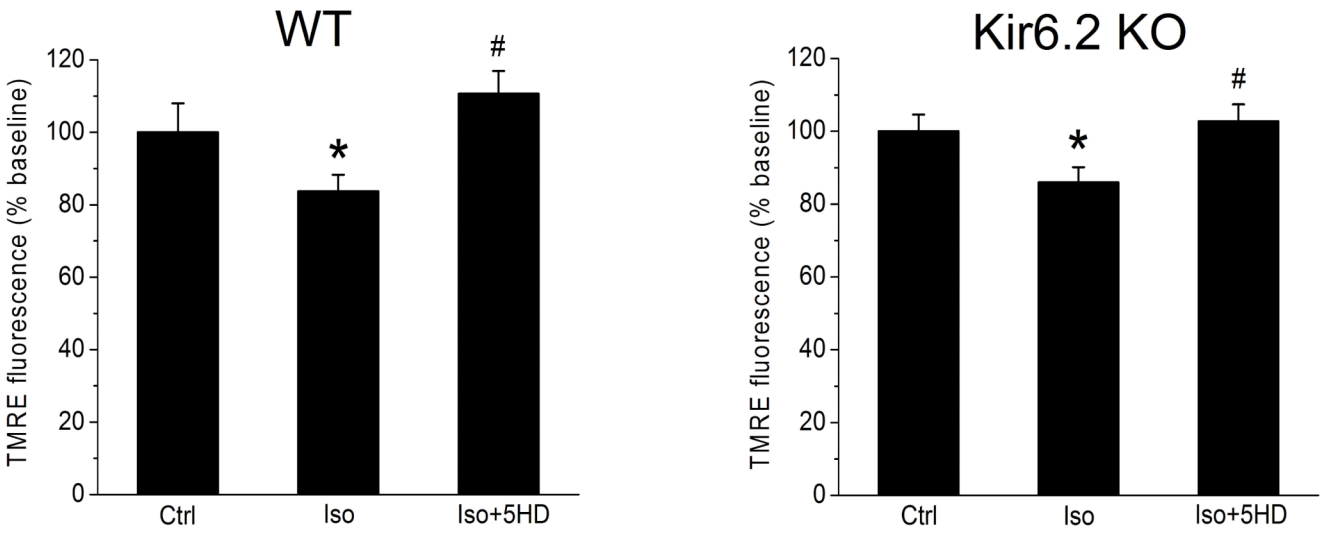

Figure 5.

Acute isoflurane increases generation of reactive oxygen species (ROS) in isolated intact mouse cardiomyocytes. (A) Representative images of CM-DCF fluorescence captured in WT and Kir6.2 KO cardiomyocytes before and during exposure to 0.5 mM isoflurane. The CM-DCF fluorescence was increased by isoflurane. (B) Summary of time dependent changes in CM-DCF fluorescence. Data were normalized to the baseline and presented as mean±SEM. Isoflurane effects on ROS production were similar in cardiomyocytes from WT (n=6) and Kir6.2 KO (n=5) mouse hearts. *P< 0.05 vs. baseline.

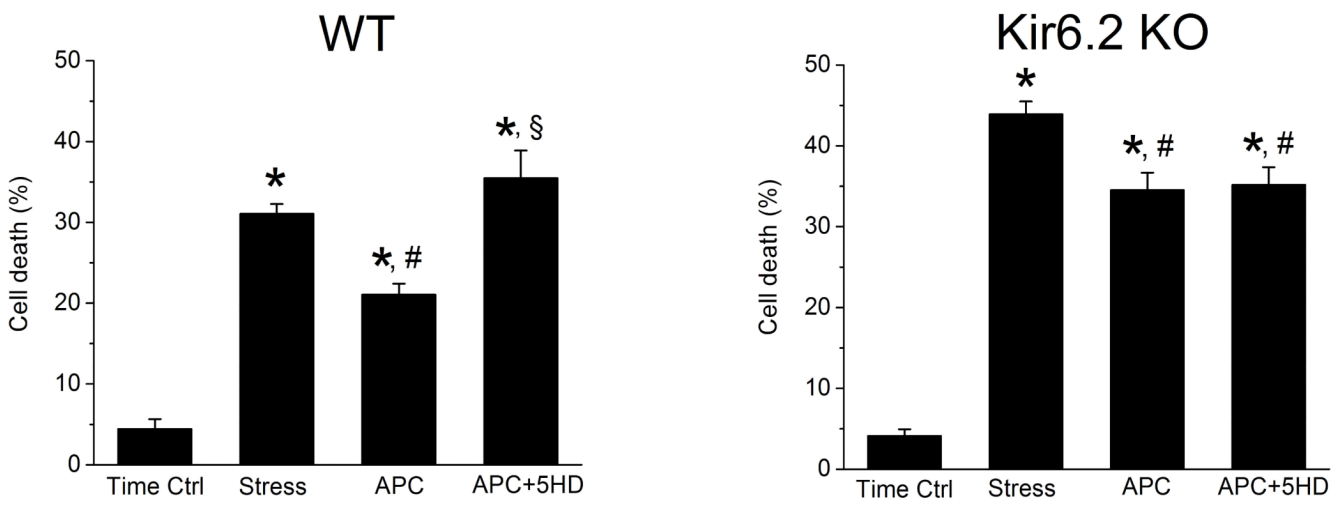

Figure 6.

Acute isoflurane induces partial depolarization of mitochondrial membrane potential in cardiomyocytes from WT (n=6) and Kir6.2 KO (n=5) hearts. Isoflurane decreased intensity of TMRE fluorescence in WT and Kir6.2 KO cardiomyocytes. The effect was abolished in the presence of 5HD, a blocker of mitoK_{ATP} channels. Ctrl, Control; Iso, isoflurane; 5HD, 5-hydroxydecanoate. Data are mean±SEM, *P< 0.05 Ctrl vs. Iso; #P< 0.05 Iso vs. Iso+5HD. *Left panel:* Summary data from WT cardiomyocytes. *Right panel:* Summary data from Kir6.2 KO cardiomyocytes.

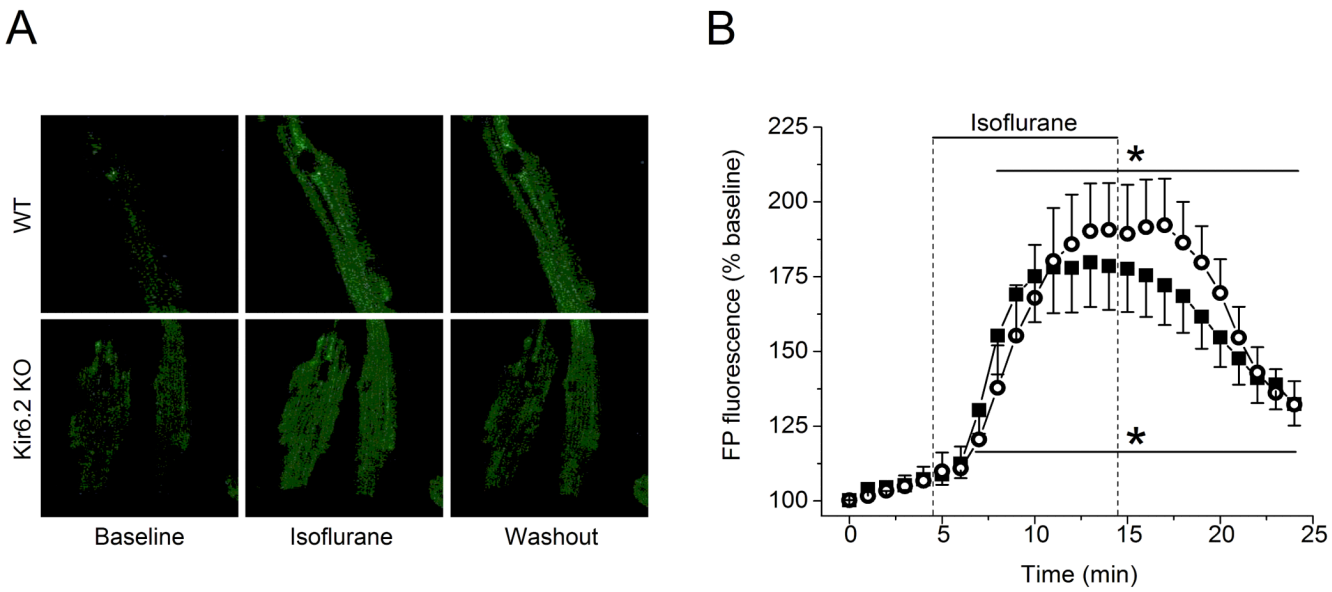

Figure 7.

Effect of APC with isoflurane on H_2O_2 induced stress and survival of isolated cardiomyocytes from WT (n=9) and Kir6.2 KO (n=6) hearts. Percent cell death was determined in four experimental groups: time control (Time Ctrl), oxidative stress with H_2O_2 (Stress), stress following cell exposure to isoflurane (APC), and stress in isoflurane-pretreated myocytes during blockade of K_{ATP} channels with 5HD (APC+5HD). Data are mean±SEM, *P< 0.05 vs. Time Ctrl; #P< 0.05 vs. Stress; §P< 0.05 vs. APC. *Left panel:* Pretreatment with isoflurane protected WT myocytes from stress. Blockade of mitoK_{ATP} channels with 5HD abolished this protection. *Right panel:* Pretreatment with isoflurane protected Kir6.2 KO myocytes from stress. However, isoflurane-induced protection of Kir6.2 KO myocytes was not altered by 5HD (APC vs. APC+5HD).