Isoflurane Preconditioning Elicits Competent Endogenous Mechanisms of Protection from Oxidative Stress in Cardiomyocytes Derived from Human Embryonic Stem Cells

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ABSTRACT

Background: Human embryonic stem cell (hESC)–derived cardiomyocytes potentially represent a powerful experimental model complementary to myocardium obtained from patients that is relatively inaccessible for research purposes. We tested whether anesthetic-induced preconditioning (APC) with isoflurane elicits competent protective mechanisms in hESC-derived cardiomyocytes against oxidative stress to be used as a model of human cardiomyocytes for studying preconditioning.

Methods: H1 hESC cell line was differentiated into cardiomyocytes using growth factors activin A and bone morphogenetic protein-4. Living ventricular hESC-derived cardiomyocytes were identified using a lentiviral vector expressing a reporter gene (enhanced green fluorescent protein) driven by a cardiac-specific human myosin light chain-2v promoter. Mitochondrial membrane potential, reactive oxygen species production, opening of mitochondrial permeability transition pore, and survival of hESC-derived cardiomyocytes were assessed using confocal microscopy. Oxygen consumption was measured in contracting cell clusters.

Results: Differentiation yielded a high percentage (~85%) of cardiomyocytes in beating clusters that were positive for cardiac-specific markers and exhibited action potentials resembling those of mature cardiomyocytes. Isoflurane depolarized mitochondria, attenuated oxygen consumption, and stimulated generation of reactive oxygen species. APC protected these cells from oxidative stress–induced death and delayed mitochondrial permeability transition pore opening.

Conclusions: APC elicits competent protective mechanisms against oxidative stress in hESC-derived cardiomyocytes, suggesting the feasibility to use these cells as a model of human cardiomyocytes for studying APC and potentially other treatments/diseases. Our differentiation protocol is very efficient and yields a high percentage of cardiomyocytes. These results also suggest a promising ability of APC to protect and improve engraftment of hESC-derived cardiomyocytes into the ischemic heart.

What We Already Know about This Topic

❖ Anesthetic-induced preconditioning (APC) is a cardioprotective strategy that increases resistance to ischemia and reperfusion by eliciting innate protective mechanisms

What This Article Tells Us That Is New

❖ APC elicits competent protective mechanisms against oxidative stress in human embryonic stem cell–derived cardiomyocytes, suggesting the feasibility to use these cells as a model of human cardiomyocytes for studying APC

The mechanisms of drug action and pathophysiology of cardiac disease are mostly studied in animals and need to be validated in human models. However, research efforts are hampered by limited access to human myocardium. We investigated...
whether cardiomyocytes derived from human embryonic stem cells (hESCs) can be used as a complimentary experimental model of human cardiomyocytes to study anesthetic-induced preconditioning (APC). APC is a cardioprotective strategy that increases resistance to ischemia and reperfusion (I/R) by eliciting innate protective mechanisms.1,2

hESCs can be differentiated in vitro into various cell types, including cardiomyocytes, and potentially represent a powerful experimental model to screen drugs and study normal and pathologic processes.3–5 These cardiomyocytes can phenotypically resemble functional human cardiomyocytes6–3 and have been tested for cell replacement therapies in the treatment of heart disease in animals, with variable success.10,11 The ability of implanted hESC-derived cardiomyocytes to repair I/R-injured myocardium critically depends on their ability to survive the stressful environment within the host tissue, which can be improved by enhancing their resistance to activation of cell death pathways using a “prosurvival cocktail.”12 Interestingly, some components of the prosurvival cocktail have effects comparable with those of APC: inhibition of mitochondrial permeability transition pore (mPTP) opening,13 antiapoptotic pathway activation,14 and opening of adenosine triphosphate-sensitive potassium channels.2

To identify the possibility that hESC-derived cardiomyocytes have a sufficiently competent response to a preconditioning stimulus to be used as an experimental model for APC, we investigated whether preconditioning with the anesthetic isoflurane elicits distinct mediators of protection in these cells: reactive oxygen species (ROS) and opening of mitochondrial adenosine triphosphate-sensitive potassium channels.2

Materials and Methods

Human Embryonic Stem Cell Culture

H1 (WA01) hESC line from WiCell Research Institute Inc. (Madison, WI) was maintained on mouse embryonic fibroblasts in hypoxic conditions (4% O2/5% CO2). Feeder cells were treated with mitomycin C (Sigma-Aldrich, St. Louis, MO) to arrest mitosis and cultured in Dulbecco modified Eagle medium (Millipore Bioscience Research Reagents, Temecula, CA) supplemented with 10% Fetal Bovine Serum (Invitrogen, Carlsbad, CA) and 1% nonessential amino acids (Millipore Bioscience Research Reagents). The hESCs were cultured in Dulbecco modified Eagle medium/Ham F12 medium (Invitrogen) supplemented with 20% knock-out serum (Invitrogen), 1% nonessential amino acids, 1% penicillin-streptomycin, 1-glutamine (Millipore Bioscience Research Reagents), β-mercaptoethanol (Sigma-Aldrich), and 4 ng/ml human recombinant basic fibroblast growth factor (Invitrogen). The colonies of hESCs were passaged every 5–7 days using a mechanical microdissection method. We used hESCs with passage numbers 39–43 for cell characterization (figs. 1 and 2), and 50–53 for APC testing (figs. 3–7).

Cardiac Differentiation of hESCs

Colonies of hESCs were mechanically dissociated into small clumps, plated onto dishes precoated with Reduced Growth Factor Matrigel (BD Biosciences, San Jose, CA), and cultured under hypoxic (4% O2) conditions. Cells were maintained pluripotent by daily feeding with medium conditioned by mouse embryonic fibroblasts supplemented with 4 ng/ml fibroblast growth factor-2 for the subsequent 7 days, followed by daily provision of RPMI/B27 medium (Invitrogen) supplemented with growth factors Activin A (50 ng/ml; R&D Systems, Minneapolis, MN) and bone morphogenetic protein-4 (10 ng/ml; R&D Systems) for the subsequent 5 days. After that, cells were placed into normoxia and growth factors were withdrawn.

Microdissection and Single Cell Dissociation

Ninety days after treatment with growth factors, the beating cell clusters were mechanically dissociated from the remaining cell aggregates under a dissecting microscope (SMZ1000; Nikon, Tokyo, Japan) and treated with 0.05% trypsin-EDTA (Invitrogen) for 4 min to dissociate individual cells, which were plated onto Matrigel-coated coverslips.

Genetic Marking of hESC-derived Cardiomyocytes with a Lentiviral Vector

Dissociated cells were transduced with a lentiviral vector encoding human myosin light chain-2v (MLC-2v)–driven enhanced green fluorescent protein (multiplicity of infection, 2.2 × 104). MLC-2v is a promoter specific for ventricular myocytes.15–17 A MLC-2v-enhanced green fluorescent protein cassette (kindly provided by Lior Gepstein, M.D., Ph.D., Professor, The Bruce Rappaport Institute in the Medical Sciences, Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel)5 was subcloned into lentiviral transfer plasmid pHR(+)c.Ub.MCSoligo.R(−)W(+). Lentiviral vector production and titering was performed as described previously.18,19

Laser-Scanning Confocal Microscopy

Four days after lentiviral vector transduction, imaging was performed using a confocal microscope (Eclipse TE2000-U; Nikon) and data were analyzed with MetaMorph 6.1 software (Molecular Devices, Sunnyvale, CA). Living hESC-derived cardiomyocytes were identified by detecting fluorescence of MLC-2v–driven enhanced green fluorescent protein–positive cells and experiments were conducted in Tyrode solution (132 mM...
NaCl, 10 mM HEPES, 10 mM glucose, 5 mM KCl, 1 mM CaCl₂, and 1.2 mM MgCl₂, pH 7.4) at room temperature. The percentage of hESC-derived cardiomyocyte death was determined after exposing cells to oxidative stress induced by 10 mM H₂O₂ (Calbiochem, La Jolla, CA) applied for 50 min, followed by perfusion with Tyrode solution for 10 min. The cells stained with red-fluorescent propidium iodide (2 μM; Sigma–Aldrich) were considered dead.20 hESC-derived cardiomyocytes were preconditioned with 0.5 mM isoflurane (≈1 minimal alveolar concentration) applied for 15 min, followed by 5-min isoflurane washout (APC). After each experiment, gas chromatography was used to test isoflurane concentrations, which varied ±10% of reported values. The tetrabromoethylrhodamine ethyl ester was used to detect mitochondrial membrane potential (ΔΨₘ) in hESC-derived cardiomyocytes. Data are normalized to the first time point in baseline (100%). For the statistical analysis, the average value of time points after the treatment has reached the maximal effect (the last five frames), and the average baseline values were used. ROS production was monitored in cells loaded with dihydroethidium (10 μM; Invitrogen). For the statistical analysis, the rate of increase in ethidium fluorescence before or after isoflurane application and in the time control was used. Opening of the mPTP was assessed as described previously in our laboratory,13 a method based on mPTP induction by photoexcitation-generated oxidative stress.21–24 The mPTP opening was detected by rapid dissipation of ΔΨₘ, observed as loss of tetrabromoethylrhodamine ethyl ester fluorescence, which is sensitive to mPTP opening inhibition.13

**Immunolabeling**

After the fixation and permeabilization, cells were treated with primary antibodies for anti-α-actinin (1:100 dilution; Sigma–Aldrich), anti–cardiac-specific troponin T (1:100; Thermo Fisher Scientific, Waltham, MA), anti-myosin light chain-2a (1:100; Synaptic Systems, Goettingen, Germany), or anti-titin (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA), after treatment with corresponding secondary antibody, Alexa Fluor 488 (1:1000; Invitrogen). Nuclei were stained with TOPRO-3 (1:1000; Invitrogen).

**Electrophysiology**

Membrane potential (Eᵥ) was measured in microdissected beating clusters using 3 M KCl-filled borosilicate glass microelectrodes (impedance, 40–60 MO) in RPMI/B27 medium. Data were processed using a Grass RPS7C polygraph (Astro-Med/Grass Inc., West Warwick, RI) and Superscope II digital data acquisition system (GW Instruments, Somerville, MA).

**Oxygen Consumption**

Spontaneously and rhythmically contracting cell clusters were separated by microdissection, and respiration of cell clusters that predominantly contain cardiomyocytes was measured using an oxygen electrode (Hansatech Instruments, Norfolk, United Kingdom) at 37°C. Isoflurane was delivered at incremental steps, and the rate of oxygen consumption after each isoflurane addition was normalized to baseline values.

**Statistical Analysis**

Data are presented as mean ± SD. Each experimental group comprises hESC-derived cardiomyocytes from at least three separate differentiations, where n indicates the number of independent experiments. For the statistical analyses, SigmaStat 3.0 software (Systat Software, Inc., San Jose, CA) was used. Statistical comparisons were performed using one-way analysis of variance or two-way repeated measures analysis of variance with Tukey or Dunnett post hoc tests where appropriate. Unpaired t test was used for comparisons between two groups where appropriate. P values are from two-tailed tests. Differences at P less than 0.05 were considered significant.

**Results**

**Differentiation and Characterization of hESC-derived Cardiomyocytes**

The presence of cardiomyocytes after cardiac differentiation of hESCs was observed as occurrence of spontaneously and rhythmically beating areas of contiguous cells in culture dishes, beginning approximately 10 days after the treatment with activin-A and bone morphogenetic protein-4 and lasting up to 1 yr (see Supplemental Digital Content 1, which is a video showing beating areas that spanned almost the entire surface of the culture dish, http://links.lww.com/ALN/A624). As shown in figure 1, immunostaining revealed an abundance of cells positive for cardiac-specific sarcomeric proteins, organized in characteristic striated pattern (fig. 1, A–D). After the dissociation from culture dishes, cardiomyocytes retained their striated appearance (fig. 1F), some continuing to exhibit spontaneous rhythmic contractions (see Supplemental Digital Content 2, which is a video showing isolated contracting cells that is a characteristic of cardiomyocytes, http://links.lww.com/ALN/A625). Short (less than 130 ms), atrial-like action potentials, and long, (more than 250 ms) ventricular-like action potentials were recorded in dissociated, contracting cell clusters, indicating electrical activity and existence of functional sarcolemmal ion channels in these cardiomyocytes.

**Genetic Marking and Labeling of Live hESC-derived Cardiomyocytes Using a Lentiviral Vector**

To identify living hESC-derived cardiomyocytes and determine differentiation efficiency, differentiated cells were genetically marked using a self-inactivating lentiviral vector containing a cardiac-specific promoter, MLC-2v, driving the expression of enhanced green fluorescent protein (fig. 2A). Lentiviral transduction efficiency, determined with ubiquitin-driven enhanced green fluorescent protein (fig. 2A), was 98 ± 1% (fig. 2B); 85 ± 3% of cells were MLC-2v–enhanced green fluorescent protein-positive (i.e., ventricular myocytes), indicating high efficiency of our differentiation protocol (fig. 2, B and C).
APC Protects hESC-derived Cardiomyocytes from Oxidative Stress

To test whether APC protects the hESC-derived cardiomyocytes from oxidative stress-induced cell death, the preconditioned and control cells were exposed to H$_2$O$_2$. The cardiomyocytes were identified as MLC-2v-enhanced green fluorescent protein-positive cells (green-fluorescent cells in fig. 3A). APC attenuated the hESC-derived cardiomyocyte death compared with control, 32 ± 16% (n = 5) versus 58 ± 15% (n = 5), respectively (fig. 3, B and C). These results correlate with those of our previous study, which showed that APC protects adult human atrial cells from oxidative stress.25

Isoflurane Depolarizes Mitochondria in hESC-derived Cardiomyocytes by Opening MitoK$_{ATP}$ Channels

Using adult rat cardiomyocytes, we have previously shown that isoflurane induces opening of mitoK$_{ATP}$ channels, causing decrease in $\Delta \Psi_m$ and thereby eliciting cardiop-
In hESC-derived cardiomyocytes, diazoxide, an opener of mitoKATP channels, decreased tetramethylrhodamine fluorescence intensity from baseline value of 99.5/110006 1.1% (n = 12) to 90.2/110006 5.4% of baseline (n = 12), indicating opening of mitoKATP channels (fig. 4). Application of 0.5 mM isoflurane decreased tetramethylrhodamine fluorescence intensity from 99.6/110006 1.7% (n = 11) to 82.2/110006 9.8% of baseline (n = 11). This was attenuated in the presence of 5-hydroxydecanoate (5-HD), an inhibitor of mitoKATP channel opening, and the baseline fluorescence decreased from 99.7/110006 1.7% (n = 11) to 94.2/110006 2.4% of baseline (n = 11), indicating that mitochondrial depolarization by isoflurane is mediated, in part, by opening of mitoKATP channels.

Oxygen Consumption by Cell Clusters Containing Cardiomyocytes Is Inhibited by Isoflurane

The rate of oxygen consumption of microdissected beating cell clusters that predominantly contained cardiomyocytes was monitored in the presence of incremental isoflurane concentration (fig. 5). At a concentration of 0.12 mM, isoflurane slightly, but not significantly, increased baseline oxygen consumption from 100.0 ± 30.1 to 106.9 ± 30.8%. However, by increasing isoflurane concentration to 0.25, 0.5, and 1.0 mM, the oxygen consumption progressively decreased to 95.4 ± 20.4, 87.3 ± 23.5, and 82.0 ± 19.4% of baseline, respectively (n = 7 in all groups). This indicates a suppression of respiration by isoflurane.

I sof l ur an e Enhances Production of ROS by hESC-derived Cardiomyocytes

Production of ROS, important signaling molecules in preconditioning of adult cardiomyocytes, was monitored in hESC-derived cardiomyocytes. As seen in figu re 6, application of 0.5 mM isoflurane significantly increased the rate of change in ethidium fluorescence intensity compared with its baseline or time control (isoflurane, 1.60 ± 0.59, n = 11; isoflurane baseline, 0.01 ± 0.62, n = 11; and time control, −0.15 ± 0.31% points/min, n = 8). This indicated that isoflurane increases ROS production in hESC-derived cardiomyocytes.
Preconditioning with Isoflurane or Hydrogen Peroxide Delays Opening of the mPTP

Cardioprotective strategies, including APC, delay opening of mPTP, a critical event in the transition toward cell death. mPTP opening-induced dissipation of $\Delta W_m$ was monitored in hESC-derived cardiomyocytes exposed to oxidative stress (fig. 7).

Compared with control, mPTP inhibitor cyclosporine A (1 $\mu$M) increased the arbitrary mPTP opening time, 100.0 $\pm$ 24.8% (n = 11) versus 119.1 $\pm$ 14.6% of control (n = 10), respectively. Similarly to cyclosporine A, APC increased arbitrary mPTP opening time, and application of 5-HD together with isoflurane abrogated this effect (control, 100.0 $\pm$ 18.2%, n = 12; APC, 139.4 $\pm$ 40.4%, n = 16; and APC + 5-HD, 95.3 $\pm$ 11.6% of control, n = 16), confirming the role of mitoK$_{ATP}$ channel opening in signal mediation of APC. Preconditioning with H$_2$O$_2$ increased arbitrary mPTP opening time to 130.5 $\pm$ 31.2% (n = 12) compared with control (100.0 $\pm$ 19.8%, n = 10).

Discussion

We have demonstrated an efficient method to differentiate cardiomyocytes from hESCs, indicated by genetic labeling using lentiviral vectors, showing that $\sim$85% of cells in beating clusters expressed cardiac-specific promoter MLC-2v. Efficient differentiation and a phenotype of a functional cardiomyocytes was also indicated by observing globally contracting cell clusters that widely expressed highly organized, cardiac-specific sarcomeric proteins and generated action potentials resembling those of mature heart cells. Moreover, we showed that preconditioning with isoflurane attenuates cell death and elicits competent mechanisms of protection in ventricular hESC-derived cardiomyocytes against oxidative stress.
stress. These included characteristic and important mediators of cardioprotection: opening of mitoK	extsubscript{ATP} channels, ROS as signaling molecules, and a delay of oxidative stress-induced mPTP opening, an endpoint of protection. Similar responses to APC in adult cardiomyocytes documented in our previous studies and hESC-derived cardiomyocytes demonstrated in this study indicate the feasibility of using hESC-derived cardiomyocytes as a model of human ventricular cardiac cells to study APC and potentially other treatments/diseases.

We showed that hESC-derived cardiomyocytes phenotypically resemble functional human cardiomyocytes by showing that these cells spontaneously and rhythmically contract, generate action potentials that, by shape and morphology, resemble functional human cardiomyocytes. Moreover, these cardiomyocytes exhibit highly organized sarcomeric structures, indicated by immunostaining for cardiac-specific troponin T, myosin light chain-2a, nonspecific sarcomeric/H9251-actinin, and titin. This is in accord with results from other laboratories that have described structural properties of hESC-derived cardiomyocytes.6,7,30 The presence of spontaneously and rhythmically beating cell clusters that extend throughout the culture dishes (see Supplemental Digital Content 1, http://links.lww.com/ALN/A624) indicate presence of pacemaker cells and cardiomyocytes that form a functional syncytium, which exhibits synchronized action potential propagation.6,31 Other laboratories demonstrated electrophysiological and functional competence of hESC-derived cardiomyocytes by electrophysiological recordings, measurements of Ca	extsuperscript{2+} transients, as well as appropriate chronotropic responses to β-receptor and muscarinic-receptor stimulation.6–9,32–34 However, the extent of these cells’ maturity requires further investigation.3 At minimum, studies describe hESC-derived cardiomyocytes as cells having characteristics of embryonic cardiomyocytes that may, with extended time in culture, differentiate into a mature cardiomyocyte phenotype.35

**Fig. 4.** Isoflurane opens mitoK	extsubscript{ATP} channels and depolarizes mitochondria in human embryonic stem cell (hESC)–derived cardiomyocytes. ΔΨ	extsubscript{m} was monitored in dissociated hESC-derived cardiomyocytes using tetramethylrhodamine ethyl ester (TMRE) fluorescence. The time point when drugs were added is indicated by the arrow. Diazoxide (50 μM) decreased TMRE fluorescence intensity, indicating mitochondrial depolarization. Application of isoflurane (Iso) also decreased TMRE fluorescence intensity, an effect that was partly blocked with 200 μM 5-hydroxydecanoate (5-HD). 2,4-Dinitrophenol (DNP; 100 μM) completely depolarized mitochondria. In group Iso+5-HD, four observations in the first time point of baseline and three observations in the second time point of baseline are missing, and in group DNP, four observations in the first five time points of baseline are missing. *P < 0.05 versus baseline; #P < 0.05 versus Iso + 5-HD.

**Fig. 5.** Isoflurane attenuates beating cell cluster oxygen consumption. The rates of oxygen consumption in contracting cell clusters after the addition of incremental isoflurane (Iso) concentration were normalized to baseline values. At the higher concentrations, isoflurane attenuated oxygen consumption. 2,4-Dinitrophenol (DNP; 100 μM) and cyanide (CN	extsuperscript{−}; 2 mM) were added to establish maximal and minimal rates of oxygen consumption. *P < 0.05 versus baseline.

**Fig. 6.** Isoflurane enhances ROS production in human embryonic stem cell (hESC)–derived cardiomyocytes. Reactive oxygen species (ROS) production in hESC-derived cardiomyocytes was measured by detecting fluorescence intensity of ethidium (Eth). Compared with the time control, isoflurane (Iso) significantly increased Eth fluorescence intensity. In group Iso, three observations in the first two time points of baseline and five observations in the last time point of treatment are missing. *P < 0.05 versus time control and baseline.

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In this study, we achieved highly efficient differentiation of human cardiomyocytes from hESCs, obtaining the unprecedented level of ~85% of cells positive for the cardiac-specific marker MLC-2v in beating cell clusters. An abundance of cardiomyocytes was corroborated by cardiac-specific immunostaining. Moreover, areas with spontaneously and rhythmically beating cells, a characteristic of cardiomyocytes, spanned the entire surface of the culture dish (see Supplemental Digital Content 1, http://links.lww.com/ALN/A624). We have applied bone morphogenetic protein-4, fibroblast growth factor, and activin-A, all endoderm-secreted growth factors, to direct differentiation into cardiac lineage, based on findings that endoderm-induced cardiomyogenic signaling regulates heart development in the embryo.36 Previously published protocols using the same growth factors were modified in this study.12,37 To be exact, pluripotent cells were exposed to activin-A at a relatively high level (50 ng/ml) for an extended period of time (5 days). We speculate that this optimized the efficient production of mesendoderm56 leading to progressive differentiation into the cardiomyogenic lineage. Our results compare highly favorably with those from other studies and approaches to differentiate cardiomyocytes, especially with a widely used method involving spontaneous differentiation termed embryoid bodies that yields a low percentage of cardiomyocytes (≤ 10%).6

Several studies have shown the functional heterogeneity of cardiomyocytes derived from hESCs and the presence of ventricular, atrial, and nodal-like cells, a ventricular phenotype being the most prevalent.7,9 In our experiments, the labeling of cells with the MLC-2v-enhanced green fluorescent protein revealed ~85% of positive cells, indicating that at least 85% of cells are of ventricular cardiomyocyte phenotype, because MLC-2v is a ventricular myocyte-specific transcription factor. We also observed only a minor proportion of cells that stained positive for the atrial myocyte-specific marker myosin light chain 2a in our immunohistochemistry experiments. All together, this indicates that, in agreement with the results from other laboratories, our differentiation protocol predominantly yields ventricular cardiomyocytes and only a small proportion of other cardiac cell types.

We have demonstrated molecular mechanisms by which preconditioning with isoflurane elicits competent defense against oxidative stress in hESC-derived cardiomyocytes. These protective mechanisms have been previously characterized in our laboratory and others using animal13,14,26,28 and, to a lesser extent, human myocardium.25,29 However, it was unclear whether hESC-derived cardiomyocytes exhibit an adequate phenotype to resist oxidative stress, a hallmark of I/R injury.39 In this study, we showed that APC successfully...
protects hESC-derived cardiomyocytes from oxidative stress, which is in accordance with the results from our previous study using adult human atrial cardiomyocytes.\textsuperscript{25,27} To investigate the phenotypic similarity of the cardioprotective mechanisms by APC between adult cardiomyocytes and hESC-derived cardiomyocytes, we tested some of the most relevant mediators of protection. We showed here that isoflurane partly depolarizes mitochondria in a 5-HD-sensitive manner, suggesting opening of mitoK\textsubscript{ATP} channels as crucial mediators of cardioprotection.\textsuperscript{26,40} This effect was almost identical to isoflurane-induced opening of mitoK\textsubscript{ATP} channels demonstrated with the similar approach in our previous study using adult rat cardiomyocytes,\textsuperscript{26} indicating a comparable response between adult and hESC-derived cardiomyocytes. We also showed here that isoflurane moderately enhances production of ROS, an important component of preconditioning signaling cascade.\textsuperscript{28,41} Our previous study demonstrated that desflurane and sevoflurane induce similar extent of ROS production in adult rat cardiomyocytes.\textsuperscript{28} The importance of ROS was confirmed here by showing that a low dose of H\textsubscript{2}O\textsubscript{2} induces preconditioning and delays mPTP opening, which is in agreement with a study by Hanouz et al.\textsuperscript{29} that indicated the critical importance of ROS signaling in APC using adult human atrial trabeculae. Anesthetic-induced increase in ROS production has been attributed to the opening of mitoK\textsubscript{ATP} channels,\textsuperscript{31} but it can also be induced by partial obstruction of the electron transport chain,\textsuperscript{42} another effect of volatile anesthetics.\textsuperscript{43} In this study, we showed that isoflurane suppresses respiration in beating cell clusters. This effect may correlate with the inhibition of the electron transport chain, but it could also reflect other effects of isoflurane, such as alternation in metabolic pathways. The importance of mitoK\textsubscript{ATP} channel opening for inducing cardioprotection by APC was verified here by showing that inhibition of channel opening by 5-HD abrogates the APC-induced delay in mPTP opening.

Our results indicate that preconditioning with isoflurane induces a delay in opening of the mPTP. This has a significant functional importance, because the opening of mPTP has been recognized as the crucial event in the transition toward cell death during I/R injury.\textsuperscript{44} Cardioprotective strategies, including APC, were found to induce a delay in mPTP opening, and mPTP blockers may decrease infarct size by 30–50\%.\textsuperscript{45} During I/R, opening of mPTP dissipates Δ\textsubscript{p}m, preventing oxidative phosphorylation, and initiates death pathways in the cell.\textsuperscript{44} Using an approach identical to that used in this study, we previously demonstrated that APC induces a similar delay in mPTP opening in adult rat cardiomyocytes,\textsuperscript{13} which further correlates with another study from our laboratory demonstrating that APC elicits cellular and mitochondrial protective mechanisms against oxidative stress in human adult cardiomyocytes.\textsuperscript{25} Taken together, all tested parameters indicate similar responses of adult cardiomyocytes and hESC-derived cardiomyocytes to APC.

Studies using hESC-derived cardiomyocytes to regenerate dysfunctional myocardium after I/R injury have had limited success as a result of factors including inefficient differentiation, poor engraftment, and survival within injured myocardium.\textsuperscript{3} However, Laflamme et al.\textsuperscript{12} demonstrated that the use of a prosurvival cocktail during implantation of hESC-derived cardiomyocytes improved cell engraftment and functional recovery of the heart. This cocktail protected graft cells from the stressful environment of (post)ischemic myocardium by blocking cellular death pathways. It is noteworthy that APC involves inhibition of the same cellular death pathways as prosurvival cocktail.\textsuperscript{13,14,41} This suggests that APC could improve cardiomyocyte engraftment, with the added advantage that, unlike the components of the cocktail, volatile anesthetics are approved for the clinical application. In fact, the recent guidelines by the American College of Cardiology/American Heart Association,\textsuperscript{46} recommended the use of volatile anesthetics based on the findings of 15 randomized trials in patients undergoing coronary bypass surgery showing that volatile anesthetics decrease troponin release and improve ventricular function compared with intravenous anesthetics.

A limitation of this study is the use of in vitro–generated cardiomyocytes that may lack some of the characteristics of adult cardiomyocytes, as discussed above. However, this study indicates competent responses of these cardiomyocytes to APC. Another limitation is the use of oxidative stress, which does not fully represent conditions during I/R injury, but it is a widely accepted model for studying reperfusion injury. Although APC exerts protection in isolated cardiomyocytes, this model does not take into account additional effects of anesthetics during APC in vivo, which includes the effects on other cell types, such as endothelial cells, or modulation of the immune response. A possible limitation is that we used hESCs passages 39 to 43 to derive cardiomyocytes for cell characterization and passages 50 to 53 for APC testing.

In conclusion, our study shows for the first time that preconditioning with anesthetic isoflurane elicits competent defensive mechanisms against oxidative stress in ventricular hESC-derived cardiomyocytes. The similarity in responses to APC between adult cardiomyocytes documented in our previous work and hESC-derived cardiomyocytes demonstrated here implies that these cardiomyocytes can be used as a valuable experimental human model to study APC, and possibly other human diseases/treatments. As a complimentary model of human cardiomyocytes, hESC-derived cardiomyocytes offer new experimental advantages, overcoming previous limitations when using human myocardium from patient surgeries. Moreover, APC may be a promising tool for protecting hESC-derived cardiomyocytes during engraftment, thereby increasing regeneration of injured myocardium. It is noteworthy that our study also demonstrates a simple and efficient modification of previously published protocols to differentiate human cardiomyocytes from hESCs.

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