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High Glucose Attenuates Anesthetic Cardioprotection in Stem-Cell-Derived Cardiomyocytes: The Role of Reactive Oxygen Species and Mitochondrial Fission

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Disclosures

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Abstract

BACKGROUND—Hyperglycemia can blunt the cardioprotective effects of isoflurane in the setting of ischemia-reperfusion injury. Previous studies suggest that reactive oxygen species (ROS) and increased mitochondrial fission play a role in cardiomyocyte death during ischemia-reperfusion injury. To investigate, the role of glucose concentration in ROS production and mitochondrial fission during ischemia-reperfusion (with and without anesthetic protection), we used the novel platform of human-induced pluripotent stem-cell-derived cardiomyocytes (iPSC-CMs).

METHODS—Cardiomyocyte differentiation from iPSC was characterized by the expression of CM-specific markers using immunohistochemistry and by measuring contractility. iPSC-CMs were exposed to varying glucose conditions (5, 11, 25 mM) for 24 hours. Mitochondrial permeability transition pore (mPTP) opening, cell viability, and ROS generation end-points were used to assess the effects of various treatment conditions. Mitochondrial fission was monitored by the visualization of fragmented mitochondria using confocal microscopy. Expression of activated dynamin-related protein 1 (Drp1), a key protein responsible for mitochondrial fission was assessed by western blot.

RESULTS—Cardiomyocytes were successfully differentiated from iPSC. Elevated glucose conditions (11 and 25 mM) significantly increased ROS generation, while only the 25 mM high glucose condition induced mitochondrial fission and increased the expression of activated Drp1 in iPSC-CMs. Isoflurane delayed mPTP opening and protected iPSC-CMs from oxidative stress in 5 and 11 mM glucose conditions to a similar level as previously observed in various isolated animal cardiomyocytes. Scavenging ROS with Trolox or inhibiting mitochondrial fission with mdivi-1 restored the anesthetic cardioprotective effects in iPSC-CMs in 25 mM glucose conditions.

CONCLUSIONS—Human iPSC-CM is a useful, relevant model for studying isoflurane cardioprotection, and can be manipulated to recapitulate complex clinical perturbations. We demonstrate that the cardioprotective effects of isoflurane in elevated glucose conditions can be restored by scavenging ROS or inhibiting mitochondrial fission. These findings may contribute to further understanding and guidance for restoring pharmacological cardioprotection in hyperglycemic patients.

INTRODUCTION

Diabetes contributes to over 240,000 deaths a year with approximately 70% of those deaths due to cardiovascular disease and another 17% due to stroke.^{1,2} Volatile anesthetics are cardioprotective in the setting of a wide variety of stressors,^{3–10} but diabetes is a notable exception, a stressor in which volatile anesthetics are not cardioprotective. The cardioprotective effects of volatile anesthetics are attenuated in the presence of diabetes via mechanisms which are largely unknown.^{3,11–13}

A substantial amount of laboratory data from various animal models demonstrates that high glucose concentrations induce an increase in mitochondrial reactive oxygen species (ROS)

production contributing to attenuation of anesthetic cardioprotection.^{11,14–18} Other data suggest that administration of antioxidants may restore the cardioprotective effects of volatile anesthetics in hyperglycemic animals.¹⁵ Also, hyperglycemia-induced ROS generation and dynamin-related protein (Drp1)-mediated mitochondrial fission have been shown to open the mitochondrial permeability transition pore (mPTP), resulting in cell death in rat cardiomyocytes.¹⁸

Most studies examining a lack of anesthetic-mediated cardioprotection in diabetes/hyperglycemia have been conducted in pre-clinical models.^{11,14,19–21} Although these studies are valuable, it is important to study *human* cardiomyocytes, since there may be significant species differences. Based on recent progress in reprogramming to an induced pluripotent stem cell (iPSC) state from somatic cells, human cardiomyocytes can be differentiated using a variety of published protocols.^{22–25} These iPSC-derived cardiomyocytes (iPSC-CMs) resemble terminally differentiated human cardiomyocytes in many aspects^{24,26–28} and provide an *in vitro* human experimental model. We previously showed cardioprotection with isoflurane in human embryonic stem cell- and iPSC-derived cardiomyocytes to a similar level as that observed in human myocardium.^{26, 27, 29} In addition, high glucose concentrations attenuated the cardioprotective effects conferred by isoflurane in iPSC-CMs.²⁶ However, the underlying mechanisms remained unknown.

Thus, the aim of this study was to investigate the role of hyperglycemia in the attenuation of isoflurane-induced cardioprotection in a relevant human model. We hypothesized that high glucose concentration-induced ROS generation and mitochondrial fission contribute to the attenuation of cardioprotection in iPSC-CMs. Additionally, we hypothesized that scavenging ROS or inhibiting mitochondrial fission in the presence of high glucose would restore isoflurane protection of iPSC-CMs.

MATERIALS AND METHODS

Human Induced Pluripotent Stem Cell Culture and Differentiation

All human cell experiments were approved by the IRB at the Medical College of Wisconsin. Human foreskin fibroblasts were reprogrammed as previously reported.^{30,31} iPSCs were a generous gift from Dr. Stephen Duncan, Ph.D. (Medical College of Wisconsin, Milwaukee, WI, USA). iPSCs were cultured as previously described.²⁷ Before initiating cardiac differentiation, iPSCs were detached with Accutase (Innovative Cell Technologies, San Diego, CA, USA) and seeded on Matrigel-coated (BD Biosciences, San Jose, CA, USA) plates in mTeSR1 medium (STEMCELL Technologies, Vancouver, BC, Canada) supplemented with 10 μ M ROCK inhibitor Y-27632 (EMD Millipore, Billerica, MA, USA). Medium was changed daily for four days and cells incubated in 4% O₂ and 5% CO₂. At ~90% confluence, the medium was changed to mTeSR1 with the addition of Matrigel for 24 hours (matrix sandwich method), and cells were incubated in 20% O₂ and 5% CO₂. Then on day 0 of differentiation, the medium was changed to Roswell Park Memorial Institute (RPMI) medium supplemented with B27 supplement (RPMI/B27) without insulin (Life Technologies, Carlsband, CA, USA) with added Matrigel and activin A (100 ng/ml) (R&D Systems, Minneapolis, MN, USA) for twenty-four hours. At day 1 the medium was changed to RPMI/B27 without insulin but with basic fibroblast growth factor (5 ng/mL) (Life

Technologies) and bone morphogenetic protein-4 (5 ng/mL) (R&D Systems). Cells were cultured in 20% O₂ and 5% CO₂ incubator for 4 days. On day 5, medium was changed to RPMI/B27 with insulin and changed every two days. Cells typically started contracting around day 12–14.²²

Microdissection and Single Cell Dissociation

One month into differentiation, contracting cardiomyocytes were mechanically dissociated using a 27-gauge needle under a dissecting microscope (SMZ1000; Nikon, Tokyo, Japan). Dissected clusters were enzymatically dispersed in 0.05% trypsin-EDTA (Invitrogen) for five minutes. Trypsin was inactivated by Dulbecco's Modified Eagle Medium containing 20% fetal bovine serum and individual cells were plated onto Matrigel-coated glass coverslips (25,000 cells/cm²).

Cardiomyocyte Differentiation Markers by Immunocytochemistry

Cells cultured on Matrigel-coated glass coverslips were fixed with 1% paraformaldehyde for 30 minutes and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and incubated with 10% donkey serum for 30 minutes, in order to reduce nonspecific staining. Cells were incubated with primary antibodies for sarcomeric α -actinin (1:100 dilution; Sigma-Aldrich) and anti-cardiac-specific troponin T (1:100; Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour at 37°C. After three washes with phosphate buffered saline (PBS), cells were incubated with fluorescently-labeled secondary antibody Alexa Fluor 594 (1:1000, Invitrogen, Waltham, MA, USA) for 1 hour at room temperature. Nuclei were counterstained with TOPRO-3 (1:1000; Invitrogen). Coverslips were then mounted onto slides and images acquired with a laser-scanning confocal microscope (Nikon Eclipse TE2000-U, Nikon, Tokyo, Japan).

Genetic Marking of iPSC-CMs with a Lentiviral Vector

Genetic marking of iPSC-CMs was conducted similarly to our previous study.²⁷ The NCX-1-eGFP cassette was subcloned into lentiviral transfer plasmid pHR(+).c.Ub.MCSoligo.R(-)W(+). The NCX-1 promoter is specific for ventricular cardiomyocytes.³² Lentiviral vector assembly and titering were performed as previously described.^{33,34} One day following differentiation, cardiomyocytes were transduced with a lentiviral vector encoding human sodium-calcium exchanger 1 (NCX-1)-driven enhanced green fluorescent protein (eGFP: multiplicity of infection, 1.8×10^4) (pNCX-1/eGFP) (Provided by Dr. Eduardo Marbán, M.D., Ph.D., Cedars-Sinai Hospital, Los Angeles, CA).

Experimental Design

Twenty-four hours following lentiviral transduction, 11 mM glucose RPMI/B27 medium was replaced with RPMI/B27 medium containing 5 mM glucose. Cells were cultured for four days with daily medium changes. In 12-well plates, the cells were divided into three groups containing 5 mM glucose with 20 mM mannitol, 11 mM glucose with 14 mM mannitol, or 25 mM of glucose, in order to normalize osmotic balance. iPSC-CMs were cultured in each condition for 24 hours.

Appropriate volumes of isoflurane stock solution (Baxter, Deerfield, IL, USA) were sonicated into culture media to achieve 0.5 mM (~1 minimum alveolar concentration; MAC) concentration as in previous studies.^{27,35} Media without isoflurane (control) was simultaneously sonicated. At the end of each experiment, isoflurane concentration was analyzed by gas chromatography. iPSC-CMs in each glucose group were then subjected to either exposure to 0.5 mM isoflurane in media or control media (without isoflurane) for 30 minutes. Following control and isoflurane exposure, the medium was exchanged for 30 minutes with culture medium containing 5, 11 or 25 mM of glucose, but without isoflurane, in order to remove any remaining isoflurane.

iPSC-CMs were then subjected to oxidative stress conditions for 2 hours using 100 μ M H₂O₂ (Calbiochem, LA Jolla, CA, USA) and 10 mM 2-deoxy-glucose (Sigma Aldrich), an inhibitor of glycolysis. Control cultures did not receive these treatments.

Trolox (250 μ M; Sigma Aldrich), a scavenger of ROS, or mdivi-1 (50 μ M; Sigma Aldrich), a dynamin related protein 1 (Drp1) inhibitor (mitochondrial division inhibitor-1), was added immediately following the two-hour oxidative stress. iPSC-CMs were then returned to their original glucose groups for four hours. Then cell viability, ROS generation, and mitochondrial fission assays were all conducted for all groups. Experimental protocols are summarized in Figure 1.

Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) activity was used as an assay of iPSC-CM cytotoxicity. iPSC-CMs were plated at 1×10^4 cells/well in 96-well plates and cultured in 100 μ L of RPMI/B27 medium supplemented with the appropriate glucose concentrations as previously discussed. LDH was measured via a colorimetric cytotoxicity assay kit according to the manufacturer's directions (Roche Diagnostics Corporation, Indianapolis, IN, USA).

Laser-Scanning Confocal Microscopy for Mitochondria Membrane Potential and mPTP Opening Assays

Four days following the lentiviral vector transduction, confocal imaging was used to identify living iPSC-CMs by NCX 1-driven eGFP expression.²⁷ Tetramethylrhodamine ethyl ester (TMRE; 30 nM; Invitrogen) was used to evaluate mitochondrial membrane potential (Ψ m). Opening of the mPTP was assessed as described previously in our laboratory,³⁶ a method based on mPTP induction by photoexcitation-generated oxidative stress.^{37–40} The mPTP opening was detected by rapid dissipation of Ψ m, observed as a loss of TMRE fluorescence, which is sensitive to mPTP opening inhibition.³⁶ The data were analyzed with Image J software.

Western Blotting

Cultured cells were washed with PBS and lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerolphosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, 1 mM PMSF). Lysates were then sonicated and centrifuged for 20 minutes at 14,000 g. Supernatant was collected and protein concentration determined using Bradford assay (Bio-Rad,

Hercules, CA, USA) according to the protocol provided by the company. Proteins (30 µg/lane) were separated on SDS/PAGE gel then transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat milk or 5% bovine serum albumin for one hour in room temperature, then probed overnight with primary antibodies against phosphoSer616 Drp1 (pSer616 Drp1) (Cell Signaling, Danvers, MA, USA), Drp1 (BD Transduction Laboratories, Lexington, KY, USA), β -Actin (Abcam, Boston, MA, USA), followed by appropriate secondary antibodies conjugated to horseradish peroxidase. Bands were visualized using enhanced chemiluminescence (Pierce, Rockford, IL, USA) and intensities were analyzed using ImageJ 1.41. Data are shown in arbitrary units (a.u).

Mitochondrial Morphology Measurement

Cells (4×10^4) were plated on glass coverslips coated with Matrigel. To visualize mitochondrial morphology, cells were incubated in 30 nM tetramethylrhodamine ethyl ester (TMRE).^{41,42} Images were captured with Nikon TE2000 inverted microscope (514 nm excitation/585 nm emission) using 60 $\times$ oil objective (NA 1.4) and analyzed with ImageJ. Images were contrast-enhanced with 0.5% saturation level, convoluted with 5 $\times$ 5 Laplacian kernel, with non-zero diagonal terms, which defines structures based on abrupt changes in fluorescence. Bandpass filtering was applied to remove structures below optical resolution limit of 0.2 µm, and then images were segmented by thresholding and binarized. The morphology of each mitochondrion was described by two factors; aspect ratio (major/minor axis of ellipse) and form factor ($4\pi \times \text{area}/\text{perimeter}^2$).⁴³ Both factors have minimum value of 1, which represents perfect circle. Whereas AR is an indicator of length, FF denotes length and branching. Hence, low values of AR and FF signify fragmented, unbranched mitochondria, and higher values mean more elongated, branched mitochondria.^{44,45} For each group, 50 random cells from three independent experiments were analyzed.

ROS Measurements

The levels of ROS production in iPSC-CMs were evaluated using the fluorescent probe dihydroethidium (DHE) (Molecular Probes, Grand Island, NY, USA). Cells were loaded with 5 µM DHE for 25 minutes at room temperature in RPMI. Images were acquired with a confocal microscope and analyzed using Image J software.

Statistical Analysis

Data are presented as mean $\pm$ SD. Each experimental group consisted of iPSC-derived CMs from at least three separate differentiations. 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 from the pooled data. Equal Variance and Shapiro-Wilk Normality Assumptions were satisfied for the residuals of each ANOVA model with a P value of 0.05 to reject. Within each glucose condition, all pairwise comparisons were conducted within the ANOVA context using post-hoc t-tests with the pooled variance estimate, followed by Holm-Sidak stepdown correction for multiple testing. Adjusted P-values are displayed in the text and all unadjusted and adjusted P-values along with 99% confidence intervals can be found in the Supplemental Digital Content. $P < 0.05$ was considered significant.

RESULTS

Characterization of Cardiomyocytes Derived from iPSCs

iPSC-CM contractility was seen within two weeks of differentiation. Immunostaining verified the presence of the cardiac proteins: cardiac-specific troponin T and sarcomeric α -actinin (Figure 2A). Dissociated cardiomyocytes were labeled with a lentiviral vector expressing eGFP (Figure 2B and 2C). Approximately 80–90% of dissociated cells expressed eGFP, indicating a high efficiency of differentiation (Figure 2D). Living eGFP expressing cells were used for subsequent confocal studies (Figure 2C).

Isoflurane Delayed mPTP Opening and Protected iPSC-CMs Against Oxidative Stress in 5 mM and 11 mM but not in 25 mM Glucose

Isoflurane-induced delay in mPTP opening directly translates to a reduction in cell death following an ischemia/reperfusion injury.^{27, 36, 46} Isoflurane treatment delayed the mPTP opening time in iPSC-CMs in the 5 mM ($P<0.001$) and 11 mM ($P<0.001$) glucose groups compared to control without isoflurane treatment. However, isoflurane-induced delay in mPTP opening was not observed in iPSC-CMs in the 25 mM glucose group ($P>0.99$) (Figure 3A).

Oxidative stress was induced in the iPSC-CMs by exposure to 100 μ M H₂O₂ with the addition of 10 mM 2-deoxy-D-glucose for 2 hours. iPSC-CMs that were pretreated with isoflurane prior to oxidative stress had less LDH release in 5 mM ($P=0.0047$) and 11 mM ($P=0.0189$) glucose groups compared to stress control groups. Isoflurane did not, however, reduce LDH release in iPSC-CMs in 25 mM glucose compared to the stress control group ($P=0.8924$) (Figure 3B).

High Glucose Induces Mitochondrial Fission, Hyperpolarization of the Mitochondrial Membrane Potential, and Generation of ROS

Increased mitochondrial fragmentation was observed when iPSC-CMs were exposed to 25 mM glucose (Figure 4A), indicating mitochondrial fission. Mitochondrial fragmentation was observed as a decrease in form factor and aspect ratio, indicating reduced mitochondrial branching and length, respectively (Figure 4B). We also observed that 24 hours of exposure to 25 mM of glucose dose-dependently hyperpolarized the mitochondrial membrane potential, observed as an increase in TMRE fluorescence (Figure 4C). Further, 25 mM glucose exposure led to hyperpolarization of the mitochondrial membrane potential compared to 5 mM glucose ($P<0.001$). Both 11 mM and 25 mM glucose concentrations increased superoxide levels compared to 5 mM ($P<0.001$, $P<0.001$, respectively) in iPSC-CMs measured with dihydroethidium (DHE) fluorescence. Further, 25 mM glucose exposure increased superoxide levels compared to 11 mM glucose concentration ($P<0.001$) (Figure 4D).

Elevated Glucose Conditions Increased the Abundance of Activated Drp1

Mitochondrial fission is primarily regulated by Drp1 phosphorylation. We observed that elevated glucose concentrations increased the abundance of pSer616 Drp1 protein (5 mM vs. 11 mM $P<0.001$; 5 mM vs. 25 mM $P<0.001$). Additionally, compared to control, H₂O₂-

induced oxidative stress increased pSer616 Drp1 ($P<0.001$), while adding the ROS scavenger Trolox resulted in reduced levels of the protein ($P<0.001$) compared to H₂O₂-induced oxidative stress, further confirming the strong correlation between ROS and mitochondrial fission (Figure 5A to 5D). Isoflurane preconditioning reduced H₂O₂-induced increase in pSer616 Drp1 only in 5 mM glucose conditions (5 mM: $P=0.0009$; 11 mM: $P=3.9685$; 25 mM $P=2.6886$) (Figure 5E and 5F).

Anesthetic Cardioprotection via Reduced ROS is Abrogated in High Glucose Conditions

Oxidative stress measured with dihydroethidium fluorescence, induced a similar level of superoxide generation in the 5 mM, 11 mM, and 25 mM glucose groups compared to control groups (Figure 6A to C). Isoflurane treatment reduced ROS levels in 5 mM and 11 mM glucose groups ($P<0.001$, $P<0.001$, respectively), but not in 25 mM glucose ($P=0.1426$). Mdivi-1 and Trolox both reduced ROS levels in all glucose conditions. We observed the greatest reduction in oxidative stress-induced ROS generation when iPSC-CMs were pretreated with isoflurane and treated with Trolox in all glucose groups.

Inhibiting Mitochondrial Fission and Scavenging ROS Restored Cardioprotection in 25 mM Glucose

iPSC-CMs treated with isoflurane prior to the oxidative stress had lower LDH release in 5 mM and 11 mM glucose groups ($P=0.00014$, $P=0.0001$, respectively), but not in 25 mM glucose ($P=0.6167$) (Figure 7A to 7C). In the 5 mM and 11 mM glucose groups, we observed that mdivi-1 and Trolox were cardioprotective individually; treatment with both Trolox and isoflurane conferred the greatest protection (Figure 7A and 7B). Isoflurane exposure did not lead to a reduced LDH release from oxidant stress in the 25 mM glucose group. However, LDH release was similar to controls when isoflurane was used in conjunction with mdivi-1 or Trolox in the 25 mM glucose group ($P<0.001$, $P<0.001$, respectively). mdivi-1 alone did not provide any level of cardioprotection (Figure 7C).

DISCUSSION

The main findings of this study in human iPSC-CMs were that high glucose concentrations attenuated isoflurane-induced cardiac protection, at least in part by increasing ROS generation and mitochondrial fission. Elevated glucose concentrations induced mitochondrial fission, mitochondrial membrane hyperpolarization, and increased ROS generation. Finally, scavenging of ROS or inhibiting mitochondrial fission restored the isoflurane cardioprotection, despite elevated glucose concentrations.

Previous studies have shown that iPSC-CMs are a viable model to investigate the underlying mechanisms of anesthetic cardioprotection,²⁶ as they respond similarly to human atrial myocytes²⁹ and human embryonic stem-cell-derived cardiomyocytes.²⁷ The iPSC used in this study were from normal subjects. The use of iPSC from subjects with specific cardiovascular diseases significantly enriches the experimental platforms available to researchers. In addition, cultured human iPSC-CMs can be exposed to countless culture conditions that mimic various disease environment conditions. Though the iPSC-CMs generated in the short differentiation protocol were immature, they still proved valuable as a

model system for studying stressed human cell responses to anesthetic-mediated protection. In this study we used a small portion of that potential to investigate how elevated glucose concentrations attenuate volatile anesthetic cardioprotection.

Mitochondria are highly dynamic organelles. They continuously move, fuse, fragment and divide. Normal balance between fusion and fission events of the mitochondrial outer membrane is required in order to maintain cellular homeostasis.⁴⁷ Unbalanced fission-fusion can affect a variety of biological processes such as apoptosis, and contribute to pathological processes such as heart failure.^{48–51} The most important mitochondrial fission protein is Drp1, which is mainly cytosolic, but upon activation, translocates to mitochondrial scission sites. When Drp1 is phosphorylated (at Ser616) mitochondrial fission occurs.⁵² Additionally, an increase in ROS production during oxidative stress conditions has been shown to activate (phosphorylated) Drp1 in human umbilical vein endothelial cells, neurons and cardiomyocytes.^{41,53–55} We found that high glucose concentrations alone increased mitochondrial fission and the ratio of phosphorylated Drp1 to total Drp1 levels in iPSC-CMs similar to earlier findings in rat neonatal cardiomyocytes.¹⁸

Mitochondrial dynamics have a key role in mitochondrial bioenergetics and function.⁵⁶ Zanna et al⁵⁷ demonstrated the correlation between mitochondrial fusion and oxidative phosphorylation. Inhibition of mitochondrial fusion resulted in reduced oxygen consumption. Loss of mitochondrial membrane potential, reduced endogenous respiration and capacity to increase respiration were observed in human dermal fibroblasts. Alterations in one of the mitochondrial membrane fusion proteins also affected mitochondrial metabolism in a similar fashion. Other evidence suggests that alterations in mitochondrial fission proteins could affect mitochondrial metabolism. Knockdown of Drp1 with RNAi in HeLa cells reduced the basal rate of oxygen consumption, decreased coupled respiration, and lowered the rate of ATP synthesis.⁵⁸ Similarly, high glucose concentration-induced mitochondrial fission and inhibition of mitochondrial fission by a dominant negative form of Drp1 markedly impaired mitochondrial ability to increase respiratory rate.⁵⁹ ROS stress during deep laser scanning-induced cellular damage also mediates mitochondrial fission in neonatal rat ventricular cardiomyocytes, human lung adenocarcinoma cells and kidney fibroblast cells.⁶⁰

ROS generation contributes to the development and progression of diabetes.^{61,62} In addition, excessive ROS generation is one of the major causes of stress in cardiomyocytes following I/R injury^{63–65} and plays a role in opening of the mPTP.⁶⁶ The mitochondrial respiratory chain is the major site of ROS production within the cell. Superoxide is a byproduct of normal respiration through one-electron reduction of dioxygen. Both *in vivo* and *in vitro* studies show increased superoxide production during high glucose exposure.^{59,67,68} The mitochondrial enzyme, manganese superoxide dismutase, converts superoxide to hydrogen peroxide which, in the presence of ferrous or cuprous ions, forms the highly reactive hydroxyl radical, which damages all classes of biomolecules. Hydrogen peroxide, on the other hand, can create a hydroxyl radical (one of the strongest oxidant agents) by interaction with ferrous ions in the catalytic Fenton reaction. There is some doubt that a similar reaction can be induced in mammalian cells, but studies involving different cells such as H9C2, endothelial cells and neurons conclusively show that H₂O₂ can induce oxidative stress and

cell death.^{69,70} The availability of free iron and copper within mitochondria is uncertain, although the reaction of superoxide with the iron sulfur center in aconitase releases ferrous iron. Excitation of fluorescence molecules using high energy laser light is known to produce ROS resulting from the interaction of the long-lived triplet state of the excited dye molecule with molecular oxygen, which in turn leads to mPTP opening, depolarization of mitochondrial membrane, cytochrome c release and cell death. We found that high glucose concentrations increased ROS generation and antioxidant Trolox used in conjunction with isoflurane reduced oxidative stress-induced ROS in all groups. These findings confirm that ROS generation is a key mediator in the attenuation of anesthetic cardioprotection in the presence of high glucose concentrations. We also observed that isoflurane was successful in reducing oxidative stress-induced pSer616 Drp1 levels in lower glucose concentrations. These results taken together indicate a strong correlation between ROS generation and mitochondrial fission in iPSC-CMs.

We found that elevated glucose concentrations attenuated the cardioprotective effects of isoflurane. Interestingly, the co-treatment of Trolox or mdivi-1 and isoflurane had a significantly greater protection in iPSC-CMs than the treatment with mdivi-1 or Trolox alone. These results show that scavenging ROS and blocking mitochondrial fission can restore the ability of cardioprotection to protect iPSC-CMs from an oxidative stress event. Our studies indicated that both ROS and fission interact and influence one another with both contributing to the attenuation of anesthetic cardioprotection in elevated prolonged high glucose conditions.

One limitation of our study was the method of induced cellular injury. We generated oxidative stress with high H₂O₂ and 2-deoxy-glucose. These exaggerated stress conditions were chosen when more physiologic hypoxia/reoxygenation conditions did not produce a damage phenotype (data not shown). Secondly, we used only 24 hours of exposure to high glucose concentrations in the attenuation of anesthetic cardioprotection in iPSC-CMs after finding that iPSC-CMs became adapted to high glucose concentrations following 48 hours and no longer responded with ROS generation and/or mitochondrial fission (data not shown).

The range of glucose concentrations used in this study was chosen based on animal studies. Of note, typical culture media for iPSC-CMs contains 11 mM glucose, which is physiologically hyperglycemic. Additional studies are needed to investigate the effects of these chronic high glucose concentrations on various iPSC-derived cell types to fully understand the mechanisms of anesthetic cardioprotection and how different disease states affect this cardioprotection. Finally, in this study we focused on the effects of high glucose concentrations on the attenuation of the cardioprotection provided by isoflurane. It would be valuable to look at other effectors of diabetes, for example, fatty acids and their role on the attenuation of the cardioprotective effects of anesthetics.

In conclusion, our study shows that 1) iPSC-CMs are useful for studying the underlying mechanisms by which high glucose concentrations attenuate volatile anesthetic cardioprotection; and 2) inhibiting mitochondrial fission or scavenging ROS restores the

cardioprotective properties of anesthetic cardioprotection in high glucose conditions in iPSC-CMs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental Outline

Induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) were cultured in three glucose concentrations (5, 11, and 25 mM). The cells then received isoflurane exposure, oxidative stress inducers, and experimental protocols all conducted in each different glucose conditions.

Figure 2. Immunolabeling and live-labeling of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs)

Confocal images of dissociated cells stained for sarcomeric proteins: sarcomeric α -actinin and cardiac-specific troponin T (A). The majority of positively labeled cells contained striation patterns with no differences between differentiations. Schematic representation of lentiviral vector pNCX-1/eGFP used for labeling cardiomyocytes (B). To determine total cell number, cells were loaded with mitochondrial marker tetramethylrhodamine ethyl ester (TMRE, red); sodium: calcium exchanger-1 (NCX-1)-enhanced green fluorescent protein (eGFP)-positive cells were counted by detecting green fluorescence, giving the number of

cardiomyocytes derived from iPSCs (C). Summarized data from six separate differentiations indicates a high percentage of iPSC-CMs in the culture (D). About 80–90% of iPSC after differentiation expressed e-GFP (D). Scale bar = 20 μm (A), and 40 μm (C).

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Figure 3. Isoflurane delayed mitochondrial permeability transition pore (mPTP) opening and protected induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) from oxidative stress in 5 mM and 11 mM glucose

mPTP opening was induced by photoexcitation-generated oxidative stress. Isoflurane delayed mPTP opening in iPSC-CMs in the presence of 5 mM and 11 mM glucose concentrations (A). Isoflurane did not delay mPTP opening in the presence of 25 mM glucose concentrations (A). * $P < 0.001$ versus control, $n=18$ cells/group. H_2O_2 -induced oxidative stress increased lactate dehydrogenase (LDH) release from iPSC-CMs in 5 mM, 11 mM and 25 mM glucose concentrations (B). In iPSC-CMs, isoflurane reduced stress-

induced LDH release in 5 mM and 11 mM glucose, but not in 25 mM glucose (B). * $P < 0.05$ versus stress, n=3 experiments/group. Ctrl = control; Iso = Isoflurane treatment; and Stress = H₂O₂ + 2-Deoxyglucose.

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Figure 4. High glucose alone induced mitochondrial fission, membrane hyperpolarization and reactive oxygen species generation

Confocal images of induced pluripotent stem cell-derived cardiomyocytes exposed to 5, 11, and 25 mM glucose. Corresponding processed sketched images are found below the confocal images (A). 25 mM glucose-induced mitochondrial fission observed as fragmentation of mitochondria and a decrease in form factor and aspect ratio; n=50 cells/group (B). High glucose induced mitochondrial membrane hyperpolarization in a concentration-dependent manner indicated by an increase in TMRE fluorescence (C). Elevated glucose induced superoxide generation observed by an increase in ethidium fluorescence, an indicator of superoxide levels (D). *P < 0.001 versus 5 mM (A–C); †P < 0.05 versus 11 mM (C). n = 6 coverslips/group.

Figure 5. High glucose-induced oxidative stress increases the abundance of activated Drp1 (pSer616 Drp1) and inhibits cardioprotective actions of isoflurane

Representative western blot images show that high glucose alone increases Drp1 phosphorylation at the Ser616 residue (A). Summarized data shows significant increase in pSer616 Drp1 expression levels when cardiomyocytes were exposed to 25 mM glucose vs. to 5 mM glucose (B). Trolox significantly reduced oxidative stress-induced increase in pSer616 Drp1 expression (C, D). Isoflurane reduced stress-induced pSer616 in 5 mM glucose alone (E, F). *P < 0.001 versus 5 mM / Ctrl; #P < 0.05 versus 5 mM / H₂O₂. n=9 differentiations/group. Ctrl=control.

Figure 6. Isoflurane reduces abundance of ROS in the setting of H₂O₂ stress in 5 and 11 mM glucose

H₂O₂-induced oxidative stress induced reactive oxygen species (ROS) in 5, 11, and 25 mM glucose concentrations compared to each of their controls (A–C). Isoflurane treatment (Iso) reduced stress-induced ROS in 5 and 11 mM glucose (A, B), but not in 25 mM glucose (C). Mdivi-1 and Trolox reduced ROS levels in each glucose condition (A–C). Interestingly Iso + Trolox reduced oxidative stress-induced ROS levels to the greatest extent in 5 and 25 mM glucose conditions compared to Iso and Trolox treatments alone (A,C). *P < 0.05 versus stress/†P < 0.001 versus Trolox treatment alone/ #P < 0.001 versus is treatment alone. n=6

coverslips/group. Ctrl=control; Stress=H₂O₂-induced oxidative stress; Iso=isoflurane treatment.

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Figure 7. Scavenging reactive oxygen species or inhibiting mitochondrial fission restored anesthetic CM protection in 25 mM glucose

H₂O₂-induced oxidative stress decreased cell viability in 5, 11, and 25 mM glucose concentrations compared to respective controls (A–C). Isoflurane (Iso) reduced stress-induced lactate dehydrogenase (LDH) release in 5 and 11 mM glucose (A, B), but not in 25 mM glucose (C). Interestingly, Trolox and mdivi-1 alone reduced LDH release in 5 and 11 mM glucose (A, B). The greatest extent of CM protection was observed when iPSC-CMs were pretreated with isoflurane and treated with Trolox (A–C) in all glucose groups. When mdivi-1 was used in conjunction with isoflurane there was a significant reduction in LDH

release compared to isoflurane and mdivi-1 treatments alone in 25 mM glucose (C). *P < 0.001 versus stress/†P < 0.05 versus Trolox treatment alone. n=6 experiments/group. Ctrl=control; Stress=H₂O₂-induced oxidative stress; Iso=isoflurane treatment.

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