Hypothermia-induced cardioprotection using extended ischemia and early reperfusion cooling

Zuo-Hui Shao,1 Wei-Tien Chang,3 Kim Chai Chan,4 Kim R. Wojciek,1,2 Chin-Wang Hsu,5 Chang-Qing Li,1 Juan Li,1 Travis Anderson,1 Yimin Qin,1,2 Lance B. Becker,1 Kimm J. Hamann,1,2* and Terry L. Vanden Hoek1*

1The Emergency Resuscitation Center, Sections of Emergency Medicine and 2Pulmonary/Critical Care, Department of Medicine, University of Chicago, Chicago, Illinois; 3Department of Emergency Medicine, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan, China; 4Emergency Department, Tan Tock Seng Hospital, Singapore; and 5Emergency Department, Tri-Service General Hospital, National Defense Medical Center, Taiwan, China

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longed ischemia. However, these results raise the question of whether ischemia could be safely extended in duration for the purpose of optimizing end-ischemia conditions (e.g., temperature) for reperfusion. In addition, the mechanism of therapeutic hypothermia protection is not completely known. However, this cardiomyocyte I/R model does demonstrate significant nitric oxide (NO) signaling and protein kinase C (PKC) and NO synthase (NOS)-mediated cardioprotective adaptation (21, 33).

Thus we hypothesized that therapeutic hypothermia would be significantly protective in this model of I/R when induced at end-ischemia and maintained for 1 h into reperfusion. Furthermore, this protection would be preserved even when end-ischemia was extended for the purpose of reaching target temperature before reperfusion. Finally, we hypothesized that hypothermia protection would be associated with PKCe-mediated NOS activation and NO production.

MATERIALS AND METHODS

**Chemicals.** Nω-nitro-L-arginine methyl ester (L-NAME), chelerythrine chloride, 2-deoxyglucose, and digitonin were obtained from Sigma (St. Louis, MO). Meristoylated PKC peptide inhibitor myr-PKCε v1-2 was purchased from Biomol (Plymouth Meeting, PA). The BD ApoAlert Caspase Profiling Plate was purchased from Becton Dickinson (Palo Alto, CA).

**Cardiomyocyte culture.** Embryonic chick ventricular cardiomyocytes from 10-day-old chicken embryo hearts were prepared, as previously described (33, 36). Experiments were performed with 3- to 5-day cell cultures, by which time a synchronously contracting layer of cells could be visualized with viability exceeding 95%.

**Perfusion system.** Coverslips with contracting cells were placed in a 1.2-ml Sykes-Moore perfusion chamber (Belco Glass, Vineland, NJ), as described in previous work (33). Tubing to these chambers was made of stainless steel and PharMed polymer (Cole-Parmer Instrument, Chicago, IL) to minimize oxygen leaks.

**Perfusate composition for simulated I/R.** Perfusate for equilibration and simulated reperfusion consisted of balanced salt solution (BSS) with 149 Torr Po2, 40 Torr PCO2, pH 7.4, 4.0 meq/l [K+], and 5.6 mM glucose. The term "simulated reperfusion" is used (shortened to "reperfusion" later) to clarify that only some aspects of reperfusion (normalization of oxygen, carbon dioxide, pH, potassium, and substrate) are studied in this model, and other aspects of I/R injury related to changes in flow cannot be studied due to the use of a flow-through system. Such a flow-through system allows for rapid changes in gas tensions and substrate supply and for maintenance of low oxygen tensions while observing the same field of cells over time. Ischemic BSS contained no glucose plus 2-deoxyglucose (20 mM) to inhibit glycolysis, 8.0 meq/l [K+], and bubbled with 80% N2-20% CO2 to produce BSS with 3–5 Torr Po2, 144 Torr PCO2, and pH 6.8. Ischemic conditions were simulated and verified as before in our cardiomyocyte model (3, 28, 33).

**Video/fluorescent microscopy.** A Nikon TE 2000-U inverted phase/epifluorescent microscope was used for cell imaging. Phase contrast Hoffman modulation optics and a charge-coupled device camera were used to monitor contractions and membrane changes over time in the same field of cells (~70 × 90 μm). Fluorescent images were acquired from a cooled Hamamatsu slow-scanning PC-controlled camera (Photometrics, Cool-SNAP), and changes in fluorescent intensity over time were quantified with MetaMorph software (Universal Imaging, Downingtown, PA).

**Viability assay.** Cell viability was assessed with the exclusion fluorescent dye propidium iodide (PI, 5 μM; Invitrogen, Gland Island, New York) measured at excitation 540 nm/emission 590 nm (5). The dye was used to quantify cell death throughout the entire experiment in a selected field of cardiomyocytes. All cells in the field studied were stained with PI at the end of the experiment by permeabilizing with digitonin (300 μM). Cell death was expressed as the PI fluorescence at any given time point relative to the maximal value seen after digitonin exposure (100%).

**Cell contraction.** Cell contractions as a useful index of function were visualized intermittently within the same field of cells, as previously reported, using phase contrast Hoffman modulation optics (34).

**Measurement of intracellular NO production.** Intracellular NO production was determined using 4,5-diaminofluorescein diacetate (DAF-2 DA, 1 μM; EMD Biosciences, San Diego, CA). DAF-2 DA is a specific NO indicator that can penetrate rapidly into the cell where it is hydrolyzed to diaminofluorescein (DAF-2) by intracellular esterases (20). DAF-2 selectively traps NO, yielding fluorescent triazolofluorescein (excitation 480 nm/emission 520 nm) (25). DAF-2 DA has proven useful in detecting rapid sequential NO generation in our cardiomyocytes (21).

**Caspase activity assay.** Cardiomyocytes exposed to I/R insult were taken at selected times, lysed, and then stored at ~80°C until analysis. The BD ApoAlert Caspase Profiling assay was performed, as previously described (28).

**DNA fragmentation analysis.** Genomic DNA was extracted using a DNA extraction kit (Qiagen, Valencia, CA) (19). Briefly, ~4 × 106 chick cardiomyocytes were harvested and washed twice with cold PBS. Buffer C1 was then added to lyse the cells and preserve the nuclei. Buffer G2 with RNase A was used to lyse the nuclei and denature proteins, and RNA was further digested into smaller fragments by incubation with Qiagen protease at 50°C for 1 h. The mixture was then entered into an equilibrated Qiagen Genomic-tip. After being washed and eluted, the DNA was precipitated by isopropanol and washed with ethanol. The extracted DNA (3–5 μg) was loaded onto a 2% agarose gel, run at 80 V for 45 min in Tris-acetate-EDTA (pH 8.3) buffer, and visualized with ethidium bromide using ultraviolet illumination and photographed by a LAS-3000 Imaging System (Fujifilm).

**Therapeutic hypothermia I/R protocols.** To establish the optimal hypothermic target temperature, additional experiments were performed using coverslips placed in sealed glass chambers filled with BSS. These chambers were placed within a water bath that controlled temperatures to 15°C, 25°C, 30°C or 37°C. Cardiomyocytes were exposed to simulated ischemia for 1 h and then reperfused for 1 h at either 15°C, 25°C, 30°C, or normothermic 37°C. All coverslips were then incubated at 37°C for another 2 h. Resulting cell death at 3-h reperfusion was then compared. As shown in Fig. 1, the hypothermia target temperature of 25°C imparted the greatest protection against reperfusion injury (P < 0.0001), although the difference between 25°C and 30°C was not statistically significant (P = 0.22). Interestingly, the target temperature of 15°C was not protective. Thus the target temperature used for further studies was 25°C.

Three distinct cooling protocols were used to explore the optimal timing for inducing hypothermia (25°C) relative to I/R (Fig. 2): 1) reperfusion cooling (RC), in which cells were subjected to 1-h normothermic (37°C) ischemia followed by 1-h hypothermic (25°C) reperfusion; 2) delayed cooling (DC), which consisted of 1-h normothermic ischemia and 15-min normothermic reperfusion followed by 1-h hypothermic reperfusion; and 3) ischemic cooling (IC), which consisted of 1-h normothermic ischemia with an extended 10- or 30-min hypothermic ischemia (i.e., total ischemia time of 70 or 90 min), followed by 1-h hypothermic reperfusion. The process of cooling from 37°C to 25°C required 4–5 min. All cooling protocols maintained the target temperature during reperfusion for 1 h and rewarmed to 37°C for an additional 2 h of reperfusion. For normothermic I/R controls, cells were subjected to ischemia (60, 70, or 90 min) followed by 3-h reperfusion, with the temperature maintained throughout at 37°C.

**Statistical analysis.** A field of ~500 cells was observed for each experiment. Treatment and control groups were used in sets containing cells isolated and cultured on the same day to eliminate variability due to cell batch. Additional coverslips were used for replicate
experiments (n, number of experiments). Results were expressed as means ± SE. For serial measurement data, a two-way, repeated-measures ANOVA was performed with Tukey post hoc analysis. The P value represents the difference between the two study groups, whereas the differences at each time point were demonstrated by marks in the graphs. P < 0.05 was considered statistically significant.

RESULTS

Timing of cooling induction: RC versus IC. As shown in Fig. 3A, 1-h ischemia/3-h reperfusion at 37°C resulted in 49.7 ± 3.4% (n = 7) cell death in this chick cardiomyocyte model. When compared with the normothermic I/R control, RC significantly decreased cell death to 22.4 ± 2.9% (n = 5, P < 0.001). However, despite similar cooling (to 25°C for 1 h) during reperfusion, the DC protocol caused 45.0 ± 8.2% cell death (n = 3), a result not significantly different from that in I/R control [P = not significant (NS); Fig. 3B].

In contrast, the IC protocol, which extended end-ischemia for 10 min to allow cooling to target temperature before reperfusion, decreased cell death to 14.3 ± 0.6% (n = 5, Fig. 3C). This was not only significantly lower than that in the normothermic I/R control with 70-min ischemia (62.0 ± 3.9%, n = 3, P < 0.001; Fig. 3C) but also lower than the cell death in I/R with 60-min ischemia (49.7 ± 3.4%, P < 0.01; Fig. 3A), despite the 10 min of lengthened ischemia. IC also improved cell survival compared with the RC group (22.4 ± 2.9%, P < 0.05; Fig. 3A). In addition, cells in the majority of IC (5/5) and RC (3/5) experiments had strong return of spontaneous synchronous contractions during reperfusion, as compared with only weak or no return of contractions in normothermic I/R controls (1/7 return of contraction in 60-min ischemic control; 0/3 in 70-min ischemic control). To test whether IC protection would be maintained by eliminating the reperfusion phase cooling, we performed IC during the last 10 min of ischemia only and started rewarming from the beginning of reperfusion. The results showed that premature rewarming of the IC protocol led to cell death of 56.6 ± 1.4%, which was not significantly different from the I/R control. This suggests that mediation of hypothermia protection necessitates not only the induction at the end-ischemic phase but maintenance of hypothermia during the early reperfusion phase, so that the protective mechanism can be completely activated and optimally sustained.

To test whether the protection conferred by hypothermia was longlasting, we further evaluated the cell survival at 24 h of reperfusion in IC and RC protocols. As shown in Fig. 4A, the differential protective effects between IC and RC became even more pronounced at 24 h of reperfusion. Whereas the protective effect conferred by IC was maintained up to 24 h (19.1 ± 4.5% vs. 56.6 ± 8.5% in I/R control; n = 3/each group, P < 0.001), RC lost most of its protection (52.9 ± 6.8%, n = 3, P = NS vs. I/R control), suggesting that IC was superior in terms of long-term protection. Moreover, IC protection was associated with attenuated DNA fragmentation at 24 h of reperfusion compared with the 24 h of I/R control group (n = 3; Fig. 4B). Whereas DNA fragmentation is not a strictly quantitative analysis, these studies suggest that hypothermia protection in the IC protocol was long lasting in the context of a primary cell culture system (these cardiomyocytes are studied at day 3–5 in culture) and that cooling reduces the apoptotic cell death due to I/R injury.

In additional IC experiments, the extended ischemia duration for cooling was lengthened further to 30 min to verify the importance of cooling relative to reperfusion (i.e., 60 min at 37°C plus 30 min at 25°C, for a total of 90-min ischemia). The results showed that, despite the 50% increase in ischemia duration, the cell death at 3-h reperfusion (28.6 ± 2.1%, n = 5) was not only significantly lower than that in normothermic I/R with 90-min ischemia (77.2 ± 3.4%, n = 4, P < 0.001) but also lower than that with 60-min ischemia (49.7 ± 3.4%, n = 7, P < 0.001; Fig. 5).

Mechanism of cooling: effect of hypothermia on NO generation. Since we previously found that cardiomyocytes can generate detectable levels of NO that mediate adaptive protection (21), we sought to study the effect of hypothermia on the production of endogenous NO. As shown in Fig. 6A, the level of DAF-2 fluorescence gradually increased during the ischemic phase in normothermic I/R. At reperfusion, a significant burst of DAF-2 fluorescence could be seen within 15 min, which then gradually decreased during the first hour of reperfusion.

Fig. 1. Optimal target temperature for therapeutic hypothermia in a chick cardiomyocyte model of ischemia-reperfusion (I/R). Multiple coverslips with cardiomyocytes were mounted in a simulated ischemia jar (37°C) for 1 h followed by 1-h reperfusion at 15°C (n = 14), 25°C (n = 25), 30°C (n = 14), and 37°C (n = 23). All coverslips were incubated at 37°C for another 2 h of reperfusion. PI, propidium iodide. Results are expressed as means ± SE. *P < 0.001 vs. normothermic I/R control.

Fig. 2. Experimental protocols for therapeutic hypothermia with the target temperature of 25°C. Cooling to target temperature occurred within 4–5 min, and hypothermia was maintained for 1 h of reperfusion before rewarming to 37°C. Two normothermic I/R controls were used, i.e., 60-min and 70-min ischemia. RC, reperfusion cooling; DC, delayed cooling; IC, ischemic cooling.
To test the effect of hypothermia on this pattern of NO generation during I/R, we first compared the RC group with the normothermic I/R control. As seen in Fig. 6A, whereas the peak of DAF-2 fluorescence at reperfusion was somewhat delayed in the RC protocol, the most significant change was the sustained, increased NO generation during subsequent reperfusion, as compared with the rapid decline in controls \( (n = 8/each \text{ group}, P < 0.001) \). Moreover, such increased DAF-2 fluorescence was continued even after rewarming. In contrast, if cooling was delayed by 15 min at reperfusion (the DC protocol), the DAF-2 fluorescence was no longer sustained after peaking, a pattern with no difference from that in normothermic I/R controls \( (n = 5/each \text{ group}; \text{Fig. 6B}) \).

Since IC exhibited the best cell protection against I/R injury, we further tested the effect of IC on NO generation during I/R. As shown in Fig. 6C, when temperature began to decrease during the last 10 min of ischemia, the increase in DAF-2 fluorescence became slightly attenuated. Also, the peak of NO at reperfusion tended to be lower and delayed, though not statistically different. After peaking, however, DAF-2 fluorescence appeared more sustained and was significantly higher than that in normothermic I/R controls, an effect that continued throughout reperfusion even after rewarming \( (n = 9/each \text{ group}, P < 0.001) \). Moreover, the level of DAF-2 fluorescence at reperfusion was also higher in IC compared with that in RC.
protection at 24 h was associated with attenuated apoptotic DNA fragmentation (Fig. 4B), we further tested the effect of hypothermia on caspase-3 activity and whether this effect could be reversed by L-NAME. As shown in Fig. 9, the

(Fig. 6B), corresponding with the better protection conferred by IC.

**Role of NOS in hypothermia-mediated NO generation.** To verify the relevance of this NO generation to hypothermia protection and to test whether it was derived from NOS, we treated cells with the nonselective NOS inhibitor L-NAME (200 μM) for 2 h before and during the entire course of the IC protocol. As seen in Fig. 7, L-NAME attenuated the increased DAF-2 fluorescence induced by hypothermia (n = 7/each group, P < 0.001; Fig. 7A) and abrogated a significant component of hypothermia protection conferred by IC (cell death 42.7 ± 1.3% in IC plus L-NAME, compared with 14.3 ± 0.6% in IC, n = 6/each group, P < 0.001; Fig. 7B). These results suggest that hypothermia protection is, in part, mediated by NOS activation with increased and sustained NO generation at reperfusion.

**Role of PKC in NO generation and hypothermia protection.** PKC has been implicated in a number of cardioprotective adaptive responses (27, 29). Specifically, PKCε is involved in the activation of NOS through the coupling of a three-tier PKCε-Akt-NOS complex (37). To test whether the hypothermia-mediated NO generation and protection seen in these cardiomyocytes were mediated via PKC activation, we used a nonselective PKC inhibitor, chelerythrine, and a specific PKCε inhibitor, myr-PKCε v1-2, in the IC protocol (14). As shown in Fig. 8A, administration of chelerythrine (2 μM) during I/R significantly attenuated the increased DAF-2 fluorescence induced by hypothermia (P < 0.001, n = 3/each group). Co-treatment of myr-PKCε v1-2 (5 μM) during I/R also attenuated the IC-induced DAF-2 fluorescence increase at reperfusion (P < 0.001, n = 7/each group; Fig. 8B). Furthermore, myr-PKCε v1-2 (5 μM) significantly reversed the protection conferred by IC (cell death 44.3 ± 9.8% vs. 15.9 ± 2.1% in IC, n = 5/each group, P < 0.001; Fig. 8C) without significantly affecting normothermic cell death (data not shown), suggesting that PKCε participates in the hypothermia-associated NO signaling and protection against I/R injury.

**Effect of hypothermia on caspase-3 activity.** Our past work suggests that a significant component of reperfusion injury seen in this cardiomyocyte model of I/R is related to caspase activation and apoptosis (28, 32, 35). Given that hypothermia
caspase-3 activity by 3-h reperfusion in the normothermic I/R group had increased $6.8 \pm 1.7$-fold from the end of ischemia ($n = 4$). In the IC group, such activation was significantly attenuated to $2.8 \pm 0.7$-fold ($n = 4, P < 0.05$). IC cells treated with L-NAME (200 μM) showed a significant reversal of attenuated caspase-3 activation with a $5.0 \pm 1.4$-fold increase by 3-h reperfusion. This level was significantly higher than that in IC ($P < 0.05$) and not statistically different from that in normothermic I/R group ($P = 0.22$).

**DISCUSSION**

In the present study of chick cardiomyocytes exposed to a severe I/R insult, therapeutic hypothermia was most protective when induced at end-ischemia and maintained for 1 h at reperfusion. Protection was evidenced by decreased cell death after 3 and 24 h of reperfusion, improved recovery of spontaneous synchronous contractions, increased NO generation at reperfusion, and attenuated both caspase-3 activation and DNA fragmentation (Figs. 3, 4, 6, 7, and 9). Hypothermia protection was lost if end-ischemia cooling was not maintained into reperfusion or if reperfusion cooling was delayed by as little as 15 min after reperfusion (Fig. 3B). These results suggest that cellular conditions (such as temperature) that occur during the transition from ischemia to reperfusion are extremely critical for the outcome after I/R. Indeed, end-ischemia cooling that increases the duration of ischemia—for the purpose of reaching
target temperature before reperfusion—appeared more protective than immediate reperfusion with rapid cooling.

Inhibition of PKC and NOS attenuated the NO increase and protective effect induced by hypothermia (Fig. 8). Thus the mechanism of therapeutic hypothermia protection appears to involve PKC-mediated NOS activation and increased NO generation, with associated attenuation of caspase-3 activation, decreased reperfusion injury, and cell death with improved recovery of spontaneous contraction.

**Optimal cooling target temperature.** This study focused on therapeutic hypothermia, i.e., cooling after warm ischemia has already occurred. Surgical and accidental hypothermia represents cooling that occurs intentionally or accidentally before the onset of organ or whole body ischemia. Our studies suggested that, after a severe ischemic insult to cardiomyocytes (expected to cause up to 50% cell death), induced therapeutic hypothermia to a target temperature of 25°C is significantly protective (Fig. 2). With regard to target temperature, it was not the primary focus of this particular study to test every possible temperature, since different cell types likely vary in response. In addition, in vivo effects (e.g., capillary flow) will likely determine optimal target temperature apart from what may be optimal at the cell level. However, we sought to find a temperature highly protective in this particular cell model that would further facilitate the study of optimal timing and mechanism of cooling. While this target temperature may be too low for application in AMI patients, it would be attainable in cardiac arrest patients placed on cardiopulmonary bypass. For example, this target temperature is consistent with the work by Nozari et al. (26), who studied the therapeutic effect of hypothermia (either 27°C or 34°C vs. normothermia) induced after 20 min of warm global cardiac ischemia in a canine model of ventricular fibrillation. In this cardiac arrest model, 27°C was found to be cardioprotective and neuroprotective. In our own mouse cardiac arrest model, 30°C was cardioprotective and neuroprotective (1). In addition, many of the studies conducted in models of AMI actually attained average heart temperatures below 30°C (17). Finally, a better understanding of hypothermia mechanisms of action would allow for enhancement of therapeutic hypothermia, no matter what target temperature is utilized.

**Optimal timing of cooling.** The reported benefit of therapeutic hypothermia for I/R injury is mixed, possibly due to the differences in timing of cooling used relative to I/R. Indeed, the current cardiomyocyte study and our mouse cardiac arrest model of postarrest cardiovascular injury and death demonstrate both significant benefit and no benefit from hypothermia, depending on minor changes in the timing of cooling (1). In these models, the benefit of hypothermia is dramatically different when its induction relative to reperfusion is altered by even minutes. These results are consistent with the work by Hale et al. (15) suggesting that end-ischemia plus early reperfusion hypothermia are important for optimizing cooling benefit. They contrast their previous positive study, in which hypothermia was initiated 10 min before reperfusion and continued for 2 h after reperfusion (15), and a past negative study, in which cooling was induced 5 min before reperfusion and continued for only 15 min into reperfusion (16). They conclude that early hypothermia induction (a number of minutes during end-ischemia) and adequate duration (possibly hours into reperfusion) may be important for achieving hypothermia benefit. In contrast to these studies that used ischemia cooling plus reperfusion cooling, the study by Maeng et al. (24) used a regional myocardial cooling technique that did not cool to target temperature before reperfusion. Their technique rapidly reperfused the myocardium (using a catheter placed in the left main coronary artery with perfusion at 150 ml/min), reaching target temperatures minutes after reperfusion. Their negative result supports the notion that, after a potentially lethal period of ischemia, the conditions of end-ischemia/early reperfusion are critical to outcome. Thus hypothermia at end-ischemia with extension into early reperfusion may be more beneficial than reperfusion cooling alone. We believe that our work extends this body of work by reporting hypothermia protocols that have both protective and nonprotective effects in the same model of I/R, highlighting the importance of hypothermia timing. In addition, it is one of the first works we are aware of that purposely controls for shorter normothermic ischemia time when using end-ischemia cooling. The finding that hypothermia is highly protective despite more prolonged ischemia time (10–30 min in this model) suggests that, after considerable normothermic ischemia has already occurred, early cooling (even if ischemia needs to be extended) may be more important than early reperfusion.

These results are consistent with cooling protocols in larger animal models that demonstrate hypothermia protection. Endovascular cooling studies of therapeutic hypothermia for AMI targeted temperatures of 32°C–34°C before reperfusion therapy, with cooling continued into the reperfusion phase (8, 11, 31). Whereas significant protection is seen in these animal models, clinical studies have shown less profound protection. This lack of translation from animal to clinical studies could conceivably be due to the incomplete cooling that was achieved before reperfusion (9). Myocardial temperature at the time of angioplasty and coronary recanalization was often well above the target temperature (average of only 1.1°C drop before reperfusion). As a result, the ischemic myocardium was reperfused at a temperature above the cooling target, with further hypothermia induced after reperfusion (9, 18).

**Mechanisms of hypothermia protection.** Our results are some of the first to suggest that hypothermia protection against I/R injury is associated with activated PKC and NOS, in-
creased NO generation, and decreased caspase-3 activation after reperfusion. This result is consistent with a number of studies favoring the protective role of NO signaling in myocardium undergoing I/R (4, 13, 23). NO can react with various protein thiol moieties to produce distinct sulfur oxidation products that may alter protein structure, function, and cooperative properties (19). For example, NO can inhibit caspase activities through S-nitrosylation of an essential active site cysteine residue that is functionally conserved among these proteases (22). By inhibition of these “initiator” caspases and “executor” caspases, NO signaling can diminish the apoptotic process related to I/R injury. Although we do not yet have direct evidence of such NO activity, NOS activation and increased NO signaling appear to play a central role in the protection induced by therapeutic hypothermia in this cardiomyocyte model of I/R injury.

The role of NO signaling in hypothermia protection is further supported by the finding that PKCe inhibition abrogates both the NO generation and the cytoprotection conferred by hypothermia. PKCe plays an essential role in mediating a number of cardioprotective effects (27, 29, 37). Specifically, PKCe inhibition and its attenuation of hypothermia-associated NO generation seen in this study are consistent with prior work suggesting a signaling pathway involving PKCe-NOS-Akt as a functional proteome module mediating NO generation and cardioprotection (37). PKCe via its phosphorylation of Akt may increase NOS activation and cardioprotection due to downstream NO signaling (37). Although the role of Akt was not specifically tested in the current study, the prominence of increased NO generation and decreased apoptosis as associated hallmarks of hypothermia protection—with both pathways known to be regulated by Akt signaling—is consistent with its involvement. Further work is needed to study its role in hypothermia protection.

Regarding the role of apoptosis in this cardiomyocyte model of I/R injury, past work has demonstrated that cytochrome c release occurs within minutes of reperfusion (as compared with extended ischemia), an event mediated by a burst of mitochondrial oxidants from complex III (3) and H$_2$O$_2$-Fe$^{2+}$-mediated oxidant stress and caspase-2 activation (28, 32, 35). Caspase-9 activation and then executioner caspase activation occur downstream of these events. Caspase inhibitors given concurrently with reperfusion significantly decrease caspase activation, attenuate cell death by 3-h reperfusion, and improve return of spontaneous synchronous contractions. Thus the association of hypothermia protection with decreased caspase activation by 3-h reperfusion is consistent with this prior work and further supports the role of ant apoptotic pathways in mediating hypothermia protection. It is possible that some of necrotic death occurs during ischemia alone, particularly during extended (4 h) ischemia, but like IC-mediated protection, caspase inhibition blocks reperfusion-induced cell death to these low (~10%) ischemic levels (see Fig. 3C and Refs. 30 and 36). This suggests, therefore, that hypothermia protects primarily by blocking apoptotic pathways.

In conclusion, therapeutic hypothermia in our cardiomyocyte model of severe postresuscitation injury is most protective when started during 10 min-extended ischemia and continued for 1 h into reperfusion. Such protection is completely lost if cooling is limited primarily to the end-ischemia or reperfusion phases. These results are consistent with our previous finding of intras ischemic cooling protection against postresuscitation injury in our mouse cardiac arrest model and with work by others demonstrating profound intras ischemic cooling protection against AMI. These studies suggest that cooling affects critical events in a transition period that encompasses both end-ischemia and early reperfusion. Thus optimal hypothermia protection against cardiac I/R injury may in some instances require prioritization of early cooling over early reperfusion.

This study is one of the first to report a purposeful extension of ischemia to better prepare cardiomyocytes for reperfusion. Such a strategy challenges the current paradigm of unconditional (i.e., regardless of ischemia time or metabolic condition of end-ischemia) early reperfusion therapy. Given this cell work and the animal AMI work by others, the question is raised of whether AMI patients would benefit more by being cooled completely to target temperature before revascularization, even if ischemia time is prolonged and reperfusion therapy delayed. These studies also raise questions about what other therapies could be used to “postcondition” ischemic cells before their reperfusion and what other end-ischemia target parameters (other than temperature) should be achieved before reperfusion.

Finally, agents that activate PKCe and NOS may be useful to enhance or replace hypothermia. Hypothetical activation of these signaling pathways contradicts the notion that cooling protection is a result of simply slowing all metabolic processes. Both the PKC and NO-signaling pathways have been implicated in preconditioning protection in these same cells (21), suggesting that therapeutic hypothermia is mediated by postconditioning pathways similar to those recently reported by others (7). Of course, translation into the in vivo setting is critical, and cooling may affect many other cell types in the heart, such as microvascular endothelial cells. This model cannot assess the “no-reflow” phenomenon associated with I/R injury. However, the prior demonstration of intras ischemic cooling protection against postresuscitation cardiovascular injury in our mouse model of cardiac arrest suggests that other cell types may be favorably impacted as well. A better understanding of these hypothermia-mediated signaling events could help monitor and possibly enhance therapeutic hypothermia protection in the future.

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