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Original Contribution

Hypothermic preconditioning of endothelial cells attenuates cold-induced injury by a ferritin-dependent process

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ABSTRACT

Hypothermia for myocardial protection or storage of vascular grafts may damage the endothelium and impair vascular function upon reperfusion/rewarming. Catalytic iron pools and oxidative stress are important mediators of cold-induced endothelial injury. Because endothelial cells are highly adaptive, we hypothesized that hypothermic preconditioning (HPC) protects cells at 0°C by a heme oxygenase-1 (HO-1) and ferritin-dependent mechanism. Storage of human coronary artery endothelial cells at 0°C caused the release of lactate dehydrogenase, increases in bleomycin-detectible iron (BDI), and increases in the ratio of oxidized/reduced glutathione, signifying oxidative stress. Hypoxia increased injury at 0°C but did not increase BDI or oxidative stress further. HPC at 25°C for 15–72 h attenuated these changes by an amount achievable by pretreating cells with 10–20 μM deferoxamine, an iron chelator, and protected cell viability. Treating cells with hemin chloride at 37°C transiently increased intracellular heme, HO-1, BDI, and ferritin. Elevated heme/iron sensitized cells to 0°C but ferritin was protective. HPC increased iron maximally after 2 h at 25°C and ferritin levels peaked after 15 h. HO-1 was not induced. When HPC-mediated increases in ferritin were blocked by deferoxamine, protection at 0°C was diminished. We conclude that HPC-mediated endothelial protection from hypothermic injury is an iron- and ferritin-dependent process.

Hypothermia is routinely used during cardiovascular surgery and transplantation to reduce myocardial oxygen consumption and slow the rate of ATP depletion in the ischemic heart. Damage to the endothelium during this time contributes to early low coronary flow [1] and vasospasm [2], secondary injury to the myocardium [3], and the development of cardiac allograft vasculopathy in heart recipients [4]. Longer periods of cold ischemia, such as in the storage of heart valves or vascular allografts, result in the loss of endothelial cells [5,6] and poor graft patency after implantation [7]. The extent to which these outcomes are a consequence of ischemia or of hypothermia per se remains unclear. Understanding the role of hypothermia in endothelial cell injuries and investigating new protective measures are therefore of clinical importance.

Cell culture experiments allow the examination of pure hypothermic effects without hypoxia, which invariably occurs during the preparation and storage of isolated organs and tissues. Studies with cultured endothelial cells have shown that hypothermia by itself impairs nitric oxide production [8], activates stress (MAPK) pathways and transcription factors [9,10], and induces apoptosis, which is observed during subsequent rewarming [11]. Importantly, it has been determined that intracellular reactive oxygen species are mediators of cold-induced endothelial cell injury, and catalytic iron pools are central to this injury process [12,13]. Sequestration of iron with deferoxamine (DFO) [13,14] or by induction of heme oxygenase (HO-1)/ferritin synthesis [15] attenuates iron-specific hypothermic injury in cultured endothelial cells, fibroblasts, and renal tubular cells, respectively. Cold-induced iron-mediated oxidative injury of the endothelium has potential clinical relevance as DFO improved coronary flow in rat hearts preserved by cold perfusion with an oxygenated medium [16], inhibited endothelial injury in cold-ischemic porcine aortic segments [17], and prevented an increase in coronary artery vascular resistance in isolated rat hearts after cold-ischemic storage [18,19].

It is well known that endothelial cells are adaptive to numerous forms of stress including heat shock, hypoxia, mechanical stress, and substrate deprivation [20,21]. We theorized that endothelial cells in tissue culture might adapt to hypothermia and that understanding the contributing mechanisms could lead to new strategies for preserving endothelial function. The objective of this study therefore was to determine if human coronary artery endothelial cells (HCAECs) could

Abbreviations: BDI, bleomycin-detectible iron; DFO, deferoxamine; EBM, endothelial basal medium; EGM, endothelial growth medium; GSH, reduced glutathione; GSSG, oxidized glutathione; t-GSH, total glutathione; HBSS, Hanks' balanced salt solution; HCAEC, human coronary artery endothelial cell; HO-1, heme oxygenase-1; HPC, hypothermic preconditioning; IRE-BP, iron responsive element-binding protein; LDH, lactate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; UW, University of Wisconsin.

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be preconditioned in vitro with mild–moderate hypothermia to induce tolerance to more severe hypothermia. We hypothesized that hypothermic preconditioning (HPC) would reduce subsequent iron-mediated oxidative injury at 0°C, a temperature commonly attained by vascular grafts or the isolated heart when stored on ice. The results of our study demonstrate that optimal preconditioning of HCAECs occurs at 25°C by an iron- and ferritin-dependent process that attenuates the subsequent increase in catalytic iron, oxidative stress, and injury at 0°C.

Materials and methods

Cell culture

Proliferating human coronary artery endothelial cells (passage 4) were obtained from Clonetics and maintained at 37°C in an atmosphere of 95% air+5% carbon dioxide using EGM2-MV medium (also from Clonetics). This medium will henceforth be referred to as endothelial growth medium or EGM. In preparation for hypothermia experiments, cells (passages 5 to 10) were seeded into culture flasks at a density of 13,000 viable cells/cm^2 (12.5- or 25-cm^2 culture flask; Falcon). After 3 to 4 days of culture, cells formed a confluent monolayer and were used for HPC or control treatments. Preconditioning was performed by maintaining confluent cells in EGM at 20, 25, or 30°C in a low-temperature CO_2 incubator, whereas control cells were maintained at 37°C. For comparative purposes, some cells were preconditioned at 20 or 30°C by placing culture flasks on a circulating water tray within the 25°C incubator and all temperatures were monitored with thermocouples placed within additional dummy flasks. All cells were given fresh medium daily during HPC or control treatments.

0°C hypothermia treatments

HPC or control cells in 12.5-cm^2 culture flasks were washed with Hanks' balanced salt solution (HBSS) and flasks received 1.5 ml of endothelial basal medium (EBM) and were stored in a circulating water bath at 0°C, a temperature commonly achieved by vascular grafts or the isolated heart when stored on ice. At the appropriate times, flasks were warmed to 37°C for 3 h because the cold-injured cells experience additional injury during rewarming. The medium from each flask was collected and cells were rinsed with HBSS and then lysed with 2 ml of 1% Triton X-100 in HBSS. The intracellular enzyme lactate dehydrogenase (LDH) released by damaged or necrotic cells was measured in the storage medium and in cell lysates using a commercially available kit (Roche Pharmaceuticals) and hypothermia-induced cell injury was expressed as the percentage release of total LDH.

Optimization of hypothermic preconditioning

To determine the effect of HPC temperature on cell damage during subsequent storage at 0°C, confluent HCAECs in 12.5-cm^2 flasks were incubated at 20, 25, 30, or 37°C for 24 h in EGM. After the HPC treatment, cells were warmed to 37°C for 3 h and then stored at 0°C in EBM for up to 96 h using the procedure previously described. The effect of HPC duration on cell injury at 0°C was determined by preconditioning cells at 25°C for 0, 14, 24, 48, or 72 h before storage at 0°C. For 48 or 72 h HPC duration, the medium was replaced with fresh EGM every 24 h. To determine if 37°C warming after HPC is required to maximize the protective response or if warming reverses the protection, cells were preconditioned at 25°C for 72 h, warmed to 37°C for up to 48 h, and then stored at 0°C. Cell damage was estimated with the LDH assay described above. Preconditioned cells in culture flasks and control cells maintained at 37°C were observed by light microscopy (Olympus CK40 inverted microscope) and photographed (MagnaFire digital camera) before and after storage at 0°C.

HPC and protection of cell viability

Protection of cell viability by HPC was determined by comparing growth curves of HPC and control cells before or after 0°C storage. Briefly, cells were rewarmed from 0 to 37°C for 3 h (or treated with EBM at 37°C/3 h for nonstored controls), trypsinized, counted with trypan blue, and diluted in EGM. Twenty thousand trypan blue-excluding cells were seeded per well in triplicates of three 24-well culture plates and cultured at 37°C for 24, 48, or 72 h. Cells were then washed with HBSS and incubated with 1 mg/ml MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl tetrazolium bromide; assay kit from Sigma) in HBSS for 4 h at 37°C. MTT is reduced by mitochondrial dehydrogenases of viable cells to produce purple formazan crystals [22]. The formazan crystals were dissolved in acidified isopropanol, the solutions were transferred to a 96-well plate, and absorbance was measured at 570 nm using a plate reader (Versamax; Molecular Devices). The absorbance was linearly proportional to the number of cells seeded between 10,000 and 60,000.

Protection by HPC and protection from oxidative or osmotic injury

The protection of cells at 0°C by HPC was compared to reductions in cold-induced oxidative injury with the iron chelator DFO or the lipophilic antioxidant vitamin E and to prevention of cold-induced osmotic injury with University of Wisconsin solution (UW). Cells were preconditioned at 25°C or maintained at 37°C for 72 h. A subset of the latter group received 50 μM DFO or vitamin E in EGM, whereas the remaining cells at 37 or 25°C received fresh EGM only. After 3 h incubation at 37 or 25°C, all cells were washed with HBSS and stored at 0°C in EBM, DFO/EBM, UW, or DFO/UW (see Fig. 4A). Because UW solution is potentially toxic [23] and decreases coronary flow in isolated rat hearts at 21–25°C [24,25], the cold storage medium, UW or EBM, was added to flasks cold, was removed at the end of cold storage and saved before rewarming flasks, and was replaced with 1.5 ml of cold EBM. The %LDH released was therefore calculated as the sum of the LDH measured in the cold storage medium plus the rewarming medium divided by the total LDH.

Effect of HPC or iron chelation on iron-mediated injury at 0°C

The effect of HPC on iron-mediated hypothermic injury was determined by comparing intracellular catalytic iron levels and LDH released by cold-stored cells that had been previously preconditioned or treated with DFO. To examine iron-mediated changes during hypothermia, confluent cells in 25-cm^2 culture flasks were preconditioned in EGM at 25°C for 24 h or maintained at 37°C for 24 h. A subset of flasks from the 37°C treatment was then treated with 0, 10, 20, or 50 μM DFO in EGM for 3 h/37°C. All cells were washed with HBSS and flasks received EBM and were stored at 0°C for up to 120 h. Flasks were removed from 0°C and cells were immediately washed with Chelex-treated HBSS at 0°C to remove iron released by damaged cells. Cells were then lysed with 300 μl of 1% Triton X-100 in Chelex-treated 20 mM Tris buffer (pH 7.4) and intracellular catalytic iron was measured in lysates with a bleomycin assay [13,26]. Confluent cells in 12.5-cm^2 flasks were similarly preconditioned or treated with DFO and stored at 0°C for up to 120 h, and the LDH released into the medium or retained by cells was measured without allowing the cells to rewarm.

Catalytic iron measurements with bleomycin

Catalytically active iron was measured using bleomycin [13,26]. Briefly, the assay involved sequential addition of 0.25 ml of calf thymus DNA (1 mg/ml), 0.05 ml of 5 mM $MgCl_2$, 0.025 ml of bleomycin sulfate (1 mg/ml), 0.10 ml of a 0.25–20 μM $FeCl_3$ standard or sample, and 0.05 ml of ascorbic acid (8 mM). The test tubes were