High-molecular-weight polyethylene glycol protects cardiac myocytes from hypoxia- and reoxygenation-induced cell death and preserves ventricular function

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Malhotra R, Valuckaite V, Staron ML, Theccanat T, D’Souza KM, Alverdy JC, Akhter SA. High-molecular-weight polyethylene glycol protects cardiac myocytes from hypoxia- and reoxygenation-induced cell death and preserves ventricular function. *Am J Physiol Heart Circ Physiol* 300: H1733–H1742, 2011. First published February 18, 2011; doi:10.1152/ajpheart.01054.2010.—Apoptosis plays a significant role in maladaptive remodeling and ventricular dysfunction following ischemia-reperfusion injury. There is a critical need for novel approaches to inhibit apoptotic cell death following reperfusion, as this loss of cardiac myocytes can progressively lead to heart failure. We investigated the ability and signaling mechanisms of a high-molecular-weight polyethylene glycol-based copolymer, PEG 15–20, to protect cardiac myocytes from hypoxia-reoxygenation (H-R)-induced cell death and its efficacy in preserving ventricular function following extended hypothermic ischemia and warm reperfusion as relevant to cardiac transplantation. Pretreatment of neonatal rat ventricular myocytes with a 5% PEG solution led to a threefold decline in apoptosis after H-R relative to untreated controls. There was a similar decline in caspase-3 activity in conjunction with inhibition of cytochrome c release from the inner mitochondrial membrane. Treatment with PEG also reduced reactive oxygen species production after H-R, and sarcolemmal lipid-raft architecture was preserved, consistent with membrane stabilization. Cell survival signaling was upregulated after H-R with PEG, as demonstrated by increased phosphorylation of Akt, GSK-3β, and ERK1/2. There was also maintenance of cardiac myocyte β-adrenergic signaling, which is critical for myocardial function. PEG 15–20 was very effective in preserving left ventricular function following prolonged hypothermic ischemia and warm reperfusion. PEG 15–20 has a potent protective antiapoptotic effect in cardiac myocytes exposed to H-R injury and may represent a novel therapeutic strategy to decrease myocardial cell death and ventricular dysfunction at the time of reperfusion during acute coronary syndrome or following prolonged donor heart preservation.

ISCHEMIC HEART DISEASE is the primary etiology for the development of heart failure (HF) (8). Loss of cardiac myocytes following ischemia-reperfusion injury has recently been shown to be attributable, in large part, to apoptosis (6, 18). Apoptosis occurs primarily after reperfusion following ischemia, whereas prolonged ischemia leads to necrosis. There is also increasing evidence that apoptosis plays an important role in both acute and chronic loss of cardiac myocytes after myocardial infarction. Studies have reported the presence of apoptotic cells in the border zone of the infarct and in remote myocardium during the early phase (15), as well as months after myocardial infarction (21), suggesting that apoptosis plays a role in maladaptive remodeling and the development of HF after ischemic injury. Apoptosis is a highly regulated program of cell death, and inhibition of this process is cardioprotective under many conditions (2, 5). As a result, apoptosis represents a potential target for therapeutic intervention. Because the regenerative capacity of the myocardium is limited, there is a critical need to prevent cardiac myocyte loss during and after ischemia and reperfusion injury.

Polyethylene glycol (PEG) has demonstrated membrane-protective effects in a variety of cells or organs against various insults. PEGs are known to decrease reactive oxygen species (ROS) production and lipid peroxidation in these injury models. Because PEGs do not scavenge superoxide anion or inhibit xanthine oxidase (12, 17), it is likely that these polymers inhibit or reduce oxidative stress primarily through preservation or restoration of membrane integrity. This could be a mechanism of protection against ROS production during ischemia-reperfusion injury at the cellular and intact organ level. In addition, the prevention of edema by PEG protects against mitochondrial swelling, which itself is an important factor contributing to the increase in ROS production. There has been little work investigating the potential efficacy of PEG in myocardial protection from ischemia-reperfusion injury. In the present study, we tested the ability of PEG 15–20 to prevent apoptotic cell death in cultured cardiac ventricular myocytes subjected to hypoxia-reoxygenation and isolated adult rat hearts that underwent prolonged cold ischemia followed by warm reperfusion to determine whether myocardial recovery could be enhanced by pretreatment with PEG. We also investigated the potential signaling mechanisms involved in PEG-mediated cardioprotection which may also be relevant to other organ systems.

MATERIALS AND METHODS

All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Chicago.

High-molecular-weight PEG 15–20. High-molecular-weight PEG 15,000–20,000 Da, referred to as PEG 15–20, was purchased from Sigma (St. Louis, MO). For imaging studies, the nascent PEG 15–20 was fused with fluorescein as custom prepared by Sigma (batch 478-004-2, 28-Nov-06) as previously described by Valuckaite et al. (22). Cardiac myocytes were pretreated with 5% PEG 15–20 for 1 h followed by gentle washing with regular DMEM/F-12 medium to remove any unbound PEG before exposure to hypoxia and reoxygenation. For ex vivo intact heart experiments, 3 ml of 1% PEG 15–20 solution in PBS was delivered into the aorta over 5 min before global ischemia.
Cell culture. Primary cultures of neonatal rat ventricular cardiac myocytes were prepared by enzymatic digestion of ventricular tissue from 1-day-old rats in a HEPES-buffered solution containing 0.1% collagenase IV, 0.1% trypsin, 15 mg/ml DNase I, and 0.1% chicken serum. The dissociated cells were collected by centrifugation and resuspended in ADS buffer (in g/l: 6.8 NaCl, 4.76 HEPES, 0.138 NaH2PO4, 0.6 glucose, 0.4 KCl, 0.205 MgSO4, and 0.0002 phenol red, pH 7.4). The cells were then selectively enriched by differential centrifugation through a discontinuous Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) gradient of densities 1.050, 1.062, and 1.082 g/ml. The enriched cardiac myocytes were plated in gelatin-coated culture dishes in DMEM/F-12 (Life Technologies, Carlsbad, CA) (1:1, vol/vol) supplemented with 5% horse serum, 3 mM pyruvic acid, 100 mM ascorbic acid, 1 mg/ml transferrin, 10 ng/ml selenium, and 100 mg/ml ampicillin. Bromodeoxyuridine at a final concentration of 0.1 mM was added during the first 36 h to prevent proliferation of cardiac fibroblasts.

Hypoxia and reoxygenation protocol. All experiments with cardiac myocytes were done after 4 days of plating. The cells were plated in DMEM/F-12 medium (1:1), which contained 5 mM glucose. For hypoxia experiments, the cells were placed in a Plexiglas chamber, and a constant stream of water-saturated 2% O2, 93% N2, and 5% CO2 was maintained over the culture. Maintenance of the desired O2 concentration was constantly monitored during incubation using a microprocessor-based oxygen sensor. To lower the partial pressure of dissolved oxygen in the medium (PO2), Oxyrase, a mixture of bacterial membrane monooxygenases and dioxygenases (Oxyrase, Ashland, OH), was added to the culture medium at a final concentration of 0.1% to ensure that the effect of Oxyrase was no longer present.

Hoechst and TUNEL staining. For morphological studies, the cardiac myocytes were grown in eight-well gelatin-coated Falcon glass culture slides (Becton Dickinson Labware, Franklin Lakes, NJ). The cells were rinsed in PBS, pH 7.4, and fixed for 30 min in 4% paraformaldehyde in PBS, pH 7.4, at room temperature. After a rinse in PBS, the cells were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate. The cells were rinsed twice in PBS and then stained with the karyophilic dye Hoechst 33258 (5 mg/ml) for 10 min at room temperature. After a final rinse in PBS, the cells were mounted in moewol, an antifade agent, and visualized under ultraviolet light with a Leitz Orthoplan microscope. Because this dye stains both apoptotic and nonapoptotic cells, we could specifically count the percentage of apoptotic cells displaying chromatin condensation and nuclear fragmentation. For statistical analysis, 100 cells were counted in five different fields. Further characterization of apoptosis was achieved using a commercially available in situ cell death detection kit to find DNA strand breaks using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reagent according to the manufacturer’s protocol (Chemicon International, Temecula, CA). The number of TUNEL-positive cells was also counted in five different fields.

Analysis of cytochrome c release. Subcellular fractionation was performed. Cardiac myocytes were rinsed with cold PBS, and mitochondrial and cytosolic (S100) fractions were prepared. Briefly, cells were resuspended in 0.25 ml of ice-cold isotonic buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM diithiothreitol, 17 mg/ml phenylmethylsulfonyl fluoride, 8 mg/ml aprotinin, 2 mg/ml leupeptin, and 5 mg/ml pepstatin, pH 7.4). The cells were disrupted by two successive sonications on ice for 20 s each with a pause of 1–2 min using a microtip generator set at 40% duty cycle (Sonicator Vibracell; Sonics and Materials, Newtown, CT). A pellet highly enriched in mitochondria was prepared by centrifugation at 10,000 g for 30 min. This pellet was resuspended in the same buffer A, and the resulting supernatant was further spun at 160,000 g for 1 h in a TLA-100 rotor in a Beckman table top ultracentrifuge (Beckman Instruments, Fullerton, CA). A pellet highly enriched in mitochondria was prepared by centrifugation at 10,000 g for 30 min. This pellet was resuspended in the same buffer A, and the resulting supernatant was further spun at 160,000 g for 1 h in a TLA-100 rotor in a Beckman table top ultracentrifuge (Beckman Instruments, Fullerton, CA). The supernatant from this final ultracentrifugation represented the cytosolic fraction.

We also performed Western blot analysis. Equivalent amounts of mitochondrial and cytosolic fractions were subjected to Western blot analysis. Briefly, the proteins were electrophoresed on 15% SDS
polyacrylamide gels, transferred to Hybond nylon membranes (Amersham Pharmacia Biotech), and immunoblotted with monoclonal antibodies specific for cytochrome c (monoclonal antibody 7H8.2C12 at 1.5 mg/ml; Pharmingen, San Diego, CA). To ensure that cytochrome c release was not caused by a physical disruption of mitochondria, both the mitochondrial and cytosolic fractions were probed with monoclonal antibodies to cytochrome oxidase (subunit IV) (monoclonal antibody 20E8-C12 at a dilution of 0.1 mg/ml; Molecular Probes, Eugene, OR), an enzyme complex bound to the outer leaflet of the inner mitochondrial membrane. The signal was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Caspase-3 activity.** Caspase-3 activity was assessed by a colorimetric assay utilizing specific substrates (Calbiochem, San Diego, CA). Control cardiac myocytes and those subjected to hypoxia-reoxygenation in the presence or absence of 5% PEG 15–20 were washed once with ice-cold PBS and collected by trypsinization followed by centrifugation. The cellular pellet was resuspended in cell lysis buffer and incubated on ice for 10 min. Lysates were centrifuged for 5 min at 13,000 revolution/min, and the supernatants were assayed for caspase-3 activity in assay buffer [50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 10 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol]. After addition of DEVD-specific caspase, substrate (2 mM) samples were incubated for 60 min at 37°C and read at 405 nm in an EL-312 Bio-Kinetics microplate reader (Bio-Tek Instruments, Winooski, VT).

**Lipid-raft coalescence.** Cardiac myocytes were treated for 1 h with 5% PEG 15–20 followed by gentle washing with regular DMEM/F-12 medium to remove any unbound PEG. The cells were then exposed to 3 h of hypoxia and 3 h of reoxygenation following by washing with medium. Lipid rafts were visualized using the Molecular Probes Vybrant Lipid raft labeling kit (Eugene, OR). Lipid rafts were visualized by fluorescence microscopy.

**Protein immunoblotting.** Equal amounts of protein extracted from cardiac myocytes prepared with radioimmune precipitation assay buffer with phosphatase inhibitors were fractionated by 12% SDS-polyacrylamide gels, transferred to Hybond nylon membranes (Amersham Pharmacia Biotech), and immunoblotted with monoclonal antibodies specific for cytochrome c (monoclonal antibody 7H8.2C12 at 1.5 mg/ml; Pharmingen, San Diego, CA). To ensure that cytochrome c release was not caused by a physical disruption of mitochondria, both the mitochondrial and cytosolic fractions were probed with monoclonal antibodies to cytochrome oxidase (subunit IV) (monoclonal antibody 20E8-C12 at a dilution of 0.1 mg/ml; Molecular Probes, Eugene, OR), an enzyme complex bound to the outer leaflet of the inner mitochondrial membrane. The signal was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

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**Protein immunoblotting.** Equal amounts of protein extracted from cardiac myocytes prepared with radioimmune precipitation assay buffer with phosphatase inhibitors were fractionated by 12% SDS-page. Antibodies against phospho Thr308 and Ser473 residues of Akt, Ser9 residue of GSK-3β, and Thr202/Tyr204 residues of ERK1/2 (Cell Signaling, Beverly, MA) were used. Blots were stripped and reprobed with total Akt, GSK-3β, or ERK1/2 antibodies, respectively, to confirm equal protein loading.

**Flow cytometry.** Intracellular ROS levels were measured by staining cells with 1 μM dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes) at 37°C for 15 min in 5% fetal bovine serum, PBS solution, followed by washing with PBS. To investigate the role of ROS in hypoxia-reoxygenation-induced cell death, cardiac myocytes were incubated with the nonfluorescent compound DCDF, which in the presence of ROS is oxidized to the highly fluorescent dichlorofluorescein (DCF). Flow cytometry was performed to quantify the DCF signal as described. Stained cells were filtered and analyzed immediately with a FACScan flow cytometer (BD Bioscience, San Jose, CA). All gain and amplifier settings were held constant for the duration of the experiment.

**MitoSOX Red staining.** Cardiac myocytes growing on coverslips were loaded with 5 μM MitoSOX Red (Invitrogen Technologies, Eugene, OR) in HBSS to detect mitochondrial superoxide followed by incubating cells for 10 min at 37°C protected from light. The cells were washed gently three times with warm buffer followed by counterstaining with DAPI and mounting in warm buffer for imaging. The MitoSOX Red mitochondrial superoxide indicator was detected using a confocal microscope at an excitation/emission maxima of 510/580 nm. The integrated density of MitoSOX staining was achieved using NIH ImageJ software analysis, and the results were represented as arbitrary fluorescence units.

**Lucigenin chemiluminescent assay.** Cardiac myocytes were suspended in 200 μl of assay buffer composed of 100 mmol/l potassium phosphate (pH 7.0), 10 μmol/l flavin adenine dinucleotide, 1 mmol/l NaNO3, and 1 mmol/l EGTA. After preincubation with 5 μmol/l lucigenin, NADPH was added to a final concentration of 500 μmol/l. The chemiluminescence was continuously monitored using a luminometer. The reaction was terminated by the addition of 100 μg/ml superoxide dismutase (SOD). The difference in average luminescence between samples with and without SOD was used to calculate the luminescence produced by O2−. Measurements were normalized for protein content.

**Fig. 2.** Cytochrome (cyto) c release and caspase-3 activity induced by hypoxia-reoxygenation. A: Western blot analysis of cytochrome c in cytosolic and membrane fractions of cardiac myocytes treated with 5% PEG 15–20 and exposed to hypoxia-reoxygenation. It also shows the cytosolic and membrane fractions probed with a monoclonal antibody specific for cytochrome oxidase IV (COX). B: caspase-3 activity in lysates of cardiac myocytes exposed to 3 h of H/R with and without PEG pretreatment. Staurosporine was used as a positive control at a final concentration of 2 μM. z-VAD-fmk, a broad spectrum caspase-3 inhibitor, was added to the medium 30 min before staurosporine (St) at a final concentration of 100 μM. N = 4 in each group. *P < 0.05 vs. Ctrl ± PEG, **P < 0.05 vs. H/R.
Intracellular cAMP quantitation. Cardiac myocytes were cultured on 12-well plates in DMEM/F-12 and assayed for intracellular cAMP accumulation during a 15-min incubation with 0.2 mM isobutylmethylxanthine, a cyclic nucleotide phosphodiesterase inhibitor, followed by stimulation with isoproterenol (10 μM) for an additional 15 min. Reactions were terminated by aspiration of culture medium and addition of 150 μl of 0.1 M hydrochloric acid (HCI) to each well. HCl extracts were assayed for cAMP content by Direct ELISA kit (Assay Designs, Ann Arbor, MI).

Cardiac physiology. Hearts were isolated and perfused in the Langendorff mode as previously described (1). All hearts were perfused with Krebs-Henseleit buffer containing 118 mM NaCl, 25 mM NaHCO3, 0.5 mM Na4-EDTA·2H2O, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4·7H2O, 2.5 mM CaCl2·2H2O, and 11 mM D-glucose (pH adjusted with 95%O2-5%CO2) for 15 min to achieve a stable baseline. After cannulation of the aorta, the coronary circulation was quickly resumed at a constant pressure of 80mmHg at 37°C. For measurement of left ventricular (LV) function, a water-filled latex balloon was inserted through the left atrium into the LV. The balloon was inflated with 1% solution of PEG 15–20 mmHg. Coronary flow was measured by collecting coronary effluent and processed for histopathological analysis utilizing the pathology core facility at The University of Chicago. Paraffin sections were stained with hematoxylin-eosin as well as processed for TUNEL staining using ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon International). Systolic function was measured as the peak rate of increase in pressure (+dP/dt) and LV-developed pressure. Diastolic function was assessed as (–dP/dt).

Statistical analysis. Analyses were conducted using Statview 4.01 software (Abacus Concepts, Berkley, CA). Experimental groups were compared using Student’s t-test or one-way ANOVA, as appropriate. The Bonferroni test was applied to all significant ANOVA results compared using Student's t-test or one-way ANOVA, as appropriate. The Bonferroni test was applied to all significant ANOVA results using SigmaStat software. P values of <0.05 were considered statistically significant. All results are expressed as means ± SE.

RESULTS

Pretreatment with PEG 15–20 decreases hypoxia-reoxygenation-induced apoptosis in cardiac myocytes in vitro. To determine the effect of 5% PEG pretreatment on apoptosis induced by hypoxia-

In PBS or PBS alone followed by 3 h of hypothermic (4°C) global ischemia and 1 h of normothermic reperfusion in Krebs-Henseleit buffer. The hearts were then fixed in buffered formalin overnight and processed for histopathological analysis utilizing the pathology core facility at The University of Chicago. Paraffin sections were stained with hematoxylin-eosin as well as processed for TUNEL staining using ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon International). Systolic function was measured as the peak rate of increase in pressure (+dP/dt) and LV-developed pressure. Diastolic function was assessed as (–dP/dt).

Fig. 4. PEG 15–20 binds to membrane lipid-raft domains and inhibits raft coalescence. A: confocal image shows binding of fluorescein-labeled PEG to cardiac myocyte plasma membranes visualized by rhodamine-labeled antifluorescin staining in red. Magnification ×1,000. B: cardiac-specific α-actinin staining shown in green. C: confocal images of control cardiac myocytes stained with red fluorescent protein fused Cholera toxin B to identify lipid rafts. D: untreated cardiac myocytes and cells pretreated with 5% PEG 15–20 (E) were exposed to 3 h each of hypoxia and reoxygenation. Nuclear DAPI staining is shown in blue in all panels. C–E: inset: merge image of nuclei and lipid rafts. (Magnification in B–E is ×400). N = 4 in each group.

**P < 0.05 vs. control and **P < 0.05 vs. H/R. D: cardiac myocytes subjected to hypoxia-reoxygenation were analyzed for O2 release by the lucigenin chemiluminescent assay, N = 3 in each group. *P < 0.05 vs. H/R without PEG.
reoxygenation in cultures of neonatal rat cardiac myocytes, we first examined cell morphology by phase-contrast microscopy and nuclear morphology by Hoechst and TUNEL staining. Cardiac myocytes pretreated with PEG 15–20 and subjected to 3 h of hypoxia followed by 3 h of reoxygenation demonstrated significantly less apoptosis compared with cells that did not receive PEG pretreatment as measured both by the TUNEL assay (17 ± 3% vs. 38 ± 5%) and Hoechst 33258 staining (14 ± 3% vs. 43 ± 4%) (Fig. 1, A and B). Cardiac myocytes maintained at normoxic conditions in the presence and absence of PEG 15–20 demonstrated a basal level of apoptosis of 7 ± 3%.

PEG 15–20 inhibits the mitochondrial cell death pathway and activation of caspase-3 in cardiac myocytes subjected to hypoxia-reoxygenation. To further investigate the protective effects of PEG 15–20 on hypoxia-reoxygenation-induced apoptosis in cardiac myocytes, we examined the mitochondrial cell death pathway and measured the release of cytochrome c from the inner mitochondrial space by immunoblotting. PEG

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**Fig. 5.** Phosphorylation of Akt, GSK-3β, and ERK1/2 is upregulated by PEG after H/R. Total cell lysates of cardiac myocytes maintained under normoxic conditions and those that underwent 3 h each of hypoxia and reoxygenation with and without PEG were immunoblotted with antibodies specific for Thr308 (A) and Ser473 (B) residues of Akt, Ser9 residue of GSK-3β (C) and Thr202/Tyr204 residues of ERK1/2 (D). Bottom: densitometric analysis of the Akt, GSK-3β, and ERK1/2 phosphorylation normalized to total respective protein. D: ERK phosphorylation for control + PEG was not different from control (P > 0.05) and not included in the corresponding histogram. N = 4 in each group. *P < 0.05 vs. H/R.
pretreatment completely inhibited hypoxia-reoxygenation-induced cytochrome $c$ release into the cytosolic fraction. Western blot analysis also showed that cytochrome oxidase, an enzyme complex bound to the inner leaflet of the outer mitochondrial membrane, was always present only in the mitochondrial membrane fraction and never in the cytosolic fraction, indicating that mitochondria remained intact and no cross contamination of cellular fractions had occurred (Fig. 2A). The release of cytochrome $c$ from the mitochondria is a well-known mechanism to trigger downstream activation of caspase-9, which in turn cleaves caspase-3, resulting in cell death. An analysis of caspase-3 activity in the lysates of cardiac myocytes revealed that PEG pretreatment led to a threefold reduction in caspase-3 activity following hypoxia-reoxygenation compared with myocytes not treated with PEG (Fig. 2B).

Cardiac myocytes pretreated with PEG 15–20 demonstrate reduced oxidative stress following hypoxia-reoxygenation. We then investigated whether pretreatment with PEG 15–20 could attenuate the generation of ROS. ROS generation was monitored by several techniques. First, staining of cells with MitoSOX Red dye that specifically detects mitochondrial ROS production demonstrated significantly less ROS production following hypoxia-reoxygenation with PEG 15–20 pretreatment. Importantly, PEG 15–20 did not stimulate an increase in ROS production under control conditions (Fig. 3A). In addition, conversion of DCDF to the fluorescent compound DCF was investigated by fluorescence-activated cell sorting (FACS) analysis. Treatment of cardiac myocytes with PEG 15–20 did not affect ROS levels under basal conditions (Fig. 3B). PEG 15–20 pretreatment, however, led to a significant shift of the fluorescence curves to the left, demonstrating attenuation of ROS production (Fig. 3C). We also utilized an alternative approach to quantitate cardiac myocyte superoxide production with a Lucigenin Chemiluminescence assay. PEG treatment had no appreciable effect on basal superoxide production in cardiac myocytes under normoxic conditions. However, pretreatment with PEG for 1 h before hypoxia-reoxygenation significantly reduced superoxide production (2.12 ± 0.56 vs. 3.85 ± 0.26 nmol of superoxide anion per minute per milligram of protein, respectively) (Fig. 3D). These results demonstrate that treatment with PEG 15–20 can decrease oxidative stress stimulated by hypoxia-reoxygenation in cardiac myocytes in vitro.

Hypoxia-reoxygenation induces lipid-raft coalescence and is prevented by PEG 15–20. Previous studies in other cell types have demonstrated that PEG 15–20 has membrane-stabilizing effects and binds specifically to lipid rafts. Also lipid-raft coalescence is a specific cellular response to several stresses and is commonly used as a specific marker of altered cellular integrity and a precursor to apoptosis. We first verified that PEG 15–20 physically binds to the cardiac myocyte membrane (Fig. 4A). This is in contrast to the cytosolic cardiac-specific $\alpha$-actinin staining (Fig. 4B). Lipid-raft coalescence was studied in cardiac myocytes before and after hypoxia-reoxygenation by confocal microscopy using the green fluorescent protein-fused Cholera toxin B. Under normoxic conditions, there was diffuse, low-intensity staining of lipid rafts by Cholera toxin B (Fig. 4C). Hypoxia-reoxygenation led to significant lipid-raft coalescence detected by focal, intense staining (Fig. 4D). Pretreatment with PEG 15–20 led to a marked decrease in lipid-raft coalescence induced by hypoxia-reoxygenation (Fig. 4E).

PEG 15–20 pretreatment is associated with upregulated cell survival signaling. Because PEG inhibited lipid-raft coalescence and apoptosis in cardiac myocytes after hypoxia-reoxygenation, we wanted to investigate and identify the signaling mechanisms that may potentially be involved in enhanced cell survival. The prosurvival phosphatidylinositol-3-kinase-Akt-GSK-3$\beta$ pathway as well as ERK1/2 activation were studied by determining the phosphorylation status of Akt residues Ser$^{473}$ and Thr$^{308}$ and the GSK-3$\beta$ Ser$^{9}$ residue as well as phosphorylation of p42/44 subunits (Thr$^{202}$/Tyr$^{204}$) of ERK1/2, respectively. Western blot analysis revealed that PEG treatment before hypoxia-reoxygenation significantly increased the ratios of p-Akt (Ser$^{473}$/Total Akt, p-Akt (Thr$^{308}$/Total Akt, p-GSK-3$\beta$/Total GSK-3$\beta$, and p-ERK/Total ERK by 65, 54, 78, and 46%, respectively, vs. hypoxia-reoxygenation alone (Fig. 5, A–D).

Restoration of $\beta$-adrenergic signaling after hypoxia-reoxygenation following pretreatment with PEG. $\beta$-Adrenergic receptor ($\beta$-AR) signaling is the most critical pathway for the regulation of myocardial function. We investigated the acute effects of hypoxia-reoxygenation on cardiac myocyte $\beta$-AR signaling by measuring basal and $\beta$-agonist-stimulated cAMP production. Hypoxia-reoxygenation led to a significant blunting of both basal and isoproterenol-stimulated cAMP production, consistent with impaired $\beta$-AR signaling (Fig. 6). In contrast, treatment with PEG before hypoxia-reoxygenation led to restoration of isoproterenol-stimulated cAMP production, which was not different from control myocytes not exposed to hypoxia and reoxygenation. These data suggest that $\beta$-AR signaling may be preserved as a result of membrane stabilization.

PEG reduces apoptosis and improves LV systolic and diastolic function after global ischemia and reperfusion. To explore the potential beneficial effects of PEG at the whole organ level, we utilized an ex vivo Langendorff perfusion model and a prolonged period of hypothermic ischemia with warm reperfusion.
erfusion as relevant to cardiac transplantation. Following a period of baseline perfusion with oxygenated Krebs solution, isolated adult rat hearts were treated with 3 ml of 1% PEG solution delivered into the aortic root, or an equal volume of PBS as control. The hearts were then stored at 4°C in PBS for 3 h followed by normothermic reperfusion for 1 h. TUNEL staining revealed a marked reduction in apoptotic cells in the LV in the PEG-treated group compared with control (Fig. 7A). Importantly, pretreatment with PEG preserved LV systolic and diastolic function following the cold ischemia and warm reperfusion period (Table 1). It appears that the beneficial effects of PEG on cardiac myocyte survival in vitro may translate into improved ventricular function following ischemia and reperfusion.

DISCUSSION

In the present study, we demonstrate that pretreatment with PEG 15–20 significantly inhibits hypoxia-reoxygenation-induced apoptosis in primary cultures of rat ventricular cardiac myocytes. Treatment with PEG led to less cytochrome c release from the inner mitochondrial membrane and decreased intracellular ROS production. PEG also upregulated prosurvival signaling through enhanced phosphorylation and activation of ERK1/2, Akt, and GSK-3β. In addition, β-AR signaling, a critical pathway regulating cardiac function, is maintained following hypoxia-reoxygenation injury with PEG treatment as measured by intracellular cAMP generation in response to β-agonist stimulation. These protective mechanisms of PEG in cardiac myocytes also appeared to lead to global myocardial protection in the intact isolated adult rat heart when subjected to hypothermic ischemia and warm reperfusion injury. Cardiac myocyte apoptosis was significantly diminished, and LV function was maintained relative to hearts that did not receive PEG pretreatment.

Our data also showed that PEG pretreatment of cardiac myocytes inhibited lipid-raft coalescence following hypoxia-reoxygenation, and this may represent a central mechanism of PEG-mediated cardioprotection. Lipid-raft coalescence in association with apoptosis is a known sequela of cell injury such as that induced by radiation in human head and neck squamous carcinoma cells (3). There is a vast literature supporting the concept that membrane lipids and proteins are found in microdomains and that certain lipids such as cholesterol and sphingolipids selectively localize in lipid raft domains (10, 16). Previous studies have demonstrated that the bioactive sphingolipid metabolite sphingosine-1-phosphate (S1P) is involved in cardiac myocyte survival signaling during proapoptotic stress in vitro and in cardioprotection achieved through ischemic preconditioning in ex vivo perfusion studies (14, 25). A recent study showed a decline in sphingosine kinase activity in the remote myocardium in the post-myocardial infarction rat heart following coronary artery ligation, and phosphorylation of Akt, a downstream prosurvival kinase activated by S1P1 receptor signaling (24), was also depressed at 1 wk post-myocardial infarction (23). PEG treatment, in our studies, led to increased phosphorylation and activation of Akt, presumably through stabilization of lipid-raft signaling. Another recent study showed that PEG 15–20 given orally to rats can protect the intestine from radiation injury (22), and a common mechanism appears to be inhibition of lipid-raft coalescence and preservation of membrane integrity. Additionally, treat-

Table 1. Left ventricular function at baseline conditions and 30 min after ischemia-reperfusion

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (B)</th>
<th>PEG (B)</th>
<th>Control (I-R)</th>
<th>PEG (I-R)</th>
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<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>323 ± 22</td>
<td>326 ± 25</td>
<td>315 ± 27</td>
<td>320 ± 26</td>
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<td>LVDP, mmHg</td>
<td>98.3 ± 12.3</td>
<td>99.2 ± 14.5</td>
<td>59.8 ± 10.5*</td>
<td>83.2 ± 14.0*</td>
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<td>+dP/dtmax, mmHg/s</td>
<td>2968.3 ± 265.4</td>
<td>2915.2 ± 281.1</td>
<td>1910.7 ± 272.8*</td>
<td>2619.8 ± 301.2*</td>
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<tr>
<td>−dP/dtmin, mmHg/s</td>
<td>2659.6 ± 311.2</td>
<td>2598.1 ± 277.8</td>
<td>1584.1 ± 294.4*</td>
<td>2143.6 ± 285.9*</td>
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<tr>
<td>Coronary flow, ml/min</td>
<td>14.9 ± 3.8</td>
<td>15.1 ± 3.3</td>
<td>10.5 ± 2.7</td>
<td>13.0 ± 2.4</td>
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</table>

Values are means ± SE. B: baseline; PEG, polyethylene glycol; I-R, following ischemia-reperfusion; LVDP, left ventricular developed pressure; dP/dt, first derivative of ventricular pressure. N = 6 in each group. *P < 0.02 vs. control (B). †P < 0.03 vs. control (I-R).
ment of rat lung microvascular endothelial cells with bovine serum albumin, which binds and activates albumin-binding glycoprotein gp60, led to phosphorylation and activation of Akt (13). Caveolae internalization appears to be an important mechanism in this process. PEG 15–20 may have a similar mechanism in the activation of cell survival signaling in cardiac myocytes. G protein-coupled receptors are also found within lipid-raft domains in addition to other proteins that regulate cAMP generation, particularly in cardiovascular cells (9, 7). Intracellular cAMP production in response to β-agonist stimulation was enhanced by PEG pretreatment in cardiac myocytes following hypoxia-reoxygenation. Preservation of β-adrenergic signaling in these myocytes may also be due to membrane stabilization resulting from inhibition of lipid-raft coalescence following treatment with PEG. Previous studies have demonstrated positive effects of cardiac-specific overexpression of adenylyl cyclase type VI with increased catecholamine-stimulated cAMP production in transgenic mice following myocardial infarction (20). These mice had less adverse LV remodeling, enhanced contractile function, and significantly lower postinfarction mortality compared with nontransgenic controls. In contrast, sustained β-adrenergic activation induces cardiac myocyte apoptosis, maladaptive remodeling, and HF (19). In the present study, basal cAMP production was not altered by PEG pretreatment under either normoxic conditions or following hypoxia-reoxygenation; however, β-agonist-stimulated cAMP production was enhanced by PEG pretreatment following hypoxia-reoxygenation.

PEG has been used both experimentally and clinically for heart preservation in the transplantation setting. The only reported clinical study utilized standard cardioplegic solution with 5% PEG 20 during the donor heart procurement in 22 patients (4). This led to a significant decline in rejection episodes during the first year after transplant compared with standard cardioplegic solution that was routinely used. Although mechanisms for this finding were not investigated, it was hypothesized that there may have been less preservation injury with the addition of PEG. Extended periods of myocardial preservation have been achieved using a novel polyethylene glycolated hemoglobin perfusate-based solution in experimental studies (11). Rabbit hearts were harvested after cold cardioplegic arrest and then underwent cold ischemic storage or continuous warm perfusion with a PEG-hemoglobin solution. LV function, measured ex vivo, was markedly better after 24 h of warm perfusion with PEG-hemoglobin solution compared with 4 h of standard hypothermic ischemic storage. These studies demonstrate that PEG has the potential to be used clinically to decrease ischemia-reperfusion injury in the heart and other organs.

Our study is the first to identify a potential mechanism for the improved cardiac function after ischemia-reperfusion injury following pretreatment with PEG. Stabilization of membrane rafts, activation of prosurvival signaling pathways, and inhibition of ROS production and apoptosis all appear to be important cellular events in PEG-mediated cardioprotection. Further investigation into the use of PEG to minimize acute ischemia-reperfusion injury in the setting of coronary artery disease and cardiac preservation for transplant is warranted.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


