

Marked Hyperglycemia Attenuates Anesthetic Preconditioning in Human-induced Pluripotent Stem Cell-derived Cardiomyocytes

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ABSTRACT

Introduction: Anesthetic preconditioning protects cardiomyocytes from oxidative stress-induced injury, but it is ineffective in patients with diabetes mellitus. To address the role of hyperglycemia in the inability of diabetic individuals to be preconditioned, we used human cardiomyocytes differentiated from induced pluripotent stem cells generated from patients with or without type 2 diabetes mellitus (DM-iPSC- and N-iPSC-CMs, respectively) to investigate the efficacy of preconditioning in varying glucose conditions (5, 11, and 25 mM).

Methods: Induced pluripotent stem cells were induced to generate cardiomyocytes by directed differentiation. For subsequent studies, cardiomyocytes were identified by genetic labeling with enhanced green fluorescent protein driven by a cardiac-specific promoter. Cell viability was analyzed by lactate dehydrogenase assay. Confocal microscopy was utilized to measure opening of the mitochondrial permeability transition pore and the mitochondrial adenosine 5'-triphosphate-sensitive potassium channels.

Results: Isoflurane (0.5 mM) preconditioning protected N-iPSC- and DM-iPSC-CMs from oxidative stress-induced lactate dehydrogenase release and mitochondrial permeability transition pore opening in 5 mM and 11 mM glucose. Isoflurane triggered mitochondrial adenosine-5'-triphosphate-sensi-

What We Already Know about This Topic

- Previous studies have demonstrated that anesthetic preconditioning protects cardiomyocytes from oxidative stress-induced injury, but is ineffective in patients with diabetes mellitus.
- Using human cardiomyocytes induced from pluripotent stem cells generated from patients with and without diabetes mellitus, the present study investigated the effect of anesthetic preconditioning in the presence of varying glucose concentrations.

What This Article Tells Us That Is New

- High glucose had detrimental effects on anesthetic preconditioning in cardiomyocytes derived from human pluripotent stem cells. Adenosine 5'-triphosphate-sensitive potassium channels are differentially effected by anesthetic preconditioning in human cardiomyocytes induced from pluripotent stem cells generated from patients with and without diabetes mellitus.

tive potassium channel opening in N-iPSC-CMs in 5 mM and 11 mM glucose and in DM-iPSC-CMs in 5 mM glucose; 25 mM glucose disrupted anesthetic preconditioning-mediated protection in DM-iPSC- and N-iPSC-CMs.

Conclusions: The opening of mitochondrial adenosine 5'-triphosphate-sensitive potassium channels are disrupted in DM-iPSC-CMs in 11 mM and 25 mM glucose and in N-iPSC-CMs in 25 mM glucose. Cardiomyocytes derived from healthy donors and patients with a specific disease, such as diabetes in this study, open possibilities in studying genotype- and phenotype-related pathologies in a human-relevant model.

THE World Health Organization estimates that diabetes mellitus affects nearly 200 million people worldwide, with 90% of them having type 2. The risk of cardiovascular disease increases dramatically for those diagnosed with diabetes.¹ Anesthetic preconditioning (APC) can reduce myocardial injury following an ischemia and reperfusion injury.²⁻⁴ However, diabetes or hyperglycemia in the nondiabetic myocardium have been shown to abolish APC in animal models.⁵⁻⁶

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Tanaka *et al.* discovered that streptozotocin-induced diabetic canines could not be efficiently protected from myocardial infarction with isoflurane preconditioning.⁵ They also observed that infarct size was directly related to blood glucose levels. Kehl *et al.* observed that moderate hyperglycemia attenuated the protective effects of 0.5 minimum alveolar concentration (MAC) isoflurane, but not 1.0 MAC isoflurane in canines. However, severe hyperglycemia attenuated the protective effects of 0.5 and 1.0 MAC isoflurane.⁶ These animal studies provided great insight into APC in diabetes mellitus; however, they could not address a genetic component of this phenomenon because of the nature of inducing diabetes in this experimental model. Moreover, it is unclear whether we could extend these mechanisms delineated in animal models to human myocardium.

Induced pluripotent stem cells (iPSCs) used in this study were generated from human skin fibroblasts by introducing pluripotency factors. Reprogramming the cells allows them to obtain characteristics of pluripotent stem cells.⁷ As such, iPSCs have the capability to differentiate into cardiomyocytes^{8–9} while maintaining their genotype. Our laboratory has previously shown that cardiomyocytes derived from human embryonic stem cells (hESC-CMs) exhibit competent responses to APC compared with various animal models and human myocardium.¹⁰ The generation of iPSCs from patient-specific-derived fibroblasts enables us to study human disease mechanisms that were nearly impossible to be investigated in the past. In this study, iPSCs derived from nondiabetic and type 2 diabetic individuals were used to generate cardiomyocytes (DM-iPSC-CMs and N-iPSC-CMs, respectively). Others have shown that differentiated cardiomyocytes behave very similar to adult cardiomyocytes on multiple levels, including exhibition of spontaneous and rhythmic contractions, and expression of cardiac specific markers.^{9–11} Thus, DM-iPSC-CMs and N-iPSC-CMs provide us with a unique *in vitro* model to investigate environmental factors, such as high glucose, that affect APC in diabetic myocardium. Because of the lack of experimentally available human myocardium, reliable type 2 diabetic animal models, and the disconnect between animal and human studies, our model has the unique ability to investigate a human disease and its ability to attenuate APC-induced cardioprotection for the first time. Our study investigates the detrimental effects of high glucose on APC-mediated cardiac protection in cardiomyocytes derived from a nondiabetic and a type 2 diabetic individual.

Materials and Methods

Human-induced Pluripotent Stem Cell Culture

Dermal fibroblasts were isolated from one type 2 diabetic and one nondiabetic patient, and these patient-specific fibroblasts were reprogrammed into iPSCs^{12–13} (DM-iPSCs and N-iPSCs, respectively). Both lines were gifts from Dr. George Daley, M.D., Ph.D. (Professor, Director, Stem Cell

Transplantation Program, Howard Hughes Medical Institute and Children's Hospital, Boston, MA) and Dr. Stephen Duncan, Ph.D. (Professor, Vice-Chairman Cell Biology, Neurobiology, and Anatomy, Director, MCW Program in Regenerative Medicine and Stem Cell Biology, Medical College of Wisconsin, Milwaukee, WI), respectively, and iPSC lines were cultured according to our previously described protocol.¹⁰ Mouse embryonic fibroblasts were inactivated with mitomycin C (Sigma-Aldrich, St. Louis, MO) and plated in Dulbecco Modified Eagle Medium (Millipore Bioscience Research Reagents, Temecula, CA) complemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% nonessential amino acids (Millipore Bioscience Research Reagents). iPSCs were maintained on inactivated mouse embryonic fibroblasts in hypoxic conditions (4% O₂, 5% CO₂) with iPSC culture medium consisting of Dulbecco Modified Eagle Medium/Ham F12 medium (Invitrogen) supplemented with 20% knockout serum (Invitrogen), 1% nonessential amino acids (Invitrogen), 1% penicillin-streptomycin (Invitrogen), L-glutamine (Millipore), β -mercaptoethanol (Sigma-Aldrich), and 4 ng/ml human recombinant basic fibroblast growth factor (Invitrogen). iPSC colonies were passaged every 6–8 days, and iPSC passages 45–65 were used in the subsequent study.

Cardiac Differentiation of N-iPSCs and DM-iPSCs

Colonies of iPSCs were mechanically dissociated into small aggregates and plated onto dishes precoated with reduced growth factor matrigel (BD Biosciences, San Jose, CA), and cultured under hypoxic (4% O₂, 5% CO₂) conditions. Cells were supplemented with iPSC medium conditioned by inactivated mouse embryonic fibroblasts for 7 days. Next, cells were grown in Roswell Park Memorial Institute (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) for 5 days. Finally, cells were plated in normoxic conditions (20% O₂) with RPMI/B27 and matured for 3 months before use in subsequent experiments.

Microdissection and Single Cell Dissociation

Three months following differentiation, cell clusters were mechanically dissociated under a dissecting microscope (SMZ1000; Nikon, Tokyo, Japan) using a 27-gauge needle and enzymatically dispersed in 0.05% trypsin-EDTA (Invitrogen) for 5 min. Trypsin was inactivated by Dulbecco Modified Eagle Medium containing 10% fetal bovine serum and individual cells were plated onto matrigel-coated coverslips at low density.

Immunofluorescence

Cells cultured on matrigel-coated glass coverslips were fixed with 1% paraformaldehyde for 30 min and then were washed three times with phosphate-buffered saline. Cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) and

blocked with 10% donkey serum for 30 min. Cells were incubated with primary antibodies for sarcomeric α -actinin (1:100 dilution; Sigma-Aldrich) or antiscardiac-specific troponin T (1:100; Thermo Fisher Scientific, Waltham, MA) for 1 h at 37°C. After three washes, cells were incubated with secondary antibody Alexa Fluor 594 (1:1,000, Invitrogen) for 1 h at room temperature. Nuclei were stained with TO-PRO-3 (1:1,000; Invitrogen). Coverslips were then mounted onto slides and images were acquired with a laser-scanning confocal microscope (Nikon Eclipse TE2000-U).

Genetic Marking of iPSC-derived Cardiomyocytes with a Lentiviral Vector

Genetic marking of iPSC-CMs was similarly conducted in our previous study.¹⁰ In brief, 3 days after dissociation, cardiomyocytes were transduced with a lentiviral vector encoding human myosin light chain-2v (MLC-2v)-driven enhanced green fluorescent protein (eGFP; multiplicity of infection, 2.2×10^4). The MLC-2v promoter is specific for ventricular myocytes.^{11,14–15} The MLC-2v-eGFP cassette (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, Israel¹⁶) was subcloned into lentiviral transfer plasmid pHR(+).c.Ub.MCSoligo.R(-)W(+). Lentiviral vector assembly and titering were performed as previously described.^{17–18}

Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) activity was assessed for determination of cell viability. LDH was measured using a colorimetric cytotoxicity assay kit according to the manufacturer's directions as an indicator of cell membrane damage (Roche Diagnostics Corporation, Indianapolis, IN). iPSC-CMs were plated at 1×10^4 cells/well in 96-well plates and cultured in 100 μ l of RPMI/B27 medium. The medium was exchanged every 2 days for 1 week. Cardiomyocytes were treated with either a control (RPMI/B27) medium or preconditioned with 0.5 mM isoflurane for 10 min and then replaced with control medium. Both control and experimental groups had a 2 h exposure of 10 mM H₂O₂ (Calbiochem, LA Jolla, CA) to induce oxidative stress. Experiments were conducted in RPMI/B27 media containing either 5 mM glucose with 20 mM mannitol, an osmotic control, 11 mM glucose with 14 mM mannitol, or 25 mM glucose throughout the experiment. Absorbance data were read in the assay plate spectrophotometrically at 490 nm (reference wavelength, 600 nm) with a microplate reader (BioTek Instruments, Inc., Winooski, VT). Only control and experimental groups that had an oxidative stress-induced LDH release were analyzed. After subtracting background absorbance of cell-free medium, total LDH released into medium was calculated.

Laser-scanning Confocal Microscopy for Mitochondrial Membrane Potential and Mitochondrial Permeability Transition Pore (mPTP) Opening Assays

Five days following lentiviral vector transduction, imaging was performed using a confocal microscope (Eclipse TE2000-U; Nikon) and data were analyzed with Image J software (National Institutes of Health, Bethesda, MD). Living iPSC-CMs were identified by detecting green fluorescent cells, indicating MLC-2v-driven eGFP expression. Experiments were conducted in either 5 mM glucose, 11 mM glucose, or 25 mM glucose. Tetramethylrhodamine ethyl ester (TMRE; 30 nM, Invitrogen) was used to detect mitochondrial membrane potential ($\Delta\Psi_m$) in iPSC-CMs for the purpose of detecting opening of mitochondrial adenosine 5'-triphosphate-sensitive potassium (mitoK_{ATP}) channels. Opening of mitoK_{ATP} channels induces a depolarization, observed as a loss of TMRE fluorescence. iPSC-CMs were exposed to either isoflurane (0.5 mM), diazoxide (100 mM; Sigma-Aldrich), a mitoK_{ATP} activator, or isoflurane plus 5-hydroxydecanoate (5-HD; 500 μ M; Sigma-Aldrich), a mitoK_{ATP} inhibitor, to determine the activity of mitoK_{ATP} channels. Data are normalized to baseline (100%). Baseline fluorescence intensity was recorded for five frames (1 frame/min) and then treatment was added and fluorescence intensity was recorded for 10 frames (1 frame/min). For statistical analysis, the average values of the baseline measurements were compared with the average values of the last five frames following treatment to determine changes in TMRE fluorescence from baseline. Rarely, cardiomyocytes would shift during the experimental protocol and were not included in the analysis. Opening of the mPTP was assessed as described previously in our laboratory,¹⁹ a method based on mPTP induction by photoexcitation-generated oxidative stress.^{20–23} The mPTP opening was detected by rapid dissipation of $\Delta\Psi_m$, observed as a loss of TMRE fluorescence, which is sensitive to mPTP opening inhibition.¹⁹

Application of Anesthetic Preconditioning

Appropriate volumes of isoflurane stock solution (Baxter, Deerfield, IL) were sonicated into media to achieve 0.5 mM (approximately 1 MAC) concentration, as done in previous studies.^{10,24} Cardiomyocytes were anesthetically preconditioned with culture media containing 0.5 mM isoflurane for 10 min followed by a washout with culture media in LDH and mPTP experiments. Culture medium containing 0.5 mM isoflurane was placed onto cardiomyocytes to trigger mitoK_{ATP} opening. At the end of each experiment, isoflurane concentration was analyzed by gas chromatography.

Statistical Analysis

Data are presented as mean \pm SD. Each experimental group consisted of iPSC-derived CMs from at least three separate differentiations; n indicates the number of independent experiments. For the statistical analyses, SigmaStat 3.0 software

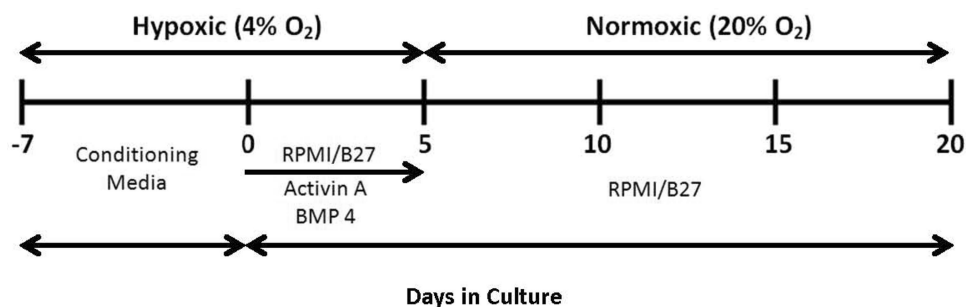


Fig. 1. Experimental scheme for cardiomyocyte differentiation of induced pluripotent stem cells (iPSCs). Nondiabetic and type 2 diabetic patient-derived pluripotent stem cells (N-iPSCs and DM-iPSCs, respectively) were expanded for 7 days with mouse fibroblast-conditioned medium on matrigel-coated dishes in hypoxic conditions (4% O₂, 5% CO₂). The addition of activin-A and bone morphogenetic protein-4 for 5 days induced cardiac differentiation. Cells were then placed into normoxic conditions and growth factors were withdrawn and supplemented with a Roswell Park Memorial Institute (RPMI)/B27 defined medium. Cardiomyocytes were allowed to mature to 3 months following differentiation before subsequent studies were conducted. BMP 4 = bone morphogenetic protein-4; RPMI = Roswell Park Memorial Institute.

(Systat Software, Inc., San Jose, CA) was used. Statistical comparisons were performed using one-way anova with Holm–Sidak correction for multiple testing over all comparisons within each cell line or unpaired Student *t* test where appropriate. *P* < 0.05 was considered significant.

Results

Differentiation and Characterization of iPSC-derived Cardiomyocytes

Cardiomyocytes differentiated from both N-iPSCs and DM-iPSCs were observed contracting in culture dishes approximately 14 days following treatment with activin-A and bone morphogenetic protein-4 (fig. 1). Contracting N-iPSC-CMs and DM-iPSC-CMs are shown in Supplemental Digital Content 1 and 2, respectively (see video, Supplemental Digital Content 1, <http://links.lww.com/ALN/A867>, and see video, Supplemental Digital Content 2, <http://links.lww.com/ALN/A868>). Immunostaining verified that dissociated cardiomyocytes from both N-iPSC and DM-iPSC populations had striation-like patterns confirmed by both cardiac-specific troponin T (figs. 2A, 2B) and sarcomeric α -actinin staining (figs. 2C, 2D).

iPSC-derived Cardiomyocytes Express eGFP Under the Control of Cardiac-specific Promoter MLC-2v

To properly identify living iPSC-CMs, determine differentiation efficiency, and ensure that subsequent confocal microscopy experiments were conducted on cardiomyocytes, we genetically labeled cells with a lentiviral vector that expressed eGFP under the transcriptional control of cardiac-specific promoter, MLC-2v (fig. 3). The differentiation efficiency was determined to be high in N-iPSC- and DM-iPSC-CMs, 67 ± 4%, 71 ± 4%, respectively (figs. 3B–D; n = 6/group). eGFP-positive cardiomyocytes were selected for subsequent confocal microscopy experiments.

APC Protects Both N-iPSC- and DM-iPSC-derived Cardiomyocytes in 5 mM and 11 mM but Not in 25 mM Glucose against Oxidative Stress

To determine whether APC could protect cardiomyocytes derived from both N-iPSCs and DM-iPSCs from oxidative stress-induced damage, we exposed control and anesthetic preconditioned cardiomyocytes to 10 mM H₂O₂ for 2 h in 5 mM, 11 mM, or 25 mM glucose environment. N-iPSC-

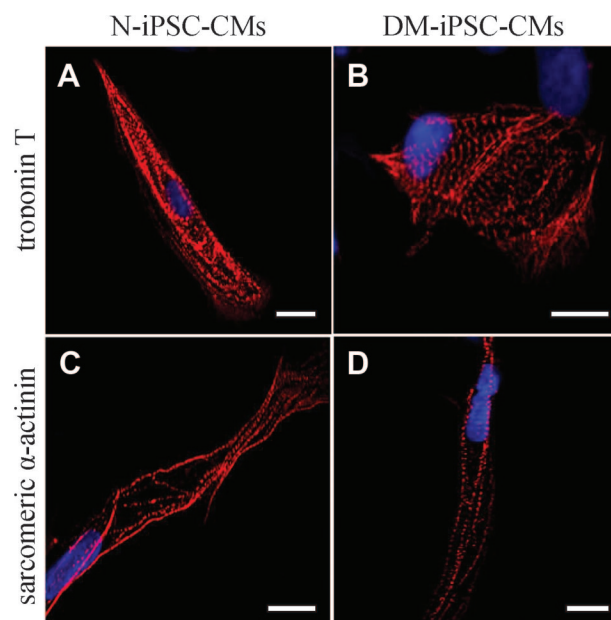


Fig. 2. Immunolabeling of nondiabetic and type 2 diabetic induced pluripotent stem cell-derived cardiomyocytes. Confocal images of dissociated cells stained for sarcomeric proteins: cardiac-specific troponin T (A, B) and sarcomeric α -actinin (C, D). The majority of positively labeled cells contained striation-like patterns with no differences between the cardiomyocytes. Scale bar = 10 μ m. DM-iPSC-CMs = type 2 diabetic induced pluripotent stem cell-derived cardiomyocytes; N-iPSC-CMs = nondiabetic induced pluripotent stem cell-derived cardiomyocytes.

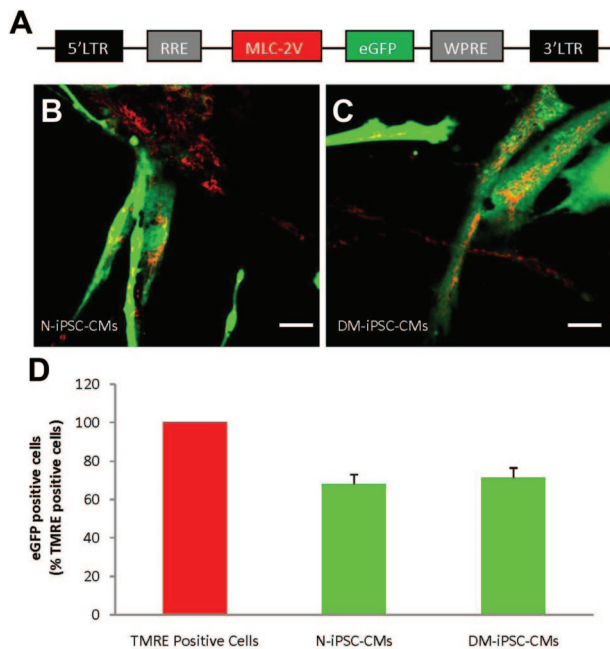


Fig. 3. Labeling of nondiabetic and type 2 diabetic induced pluripotent stem cell-derived cardiomyocytes (N-iPSC- and DM-iPSC-CMs, respectively) using a lentiviral vector. Schematic representation of lentiviral vector pHR(+)-C.mlc-2v-egfp.r(-)w(+) used for labeling cardiomyocytes (A). To determine total cell number, cells were loaded with mitochondrial marker tetramethylrhodamine ethyl ester (red); myosin light chain-2v-enhanced green fluorescent protein-positive cells were counted by detecting green fluorescence, giving the number of cardiomyocytes derived from each cell line (B, C, respectively). Summarized data from six separate differentiations, exhibiting a high percentage of induced pluripotent stem cell-derived cardiomyocytes (D). Scale bar = 40 μ M. DM-iPSC-CMs = type 2 diabetic induced pluripotent stem cell-derived cardiomyocytes; eGFP = enhanced green fluorescent protein; MLC-2v = myosin light chain-2v; N-iPSC-CMs = nondiabetic induced pluripotent stem cell-derived cardiomyocytes; TMRE = tetramethylrhodamine ethyl ester.

CMs that were preconditioned with isoflurane before H_2O_2 exposure had a significantly lower LDH release in 5 mM glucose compared with stress control, $128 \pm 4\%$ versus $152 \pm 4\%$, respectively (fig. 4A; $n = 7$ /group) and in 11 mM glucose compared with stress control, $131 \pm 6\%$ versus $151 \pm 6\%$, respectively (fig. 4A; $n = 8$ /group). APC did not reduce LDH release in N-iPSC-CMs in 25 mM glucose compared with the stress control ($151 \pm 9\%$ vs. $150 \pm 7\%$, respectively; fig. 4A; $n = 7$ /group). DM-iPSC-CMs also had a reduction in LDH release when preconditioned with isoflurane compared with the stress control in 5 mM glucose ($119 \pm 3\%$ vs. $148 \pm 3\%$, respectively; fig. 4B; $n = 7$ /group) and in 11 mM glucose ($124 \pm 4\%$ vs. $146 \pm 3\%$, respectively; fig. 4B; $n = 8$ /group). However, the APC-induced reduction in LDH release in DM-iPSC-CMs was lost in the presence of 25 mM glucose (fig. 4B; $n = 7$ /group).

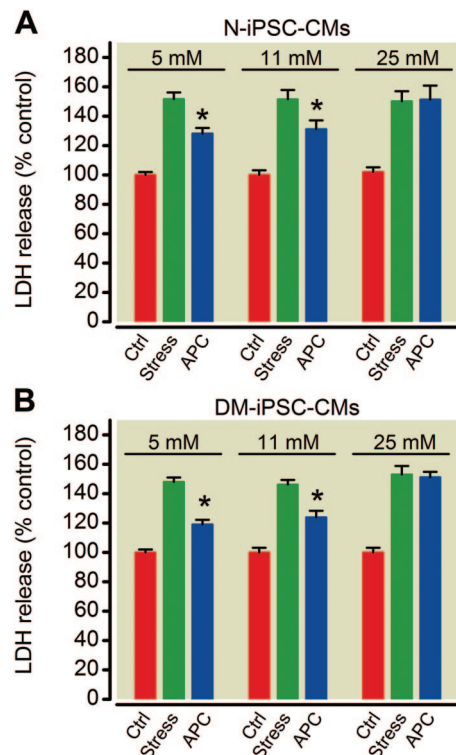


Fig. 4. Anesthetic preconditioning protects nondiabetic and type 2 diabetic induced pluripotent stem cell-derived cardiomyocytes (N-iPSC- and DM-iPSC-CMs, respectively) in 5 mM and 11 mM glucose from oxidative stress. H_2O_2 -induced oxidative stress increased lactate dehydrogenase release from N-iPSC- (A) and DM-iPSC-CMs (B) in a 5 mM, 11 mM, and 25 mM glucose environment. In both N-iPSC- and DM-iPSC-derived cardiomyocytes, anesthetic preconditioning reduced lactate dehydrogenase release in 5 mM and 11 mM glucose, but not in 25 mM glucose. * $P < 0.05$ versus stress. APC = anesthetic preconditioning; Ctrl = control; LDH = lactate dehydrogenase; N-iPSC-CMs = nondiabetic induced pluripotent stem cell-derived cardiomyocytes (DM-iPSC-CMs).

Preconditioning with Isoflurane Delays Opening of the mPTP in Both N-iPSC- and DM-iPSC-derived Cardiomyocytes in 5 mM and 11 mM but Not in 25 mM Glucose

APC delays opening of the mPTP and thus reduces cell death in ischemia-reperfusion injury.^{10,19,25} Opening of the mPTP causes a rapid collapse in $\Delta\Psi_m$, which can be detected following rapid loss of TMRE fluorescence intensity in iPSC-CMs (fig. 5A). APC increased the mPTP opening time in N-iPSC-CMs in 5 mM glucose compared with control, $139 \pm 6\%$ versus $100 \pm 5\%$ (fig. 5B; $n = 22$ cells/group). APC also increased mPTP opening time in DM-iPSC-CMs in 5 mM glucose compared with control, $128 \pm 4\%$ versus $100 \pm 5\%$ (fig. 5C; $n = 25$ cells/group). In addition, APC increased mPTP opening time in N-iPSC-CMs in 11 mM glucose, $131 \pm 7\%$ versus $100 \pm 5\%$ (fig. 5B; $n = 32$), and in DM-iPSC-CMs in 11 mM glucose, $122 \pm 9\%$ versus $100 \pm 5\%$, respectively (figs. 5B and 5C; $n = 30$ cells/group). The APC-induced delay in mPTP opening was lost

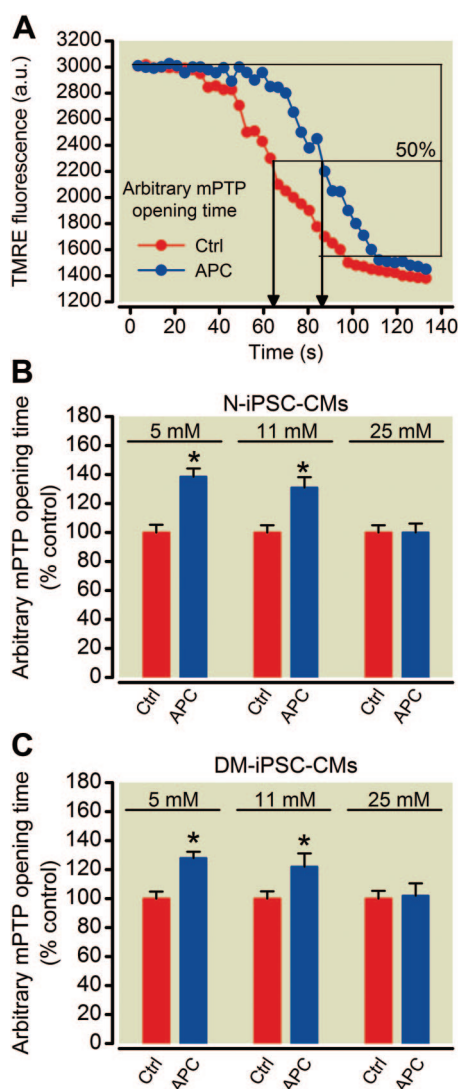


Fig. 5. Preconditioning delays mitochondrial permeability transition pore (mPTP) opening in nondiabetic and type 2 diabetic induced pluripotent stem cell-derived cardiomyocytes (N-iPSC- and DM-iPSC-CMs, respectively) in a 5 mM and 11 mM glucose environment. mPTP opening was induced by photoexcitation-generated oxidative stress and detected by rapid dissipation of tetramethylrhodamine ethyl ester fluorescence. Representative signal traces from control and anesthetic preconditioning (A). Arbitrary mPTP opening time was determined as the time when tetramethylrhodamine ethyl ester fluorescence intensity decreased by half between initial and residual fluorescence intensity. Anesthetic preconditioning delayed mPTP opening in N-iPSC-CMs in 5 mM and 11 mM glucose environments (B). Anesthetic preconditioning was not able to delay mPTP opening in 25 mM glucose; it delayed mPTP opening in DM-iPSC-CMs in 5 mM and 11 mM glucose, but not 25 mM glucose (C). * $P < 0.05$ versus control. APC = anesthetic preconditioning; Ctrl = control; DM-iPSC-CMs = type 2 diabetic induced pluripotent stem cell-derived cardiomyocytes; mPTP = mitochondrial permeability transition pore; N-iPSC-CMs = nondiabetic induced pluripotent stem cell-derived cardiomyocytes; TMRE = tetramethylrhodamine ethyl ester.

in N-iPSC-CMs in 25 mM glucose, $100 \pm 6\%$ versus $100 \pm 5\%$ (fig. 5B; $n = 25$ cells/group), and in DM-iPSC-CMs when the cells were exposed to 25 mM glucose, $102 \pm 9\%$ versus $100 \pm 6\%$ (fig. 5C; $n = 24$ cells/group).

Isoflurane Triggers Opening of the mitoK_{ATP} Channels and Depolarizes Mitochondria in iPSC-derived Cardiomyocytes

Isoflurane induced opening of mitoK_{ATP} channels, causing a mild mitochondrial depolarization, which is observed as a partial loss of TMRE fluorescence intensity; the addition of 5-HD, a mitoK_{ATP} inhibitor, attenuated a portion of this depolarization (fig. 6A). Diazoxide, a mitoK_{ATP} opener, triggered opening of the mitoK_{ATP} channels and a subsequent loss of $\Delta\Psi_m$ in N-iPSC-CMs in 5 mM ($n = 56$ cells/group), 11 mM ($n = 60$ cells/group), and 25 mM glucose ($n = 63$ cells/group) compared with baseline (fig. 6B). Diazoxide also opened mitoK_{ATP} channels in DM-iPSC-CMs in 5 mM ($n = 62$ cells/group), 11 mM ($n = 65$ cells/group), and 25 mM glucose ($n = 68$ cells/group) compared with baseline (fig. 6C). The addition of isoflurane (0.5 mM) decreased TMRE fluorescence, indicating a mitochondrial depolarization in N-iPSC-CMs in 5 mM glucose (decrease by $34 \pm 5\%$), which was attenuated in the presence of 500 mM 5-HD (decrease by $18 \pm 5\%$) (fig. 6B; $n = 56$ cells/group). Isoflurane induced a mitochondrial depolarization in DM-iPSC-CMs in 5 mM glucose (decrease by $21 \pm 4\%$), which was attenuated in the presence of 500 mM 5-HD (decrease by $9 \pm 4\%$) (fig. 6C; $n = 62$ cells/group). This indicated opening of mitoK_{ATP} channels by isoflurane. However, N-iPSC-CMs in 25 mM glucose had the same extent of decrease in TMRE fluorescence between isoflurane and isoflurane + 5-HD treatment, indicating the inability of isoflurane to open mitoK_{ATP} channels (fig. 6B; $n = 63$ cells/group). Isoflurane did not open mitoK_{ATP} channels to a greater extent than when isoflurane + 5-HD was present in DM-iPSC-CMs in 11 mM and 25 mM glucose (fig. 6C; $n = 68$ cells/group). The ability of isoflurane-induced mitochondrial depolarization in the presence of mitoK_{ATP} channel blocker suggests that isoflurane decreases $\Delta\Psi_m$ in part by other mechanisms.²⁶

Discussion

In the present study, we investigated the effects of APC on iPSC-derived cardiomyocytes from a healthy individual and a type 2 diabetic patient in varying glucose conditions. Our major findings are summarized as follows. 1) iPSCs reprogrammed from nondiabetic and type 2 diabetic human fibroblasts could be differentiated into cardiomyocytes with directed differentiation, as supported by visualization of contracting cell regions; expression of cardiac-specific, highly organized contractile myofilaments; and positive genetic labeling with cardiac-specific marker (MLC 2v-eGFP). 2) Preconditioning with isoflurane reduced LDH release and delayed mPTP opening in both N-iPSC-CMs and unexpectedly in DM-iPSC-CMs in 5 mM and 11 mM, but not 25 mM glucose.

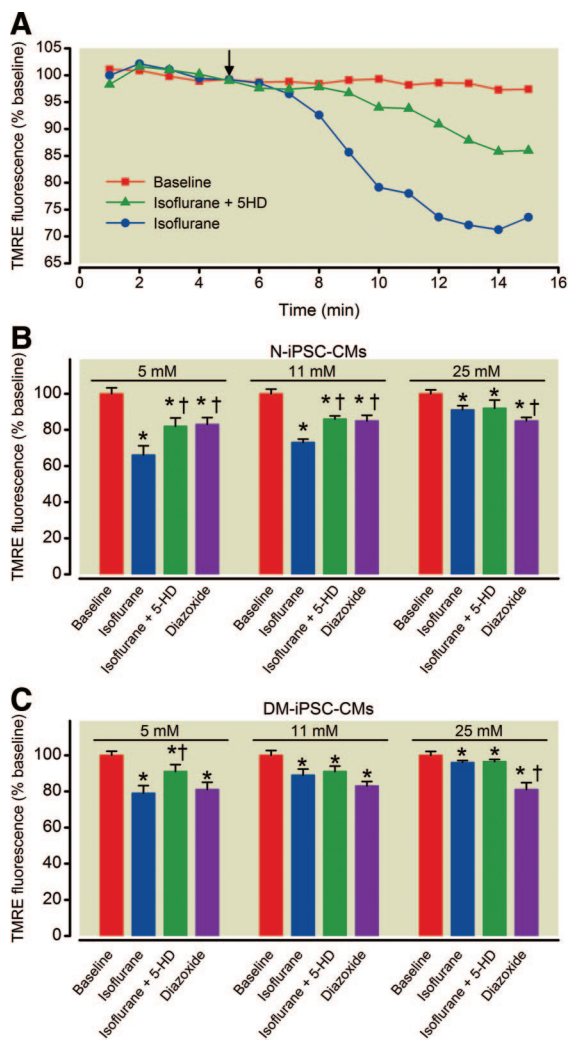


Fig. 6. Isoflurane opens mitochondrial potassium adenosine-5'-triphosphate-sensitive (mitoK_{ATP}) channels to the greatest extent in nondiabetic induced pluripotent stem cell-derived cardiomyocytes (N-iPSC-CMs). Mitochondrial membrane potential was monitored in dissociated iPSC-CMs. Representative signal traces from the addition (arrow) of isoflurane alone or in the presence of mitoK_{ATP} blocker 5-hydroxydecanoate (5-HD) in N-iPSC-CMs in 11 mM glucose (A). Isoflurane opened mitoK_{ATP} channels and caused a loss of tetramethylrhodamine ethyl ester fluorescence indicative of a mitochondrial depolarization. 5-hydroxydecanoate partly blocked the isoflurane-induced mitochondrial depolarization. Diazoxide depolarized the mitochondria in both N-iPSC-CMs and DM-iPSC-CMs in a 5 mM, 11 mM, and 25 mM glucose environment. Isoflurane induced the greatest mitochondrial depolarization in N-iPSC-CMs in a 5 mM glucose environment. 5-hydroxydecanoate partly attenuated the isoflurane-induced mitochondrial depolarization (B). Isoflurane-induced mitochondrial depolarization was reduced in DM-iPSC-CMs compared with N-iPSC-CMs in their respective glucose conditions (C). **P* < 0.05 versus baseline; †*P* < 0.05 versus isoflurane treatment. 5-HD = 5-hydroxydecanoate; DM-iPSC-CMs = type 2 diabetic induced pluripotent stem cell-derived cardiomyocytes; N-iPSC-CMs = nondiabetic induced pluripotent stem cell-derived cardiomyocytes; TMRE = tetramethylrhodamine ethyl ester.

3) Isoflurane triggered the opening of mitoK_{ATP} channels in N-iPSC-CMs in 5 mM and 11 mM glucose and in DM-iPSC-CMs, but only in 5 mM glucose. In summary, both N-iPSC- and DM-iPSC-CMs could be protected in 5 mM glucose, whereas 11 mM glucose attenuated isoflurane-induced mitoK_{ATP} opening in DM-iPSC-CMs, and 25 mM glucose abrogated all cardioprotective effects provided by APC in both DM-iPSC- and N-iPSC-CMs, indicating that 25 mM glucose was the predominant mechanism in the attenuation of APC in diabetic individuals.

We have shown that preconditioning with isoflurane attenuates cell damage and triggers cardioprotective mechanisms in iPSC-CMs against oxidative stress in 5 mM and 11 mM glucose. We recorded isoflurane-induced mitoK_{ATP} opening and a delay in oxidative stress-induced mPTP opening in N-iPSC-CMs in 5 mM and 11 mM glucose. iPSC-CMs responded similarly to APC as in studies of adult cardiomyocytes²⁷ and hESC-CMs,¹⁰ indicating that patient-specific iPSC-derived cardiomyocytes can be a vital tool in understanding human diseases not only at the genetic level, but can also test how environmental conditions play a role in a disease phenotype.

We achieved a high rate of differentiation efficiency; approximately 70% of cells expressed eGFP driven by the cardiomyocyte-specific promoter MLC-2v (fig. 3). N-iPSC-CMs and DM-iPSC-CMs both had striation-like patterns (fig. 2), indicating structural integrity similar to adult human cardiomyocytes and hESC-CMs.²⁸ We utilized growth factors to differentiate our iPSCs into cardiomyocytes based on the presence of these factors in regulating heart development in the embryo.²⁸ A previously published cardiac differentiation protocol for the induction of human embryonic stem cells and differentiation into cardiomyocytes was used for iPSC differentiation.¹⁰ We are confident that cardiomyocytes derived from iPSCs can serve as a powerful tool to understand different human diseases and how environment plays a role in attenuating the cardioprotection provided by APC. Cardiomyocytes derived from iPSCs share many characteristics of human adult cardiomyocytes: contractions, cardio-specific immunostaining that illustrates the highly organized contractile sarcomeres, atrial and ventricular-like action potentials, and the exhibition of functioning APC mediators.

iPSC-derived cardiomyocytes have many unique advantages over models that are currently available to study human diseases. The first shortcoming of many disease animal models is that the model itself does not replicate the complexity of the disease in its entirety. Specifically, the streptozotocin-induced diabetic models do not alter the genome of the model. Many of the type 2 diabetic murine models,²⁹ although more relevant, do not exhibit all the components of type 2 diabetes in humans. In addition, there are many shortcomings of using an animal model compared with a human disease.³⁰ As stem cell biology advances, pluripotent stem cell-derived cell types are being utilized in drug discovery and toxicity screening.^{31–32} The use of pluripotent stem cells to investigate disease models have become more prevalent as well.^{33–35}

Clearly, a human-type model will have many advantages over animal models as we continue to study human diseases.

Our laboratory has extensively investigated APC in animal models,^{3,19,25} human myocardium,²⁷ and in hESC-CMs.¹⁰ It was unclear whether iPSC-CMs could be protected from oxidative stress and what role glucose had in disabling the cardioprotection provided by preconditioning with isoflurane. In this study we showed that cardiomyocytes derived from both N-iPSCs and DM-iPSCs could be preconditioned with isoflurane; however, the presence of 25 mM glucose obliterated the APC-induced cardioprotective effects in each respective cell line. To further investigate the underlying mechanisms of the protection provided by APC in iPSC-CMs, we tested $\text{mitoK}_{\text{ATP}}$ channel activity and mPTP opening. We showed that isoflurane partly depolarized mitochondria in a 5-HD-sensitive manner to the greatest extent in N-iPSC-CMs in 5 mM and 11 mM glucose and in DM-iPSC-CMs in 5 mM glucose. This isoflurane-induced depolarization suggests that opening of the $\text{mitoK}_{\text{ATP}}$ channels plays a role in cardioprotection.^{3,36} N-iPSC-CMs in 25 mM glucose and DM-iPSC-CMs in 11 mM and 25 mM glucose did not show a significant difference in isoflurane-induced $\text{mitoK}_{\text{ATP}}$ opening compared with groups treated with the $\text{mitoK}_{\text{ATP}}$ channel blocker, 5-HD. This is not surprising, as others have indicated that both diabetes and hyperglycemia have shown to attenuate isoflurane-induced $\text{mitoK}_{\text{ATP}}$ opening.^{37–38}

It is well known that isoflurane acts on the electron transport chain, specifically complex I,²⁶ and the isoflurane-induced inhibition of this complex could explain the mitochondrial depolarization that we observed in the 5-HD groups. However, APC protected DM-iPSC-CMs in 11 mM glucose from oxidative stress and caused an APC-induced delay in mPTP opening, suggesting that isoflurane may elicit cell protection *via* parallel pathways that do not fully depend on the opening of $\text{mitoK}_{\text{ATP}}$ channels. In addition, isoflurane may induce $\text{mitoK}_{\text{ATP}}$ channel opening in a few channels or at levels that we could not accurately record with confocal microscopy. Further studies are needed to understand the role of $\text{mitoK}_{\text{ATP}}$ channels in cardioprotective strategies in iPSC-CMs, especially at moderately high glucose levels where APC provided protection without the measureable aid of $\text{mitoK}_{\text{ATP}}$ channels opening.

In addition, we investigated the effects of preconditioning on the delay of the mPTP opening. Typically, the mPTP opens during an ischemia-reperfusion injury resulting in an increase in the permeability of large solutes in the inner membrane of the mitochondria. mPTP opening dissipates $\Delta\Psi_{\text{m}}$, initiating cell death pathways.³⁹ Preconditioning cardiomyocytes with volatile anesthetics has been shown to delay the opening of the mPTP.^{40–41} Delaying the mPTP opening preserves the mitochondrial bioenergetics and overall improves the cellular and mitochondrial function. Our results indicate that APC delays opening of the mPTP in N-iPSC- and DM-iPSC-CMs in 5 mM and 11 mM glucose, but not in 25 mM glucose conditions, which is in line with LDH experiments

suggesting that iPSC-CMs derived from type 2 diabetic patients can be preconditioned and high glucose conditions may be playing a key role in attenuating cardioprotective effects provided by APC in these patients. The presence of 25 mM glucose alone attenuated APC-mediated delay in mPTP opening. Studies in rat cardiomyocytes have shown that pyruvate, a product of glycolysis, obliterated APC-induced delay in mPTP opening in $\Delta\Psi_{\text{m}}$ -dependent manner.²⁴ The delay in mPTP opening in N-iPSC-CMs in 5 mM and 11 mM glucose was similar to observations made in rat cardiomyocytes,¹⁹ human adult cardiomyocytes,²⁷ and hESC-CMs.¹⁰

A limitation of our study is the use of *in vitro* generated cardiomyocytes. Our iPSC-CMs may lack some of the characteristics of adult cardiomyocytes. However, with the parameters that we investigated, N-iPSC-CMs responded to APC similarly as other rat, rabbit, guinea pig, canine, human, and hESC-CM models that our department has previously investigated. To induce an injury, we generated oxidative stress with H_2O_2 or photoexcitation, which does not fully represent the conditions of an ischemia and reperfusion event. Specifically, the use of 10 mM H_2O_2 to induce an oxidative stress environment in our iPSC-CMs was excessively higher than H_2O_2 levels used in animal studies. We have observed that our stem cell-derived cardiomyocytes are more resistant to oxidative stress-induced injury. Further studies are needed to investigate the mechanisms involved in the resistance of iPSC-CMs to oxidative stress. Nevertheless, H_2O_2 -induced injury is widely accepted for studying reperfusion injury as it mimics the most important type of stress during this injury: oxidative stress. Finally, we solely investigated the effects of APC on cardiomyocytes. APC not only protects cardiomyocytes directly, but also indirectly through its action on other cell types in the heart, such as endothelial cells,⁴² or by modulation of inflammatory response.⁴³

In conclusion, for the first time our study shows that preconditioning with isoflurane protects cardiomyocytes derived from nondiabetic iPSCs from oxidative stress in 5 mM and 11 mM glucose. 25 mM glucose had detrimental effects on cardioprotection provided by APC in both nondiabetic and type 2 diabetic patient-derived cardiac lines. Unexpectedly, type 2 diabetic patient-specific iPSCs could be anesthetically preconditioned in 5 mM and 11 mM glucose. Additional studies and cell lines are needed to further understand the role of genetics in the disease phenotype, and identify potential components that may be contributing to the inability of human diabetic patients to be preconditioned.

The similarity between our study conducted on iPSC-CMs to our previous work in animal models, human cardiomyocytes, and hESC-CMs proves that cardiomyocytes derived from patient-specific iPSCs are a suitable model to further understand preconditioning in a human model and the underlying mechanisms by which disease phenotypes alter the protection provided by APC. Development of iPSC-derived ventricular cardiomyocytes from healthy individuals and patients with various diseases in conjunction with mod-

ulating external factors, such as varying glucose levels, provides a powerful tool for translational research.

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