Anesthesiology 2010; 113:906–16

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

Ana Sepac, M.D.,* Filip Sedlic, M.D., Ph.D.,* Karim Si-Tayeb, Ph.D.,† John Lough, Ph.D.,‡ Stephen A. Duncan, D.Phil.,‡ Martin Bienengraeber, Ph.D.,§ Frank Park, Ph.D.,‖ Jinhee Kim, M.D., Ph.D.,# Zeljko J. Bosnjak, Ph.D.**

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

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of the article on the Journal's Web site (www.anesthesiology.org).

* Postdoctoral Fellow, § Assistant Professor, # Visiting Professor, ** Professor and Vice Chairman of Research, Department of Anesthesiology, † Postdoctoral Fellow, ‡ Professor, Department of Cell Biology, Neurobiology and Anatomy, ‖ Associate Professor, Department of Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin.

Received from the Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin. Submitted for publication February 23, 2010. Accepted for publication June 28, 2010. Supported by Grant nos. P01-GM066730 and R01-HL034708 (to Dr. Bosnjak) and DK55743, DK087377, and HL094857 (to Dr. Duncan) from the National Institutes of Health (Bethesda, Maryland) and an endowment (to Drs. Duncan and Lough) from Advancing Healthier Wisconsin (Milwaukee, Wisconsin). Drs. Sepac and Sedlic contributed equally to this work. Presented at the American Society of Anesthesiologists meeting, New Orleans, Louisiana, October 19, 2009, and the Experimental Biology meeting, New Orleans, Louisiana, April 19, 2009.

Address correspondence to Dr. Sedlic: Department of Anesthesiology, Medical College of Wisconsin, 8701 Watertown Plank Rd, Milwaukee, Wisconsin 53226. fsedlic@mcw.edu; sedlic_filip@yahoo.com.

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 cardiomyocytes[6–9] 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 ($mitoK_{ATP}$) channels as signal mediators and a delay in mPTP opening as an endpoint of protection. The model was validated by comparing the obtained results with our previous work using adult human and adult animal cardiomyocytes. We achieved a high purity of differentiated cardiomyocytes (~85% in beating areas). This study is the first to demonstrate that APC elicits characteristic endogenous cytoprotective mechanisms against oxidative stress in hESC-derived cardiomyocytes. Our results suggest that these cardiomyocytes could be used as an experimental model to study APC and potentially other treatments/diseases in human cardiomyocytes. Our study implies that APC could be also used to protect hESC-derived cardiomyocytes and thereby increase their engraftment into the injured myocardium.

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% O_2/5% CO_2). 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, L-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% O_2) 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, 2.2×10^4). 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 $CaCl_2$, and 1.2 mM $MgCl_2$, 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 H_2O_2 (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 tetramethylrhodamine ethyl ester (30 nM; Invitrogen) was used to detect mitochondrial membrane potential ($\Delta\Psi_m$) 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 $\Delta\Psi_m$, observed as loss of tetramethylrhodamine ethyl ester fluorescence, which is sensitive to mPTP opening inhibition.[13]

Immunolabeling

After the fixation and permabilization, 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 (E_m) was measured in microdissected beating clusters using 3 M KCl-filled borosilicate glass microelectrodes (impedance, 40–60 MΩ) in RPMI/B27 medium. Data were processed using a Grass RPS7C polygraph (Astro-Med/Grass Inc., West Warvick, 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, 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).

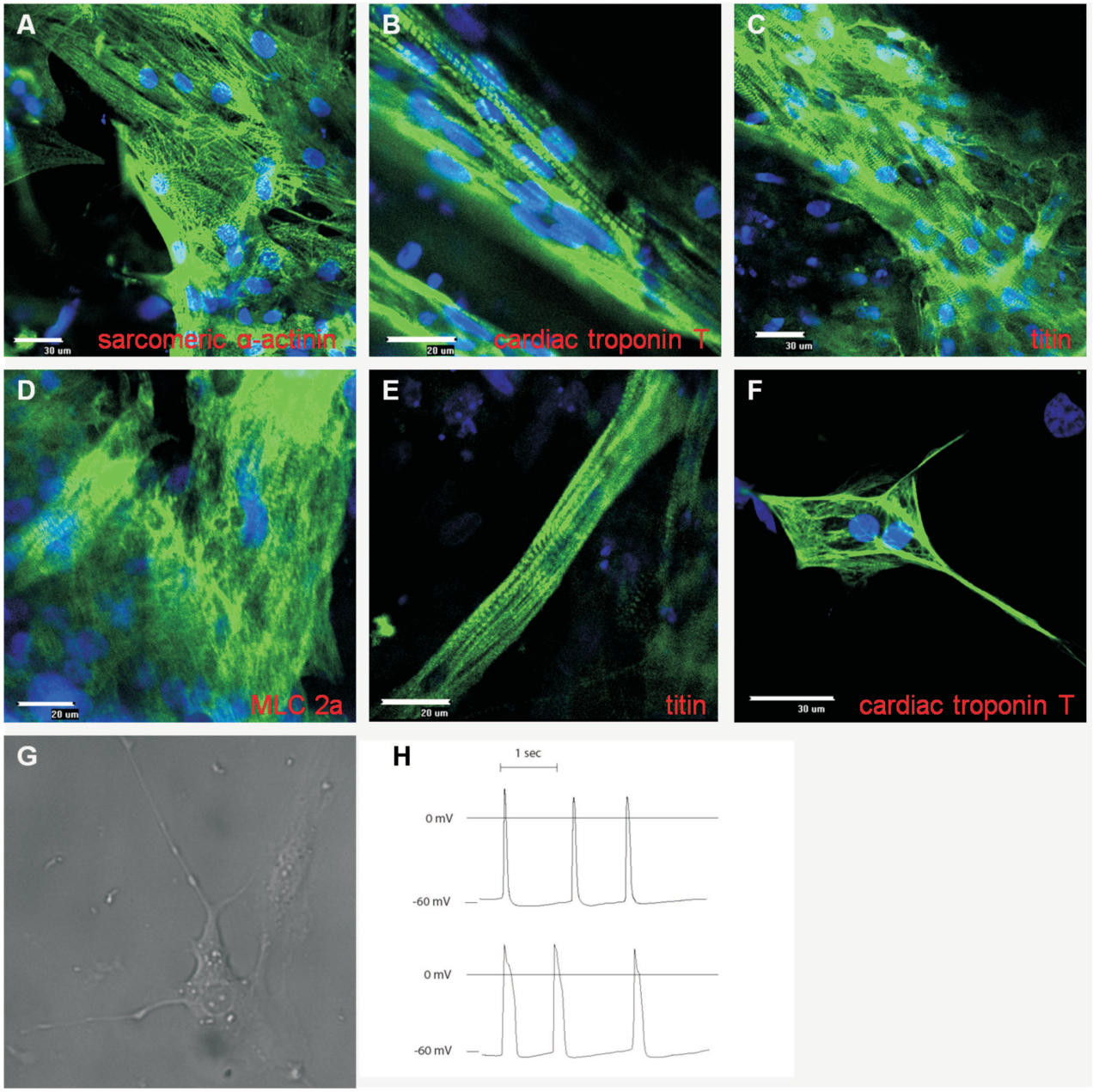

Fig. 1. Immunolabeling and electrophysiological characterization of human embryonic stem cell–derived cardiomyocytes. Confocal images of nondissociated cells after 90 days from treatment with growth factors and stained for cardiac sarcomeric proteins: (*A*) sarcomeric α-actinin, (*B*) cardiac-specific troponin T, (*C*) titin, and (*D*) cardiac-specific myosin light chain-2a (MLC-2a). A large percentage of positive cells is observed, including striated patterns, indicating highly organized sarcomeres. (*E*) Occasional occurrence of cells with rod-shaped morphology that resemble adult cardiomyocytes. (*F*) Dissociated cell stained for cardiac-specific troponin T showing that cells maintain sarcomeric organization after dissociation. (*G*) A dissociated cell that is spontaneously and rhythmically contracting. (*H*) Representative recordings of action potentials: shorter, atrial-like (*top trace*), and longer, ventricular-like (*bottom trace*).

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_2O_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 cardiopro-

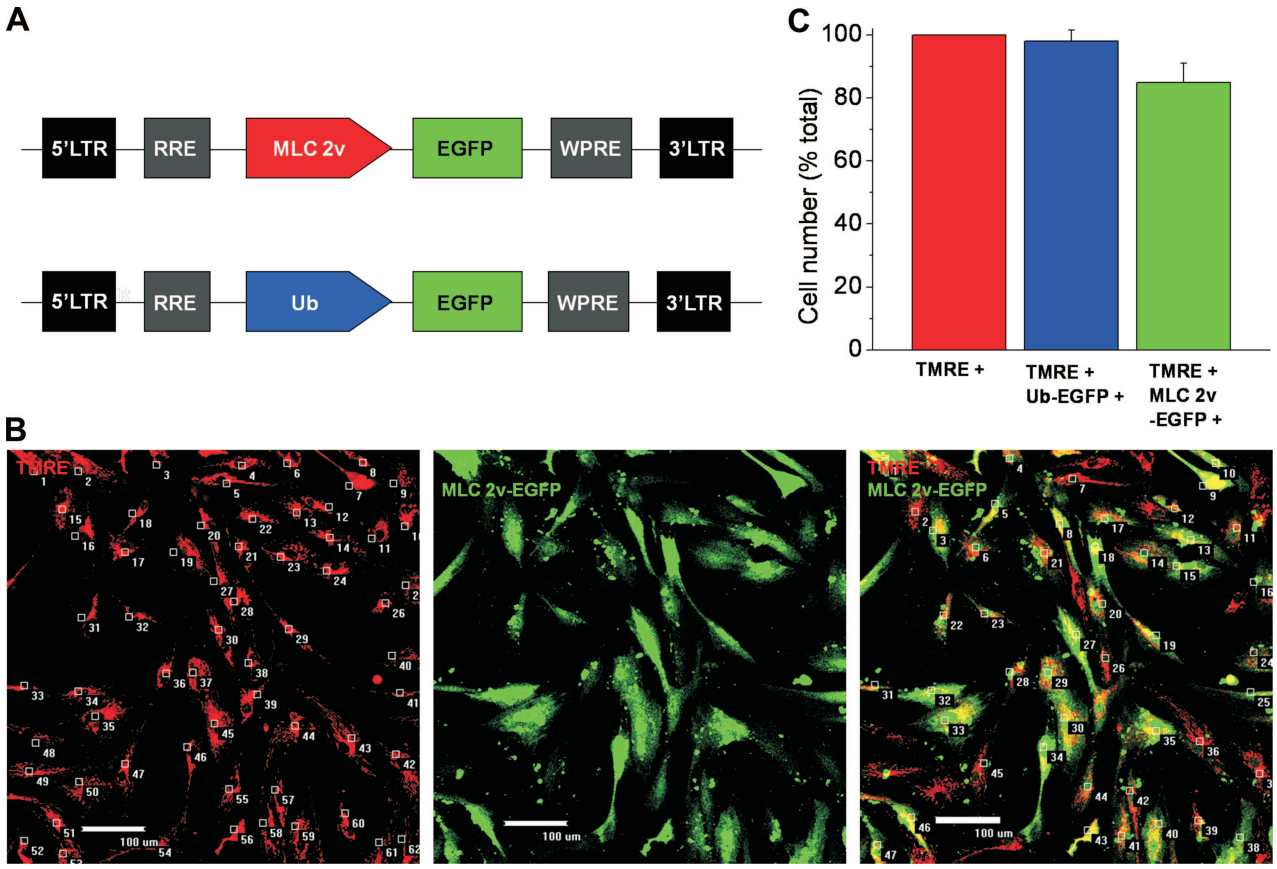

Fig. 2. Labeling and counting of human embryonic stem cell (hESC)–derived cardiomyocytes using lentiviral vector. (*A*) Schematic representation of lentiviral vectors, pHR(+)c.MLC-2v.EGFP.R(−)W(+) and pHR(+)c.Ub.EGFP.R(−)W(+) used for identifying cardiomyocytes and determining transduction efficiency, respectively. (*B*) To determine the total cell number in beating clusters, after dissociation cells were loaded with tetramethylrhodamine ethyl ester (TMRE; red) to visualize cell bodies. Myosin light chain-2v (MLC-2v)–enhanced green fluorescent protein (EGFP)–positive cells were counted by detecting green fluorescence, giving the number of ventricular myocytes. (*C*) Summarized data from five separate differentiation experiments show high percentage of hESC-derived cardiomyocytes. Ub = ubiquitin.

tection.[26] In hESC-derived cardiomyocytes, diazoxide, an opener of mitoK$_{ATP}$ channels,[27] decreased tetramethylrhodamine ethyl ester fluorescence intensity from baseline value of 99.5 ± 1.1% (n = 12) to 90.2 ± 5.4% of baseline (n = 12), indicating opening of mitoK$_{ATP}$ channels (fig. 4). Application of 0.5 mM isoflurane decreased tetramethylrhodamine ethyl ester fluorescence intensity from 99.6 ± 1.7% (n = 11) to 82.2 ± 9.8% of baseline (n = 11). This was attenuated in the presence of 5-hydroxydecanoate (5-HD), an inhibitor of mitoK$_{ATP}$ channel opening,[27] and the baseline fluorescence decreased from 99.7 ± 1.7% (n = 11) to 94.2 ± 2.4% of baseline (n = 11), indicating that mitochondrial depolarization by isoflurane is mediated, in part, by opening of mitoK$_{ATP}$ 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.

Isoflurane Enhances Production of ROS by hESC-derived Cardiomyocytes

Production of ROS, important signaling molecules in preconditioning of adult cardiomyocytes,[28,29] was monitored in hESC-derived cardiomyocytes. As seen in figure 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.

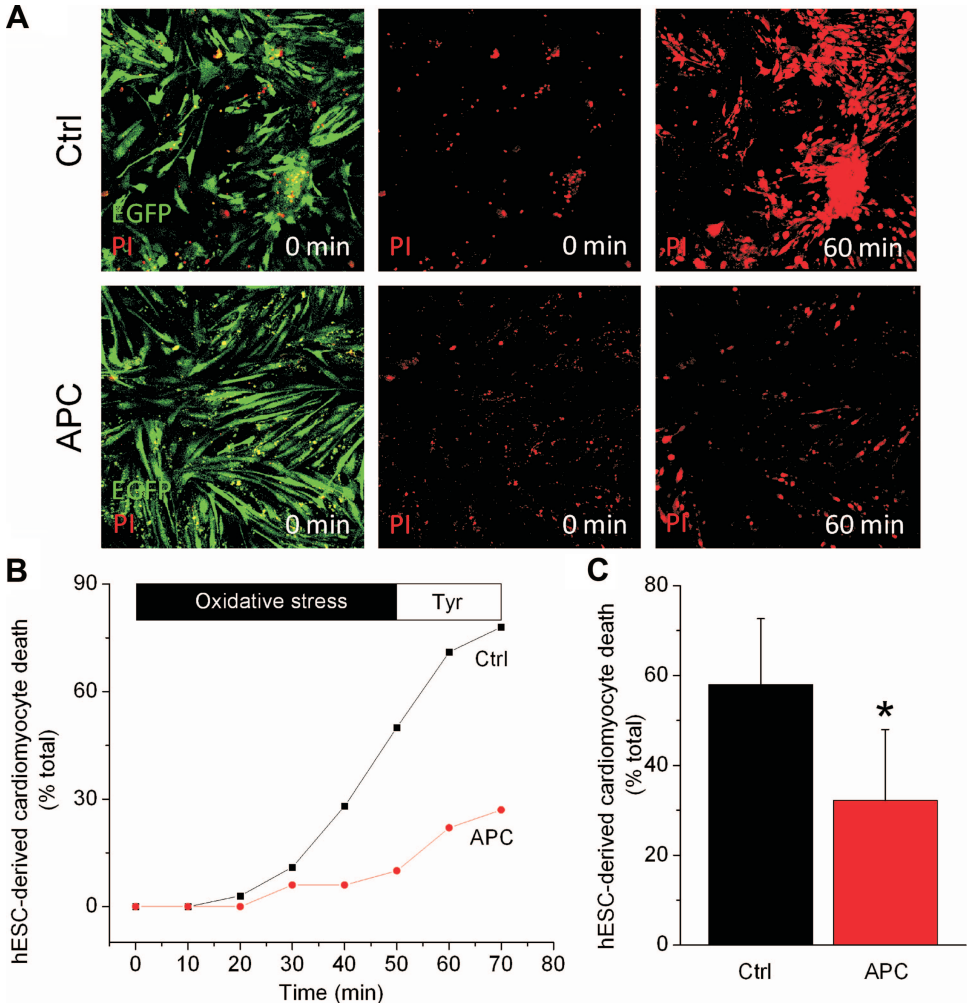

Fig. 3. Human embryonic stem cell (hESC)–derived cardiomyocytes are protected from oxidative stress by anesthetic-induced preconditioning (APC). (*A*) Myosin light chain-2v (MLC-2v)–enhanced green fluorescent protein-positive cells (*i.e.*, hESC-derived cardiomyocytes) were identified by green fluorescence using confocal microscopy. After exposure to oxidative stress and compared with control (Ctrl), APC decreased the number of hESC-derived cardiomyocytes that stained positive for red-fluorescent propidium iodide (PI), an indication of cell death. (*B*) The rate of increase in number of PI-positive cells, expressed as percentage of total number of hESC-derived cardiomyocytes, is attenuated in APC group compared with Ctrl. (*C*) Summarized values after the application of H_2O_2, after 10 min of perfusion with Tyrode solution. * $P < 0.05$ *versus* Ctrl.

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 $\Delta\Psi_m$ was monitored in hESC-derived cardiomyocytes exposed to oxidative stress (fig. 7).

Compared with control, mPTP inhibitor cyclosporine A (1 μM) increased the arbitrary mPTP opening time, 100.0 ± 24.8% (n = 11) *versus* 119.1 ± 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 ± 18.2%, n = 12; APC, 139.4 ± 40.4%, n = 16; and APC + 5-HD, 95.3 ± 11.6% of control, n = 16), confirming the role of mitoK$_{ATP}$ channel opening in signal mediation of APC. Preconditioning with H_2O_2 increased arbitrary mPTP opening time to 130.5 ± 31.2% (n = 12) compared with control (100.0 ± 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 ~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

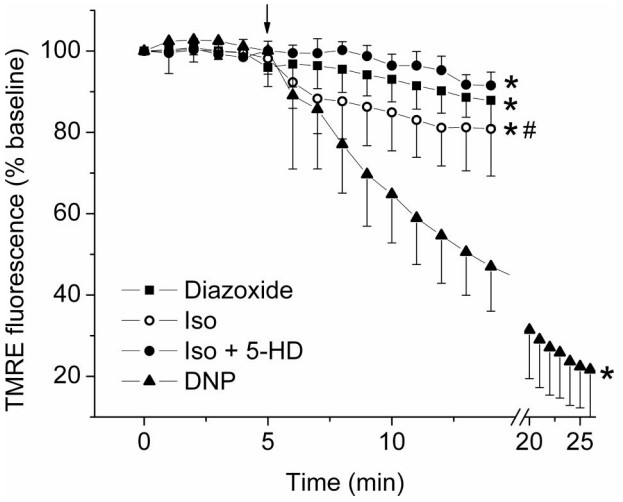

Fig. 4. Isoflurane opens mitoK$_{ATP}$ channels and depolarizes mitochondria in human embryonic stem cell (hESC)–derived cardiomyocytes. $\Delta\Psi_m$ was monitored in dissociated hESC-derived cardiomyocytes using tetramethylrhodamine ethyl ester (TMRE) fluorescence. The time point when drugs were added 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.

stress. These included characteristic and important mediators of cardioprotection: opening of mitoK$_{ATP}$ 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

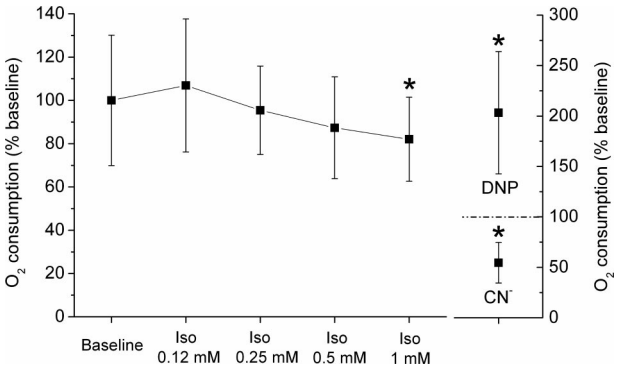

Fig. 5. Isoflurane attenuates beating cell cluster oxygen consumption. The rates of oxygen consumption in contracting cell clusters after the addition of incremental isoflurane (Iso) concentration were normalized to baseline values. At the higher concentrations, isoflurane attenuated oxygen consumption.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

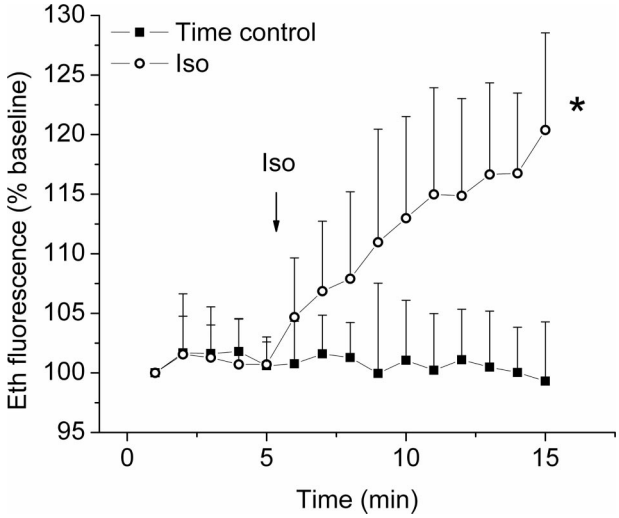

Fig. 6. Isoflurane enhances ROS production in human embryonic stem cell (hESC)–derived cardiomyocytes. Reactive oxygen species (ROS) production in hESC-derived cardiomyocytes was measured by detecting fluorescence intensity of ethidium (Eth). Compared with the time control, isoflurane (Iso) significantly 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.

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 α-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^{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]

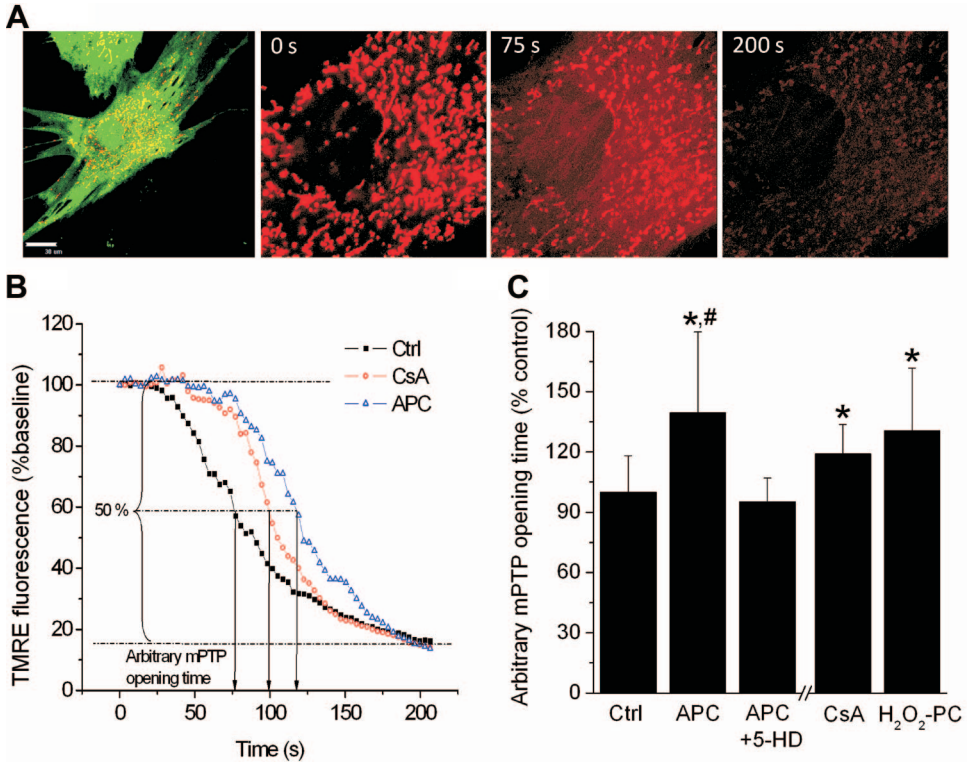

Fig. 7. Preconditioning delays mitochondrial permeability transition pore (mPTP) opening in human embryonic stem cell–derived cardiomyocytes. (*A*) mPTP opening was induced by photoexcitation-generated oxidative stress and detected by rapid dissipation of tetramethylrhodamine ethyl ester (TMRE) fluorescence. (*B*) Representative signal traces from control (Ctrl), cyclosporine A (CsA)–treated cells, and anesthetic-induced preconditioning (APC). Arbitrary mPTP opening time was determined as the time when TMRE fluorescence intensity decreased by half between initial and residual fluorescence intensity. (*C*) mPTP blocker CsA and APC increased arbitrary mPTP opening time, which was blocked in presence of 5-hydroxydecanoate (APC + 5-HD). Preconditioning with 40 μM H_2O_2 (H_2O_2-PC) also delayed mPTP opening. * $P < 0.05$ *versus* Ctrl; # $P < 0.05$ *versus* APC + 5-HD.

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 mesendoderm[38] 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 animal[13,14,26,28] 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.[25] 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$_{ATP}$ channels as crucial mediators of cardioprotection.[26,40] This effect was almost identical to isoflurane-induced opening of mitoK$_{ATP}$ channels demonstrated with the similar approach in our previous study using adult rat cardiomyocytes,[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.[28,41] Our previous study demonstrated that desflurane and sevoflurane induce similar extent of ROS production in adult rat cardiomyocytes.[28] The importance of ROS was confirmed here by showing that a low dose of H_2O_2 induces preconditioning and delays mPTP opening, which is in agreement with a study by Hanouz *et al.*[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$_{ATP}$ channels,[41] but it can also be induced by partial obstruction of the electron transport chain,[42] another effect of volatile anesthetics.[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$_{ATP}$ 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.[44] Cardioprotective strategies, including APC, were found to induce a delay in mPTP opening, and mPTP blockers may decrease infarct size by 30–50%.[45] During I/R, opening of mPTP dissipates $\Delta\Psi_m$, preventing oxidative phosphorylation, and initiates death pathways in the cell.[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,[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.[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.[3] However, Laflamme *et al.*[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.[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.[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 cardiac 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.

The authors thank Anna Stadnicka, Ph.D. (Associate Professor, Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin), and Wai-Meng Kwok, Ph.D. (Professor, Department of

Anesthesiology, Medical College of Wisconsin), for helpful scientific discussions; Aniko Szabo, Ph.D. (Associate Professor, Department of Population Health, Medical College of Wisconsin), for help with the statistical analyses; and Steve Contney, M.S. (Research Scientist, Department of Anesthesiology, Medical College of Wisconsin), and Sara Reszczynski (Technician, Department of Anesthesiology, Medical College of Wisconsin) for technical assistance.

References

1. Zaugg M, Schaub MC: Signaling and cellular mechanisms in cardiac protection by ischemic and pharmacological preconditioning. J Muscle Res Cell Motil 2003; 24:219–49
2. Stadnicka A, Marinovic J, Ljubkovic M, Bienengraeber MW, Bosnjak ZJ: Volatile anesthetic-induced cardiac preconditioning. J Anesth 2007; 21:212–9
3. van Laake LW, Hassink R, Doevendans PA, Mummery C: Heart repair and stem cells. J Physiol 2006; 577:467–78
4. Wobus AM, Boheler KR: Embryonic stem cells: Prospects for developmental biology and cell therapy. Physiol Rev 2005; 85:635–78
5. Caspi O, Itzhaki I, Kehat I, Gepstein A, Arbel G, Huber I, Satin J, Gepstein L: *In vitro* electrophysiological drug testing using human embryonic stem cell derived cardiomyocytes. Stem Cells Dev 2009; 18:161–72
6. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L: Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 2001; 108:407–14
7. Mummery C, Ward-van Oostwaard D, Doevendans P, Spijker R, van den Brink S, Hassink R, van der Heyden M, Opthof T, Pera M, de la Riviere AB, Passier R, Tertoolen L: Differentiation of human embryonic stem cells to cardiomyocytes: Role of coculture with visceral endoderm-like cells. Circulation 2003; 107:2733–40
8. Dolnikov K, Shilkrut M, Zeevi-Levin N, Gerecht-Nir S, Amit M, Danon A, Itskovitz-Eldor J, Binah O: Functional properties of human embryonic stem cell-derived cardiomyocytes: Intracellular Ca^{2+} handling and the role of sarcoplasmic reticulum in the contraction. Stem Cells 2006; 24:236–45
9. He JQ, Ma Y, Lee Y, Thomson JA, Kamp TJ: Human embryonic stem cells develop into multiple types of cardiac myocytes: Action potential characterization. Circ Res 2003; 93:32–9
10. Laflamme MA, Zbinden S, Epstein SE, Murry CE: Cell-based therapy for myocardial ischemia and infarction: Pathophysiological mechanisms. Annu Rev Pathol 2007; 2:307–39
11. Caspi O, Huber I, Kehat I, Habib M, Arbel G, Gepstein A, Yankelson L, Aronson D, Beyar R, Gepstein L: Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. J Am Coll Cardiol 2007; 50:1884–93
12. Laflamme MA, Chen KY, Naumova AV Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S, O'Sullivan C, Collins L, Chen Y, Minami E, Gill EA, Ueno S, Yuan C, Gold J, Murry CE: Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 2007; 25:1015–24
13. Pravdic D, Sedlic F, Mio Y, Vladic N, Bienengraeber M, Bosnjak ZJ: Anesthetic-induced preconditioning delays opening of mitochondrial permeability transition pore *via* protein Kinase C-epsilon-mediated pathway. ANESTHESIOLOGY 2009; 111:267–74
14. Raphael J, Abedat S, Rivo J, Meir K, Beeri R, Pugatsch T, Zuo Z, Gozal Y: Volatile anesthetic preconditioning attenuates myocardial apoptosis in rabbits after regional ischemia and reperfusion *via* Akt signaling and modulation of Bcl-2 family proteins. J Pharmacol Exp Ther 2006; 318: 186–94
15. Huber I, Itzhaki I, Caspi O, Arbel G, Tzukerman M, Gepstein A, Habib M, Yankelson L, Kehat I, Gepstein L: Identification and selection of cardiomyocytes during human embryonic stem cell differentiation. FASEB J 2007; 21: 2551–63
16. Henderson SA, Spencer M, Sen A, Kumar C, Siddiqui MA, Chien KR: Structure, organization, and expression of the rat cardiac myosin light chain-2 gene. Identification of a 250-base pair fragment which confers cardiac-specific expression. J Biol Chem 1989; 264:18142–8
17. Zhu H, Garcia AV, Ross RS, Evans SM, Chien KR: A conserved 28-base-pair element (HF-1) in the rat cardiac myosin light-chain-2 gene confers cardiac-specific and alpha-adrenergic-inducible expression in cultured neonatal rat myocardial cells. Mol Cell Biol 1991; 11:2273–81
18. Park F: Correction of bleeding diathesis without liver toxicity using arenaviral-pseudotyped HIV-1-based vectors in hemophilia A mice. Hum Gene Ther 2003; 14:1489–94
19. Park F, Sweeney WE, Jia G, Roman RJ, Avner ED: 20-HETE mediates proliferation of renal epithelial cells in polycystic kidney disease. J Am Soc Nephrol 2008; 19:1929–39
20. Vanden Hoek TL, Shao Z, Li C, Schumacker PT, Becker LB: Mitochondrial electron transport can become a significant source of oxidative injury in cardiomyocytes. J Mol Cell Cardiol 1997; 29:2441–50
21. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ: Reactive oxygen species (ROS)-induced ROS release: A new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. J Exp Med 2000; 192:1001–14
22. Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, Sollott SJ: Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J Clin Invest 2004; 113: 1535–49
23. Hüser J, Rechenmacher CE, Blatter LA: Imaging the permeability pore transition in single mitochondria. Biophys J 1998; 74:2129–37
24. Hüser J, Blatter LA: Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore. Biochem J 1999; 343:311–7
25. Mio Y, Bienengraeber MW, Marinovic J, Gutterman DD, Rakic M, Bosnjak ZJ, Stadnicka A: Age-related attenuation of isoflurane preconditioning in human atrial cardiomyocytes: Roles for mitochondrial respiration and sarcolemmal adenosine triphosphate-sensitive potassium channel activity. ANESTHESIOLOGY 2008; 108:612–20
26. Ljubkovic M, Mio Y, Marinovic J, Stadnicka A, Warltier DC, Bosnjak ZJ, Bienengraeber M: Isoflurane preconditioning uncouples mitochondria and protects against hypoxia-reoxygenation. Am J Physiol Cell Physiol 2007; 292: C1583–90
27. Kowaltowski AJ, Seetharaman S, Paucek P, Garlid KD: Bioenergetic consequences of opening the ATP-sensitive K(+) channel of heart mitochondria. Am J Physiol Heart Circ Physiol 2001; 280:H649–57
28. Sedlic F, Pravdic D, Ljubkovic M, Marinovic J, Stadnicka A, Bosnjak ZJ: Differences in production of reactive oxygen species and mitochondrial uncoupling as events in the preconditioning signaling cascade between desflurane and sevoflurane. Anesth Analg 2009; 109:405–11
29. Hanouz JL, Zhu L, Lemoine S, Durand C, Lepage O, Massetti M, Khayat A, Plaud B, Gérard JL: Reactive oxygen species mediate sevoflurane- and desflurane-induced preconditioning in isolated human right atria *in vitro*. Anesth Analg 2007; 105:1534–9
30. Snir M, Kehat I, Gepstein A, Coleman R, Itskovitz-Eldor J, Livne E, Gepstein L: Assessment of the ultrastructural and

proliferative properties of human embryonic stem cell-derived cardiomyocytes. Am J Physiol Heart Circ Physiol 2003; 285:H2355–63

31. Kehat I, Gepstein A, Spira A, Itskovitz-Eldor J, Gepstein L: High-resolution electrophysiological assessment of human embryonic stem cell-derived cardiomyocytes: A novel *in vitro* model for the study of conduction. Circ Res 2002; 91:659–61
32. Zhu WZ, Santana LF, Laflamme MA: Local control of excitation-contraction coupling in human embryonic stem cell-derived cardiomyocytes. PLoS One 2009; 4:e5407.
33. Brito-Martins M, Harding SE, Ali NN: Beta(1)- and beta(2)-adrenoceptor responses in cardiomyocytes derived from human embryonic stem cells: Comparison with failing and non-failing adult human heart. Br J Pharmacol 2008; 153: 751–9
34. Kehat I, Khimovich L, Caspi O, Gepstein A, Shofti R, Arbel G, Huber I, Satin J, Itskovitz-Eldor J, Gepstein L: Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. Nat Biotechnol 2004; 22: 1282–9
35. Sartiani L, Bettiol E, Stillitano F, Mugelli A, Cerbai E, Jaconi ME: Developmental changes in cardiomyocytes differentiated from human embryonic stem cells: A molecular and electrophysiological approach. Stem Cells 2007; 25:1136–44
36. Lough J, Sugi Y: Endoderm and heart development. Dev Dyn 2000; 217:327–42
37. Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM, Field LJ, Keller GM: Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature 2008; 453:524–8
38. Liu H, Dalton S, Xu Y: Transcriptional profiling of definitive endoderm derived from human embryonic stem cells. Comput Syst Bioinformatics Conf 2007; 6:79–82
39. Vanden Hoek TL, Qin Y, Wojcik K, Li CQ, Shao ZH, Anderson T, Becker LB, Hamann KJ: Reperfusion, not simulated ischemia, initiates intrinsic apoptosis injury in chick cardiomyocytes. Am J Physiol Heart Circ Physiol 2003; 284:H141–50
40. Marinovic J, Bosnjak ZJ, Stadnicka A: Distinct roles for sarcolemmal and mitochondrial adenosine triphosphate-sensitive potassium channels in isoflurane-induced protection against oxidative stress. ANESTHESIOLOGY 2006; 105: 98–104
41. Tanaka K, Weihrauch D, Ludwig LM, Kersten JR, Pagel PS, Warltier DC: Mitochondrial adenosine triphosphate-regulated potassium channel opening acts as a trigger for isoflurane-induced preconditioning by generating reactive oxygen species. ANESTHESIOLOGY 2003; 98:935–43
42. Batandier C, Guigas B, Detaille D, El-Mir MY, Fontaine E, Rigoulet M, Leverve XM: The ROS production induced by a reverse-electron flux at respiratory-chain complex 1 is hampered by metformin. J Bioenerg Biomembr 2006; 38: 33–42
43. Hanley PJ, Ray J, Brandt U, Daut J: Halothane, isoflurane and sevoflurane inhibit NADH:ubiquinone oxidoreductase (complex I) of cardiac mitochondria. J Physiol 2002; 544: 687–93
44. Murphy E, Steenbergen C: Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. Physiol Rev 2008; 88:581–609
45. Argaud L, Gateau-Roesch O, Muntean D, Chalabreysse L, Loufouat J, Robert D, Ovize M: Specific inhibition of the mitochondrial permeability transition prevents lethal reperfusion injury. J Mol Cell Cardiol 2005; 38:367–74
46. Fleisher LA, Beckman JA, Brown KA, Calkins H, Chaikof E, Fleischmann KE, Freeman WK, Froehlich JB, Kasper EK, Kersten JR, Riegel B, Robb JF, Smith SC Jr, Jacobs AK, Adams CD, Anderson JL, Antman EM, Buller CE, Creager MA, Ettinger SM, Faxon DP, Fuster V, Halperin JL, Hiratzka LF, Hunt SA, Lytle BW, Nishimura R, Ornato JP, Page RL, Tarkington LG, Yancy CW, American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines on Perioperative Cardiovascular Evaluation for Noncardiac Surgery), American Society of Echocardiography, American Society of Nuclear Cardiology, Heart Rhythm Society, Society of Cardiovascular Anesthesiologists, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society for Vascular Surgery: ACC/AHA 2007 guidelines on perioperative cardiovascular evaluation and care for noncardiac surgery: A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines on Perioperative Cardiovascular Evaluation for Noncardiac Surgery): Developed in collaboration with the American Society of Echocardiography, American Society of Nuclear Cardiology, Heart Rhythm Society, Society of Cardiovascular Anesthesiologists, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, and Society for Vascular Surgery. Circulation 2007; 116:e418–99