Limitations of Mild, Moderate, and Profound Hypothermia in Protecting Developing Hippocampal Neurons After Simulated Ischemia

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Mild hypothermia (33°C–34°C) after cerebral ischemia in intact animals or ischemia-like conditions *in vitro* reduces neuron death. However, it is now clear that more profound hypothermia or delayed hypothermia may not provide significant protection. To further define the limitations of hypothermia after cerebral ischemia, we used hippocampal slice cultures to examine the effects of various degrees, durations, and delays of hypothermia on neuron death after an ischemia-like insult. Organotypic cultures of the hippocampus from 7- to 8 day-old rat pups were cooled to 32°C, 23°C, 17°C, or 4°C immediately or after a 2–4 hour delay from an injurious insult of oxygen and glucose deprivation (OGD). Cell death in CA1, CA3 and dentate regions of the cultures was assessed 24 hours later with SYTOX[®] or propidium iodide, both of which are fluorescent markers labeling damaged cells. OGD caused extensive cell death in CA1, CA3, and dentate regions of the hippocampal cultures. Hypothermia (32°C, 23°C and 17°C) for 4–6 hours immediately after OGD was protective at 24 hours, but when hypothermia was applied for longer periods or delayed after OGD, no protection or increased death was seen. Ultra-profound hypothermia (4°C) increased cell death in all cell areas of the hippocampus even when after a milder insult of only hypoxia. In an *in vitro* model of recovery after an ischemia-like insult, mild to profound hypothermia is protective only when applied without delay and for limited periods of time (6–8 hours). Longer durations of hypothermia, or delayed application of the hypothermia can increase neuron death. These findings may have implications for clinical uses of therapeutic hypothermia after hypoxic or ischemic insults, and suggest that further work is needed to elucidate the limitations of hypothermia as a protective treatment after ischemic stress.

Introduction

INDUCED MILD HYPOTHERMIA (MH, core temperature 32°C–34°C) is currently used to treat neonates with moderate to severe hypoxic-ischemic encephalopathy (HIE) (Kattwinkel *et al.*, 2010). To date, there have been six randomized, controlled trials showing that MH (33°C–34°C) reduces both mortality and disability (Higgins *et al.*, 2006; Azzopardi *et al.*, 2008; Gunn *et al.*, 2008; Simbruner *et al.*, 2010; Zhou *et al.*, 2010; Jacobs Se *et al.*, 2011). A meta-analysis of these trials showed that MH significantly reduces the risk of death or disability at 18 months (Edwards *et al.*, 2010; Alkharfy, 2013). The beneficial effects of therapeutic hypothermia (TH) persist after the treatment (Shankaran *et al.*, 2005), and the benefits may be permanent (Colbourne and Corbett, 1995). Despite these benefits, the broader clinical application of hypothermia is limited because of concerns about side effects of increased

infections, organ system dysfunction, coagulopathy, and the intrinsically poor tolerance of non-hibernating mammalian tissues to hypothermia (Hochachka, 1986). As hypothermia becomes more widely used, understanding these limitations is increasingly important. Among the unresolved issues related to TH are the optimal temperature profiles for protecting the newborn brain from HIE and defining the types or severity of HIE most amenable for treatment.

The benefits and limitations of hypothermia are also relevant to adults who have suffered cerebral ischemia. Although MH (32°C–34°C) is currently the only proven therapy that improves neurologic outcome after cardiac arrest in adults (Bernard *et al.*, 2002; Hypothermia after Cardiac Arrest Study Group, 2002; Busch *et al.*, 2006), and deep hypothermia (<25°C) is routinely used during high-risk surgery to protect the brain and other organs from ischemic insults (Yenari *et al.*, 2008; Mackensen *et al.*, 2009), there appears to be an inverse

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relationship between the duration of active cooling and outcomes.

Frequent clinical complications associated with TH (Sugerman and Abella, 2009; Groysman *et al.*, 2011; Drabek and Kochanek, 2012) currently limit the application of lower temperatures and durations of hypothermia in both adults and children (DeLeon *et al.*, 1990; Levin *et al.*, 2005). Specifically, deep or profound hypothermia (temperatures $<20^{\circ}\text{C}$) is associated with neurologic injury, a concern dating from the first application of hypothermia in cardiac surgery in the 1950s and 1960s [reviewed in Watanabe *et al.* (1982)]. Reports of neurological injury from deep hypothermia include those of Egerton *et al.* (1963), who reported that 15 of 16 patients cooled to 8°C – 12°C sustained brain damage, and Khaladj *et al.* (2008), who described a 10% rate of neurologic complications with cold cerebral perfusion. Deep hypothermia is associated with a high incidence of neurologic complications in children (Levin *et al.*, 2005) with the lowest temperatures ($<16^{\circ}\text{C}$) specifically associated with poor outcomes (Visconti *et al.*, 2006). Although greater therapeutic effects might be expected from more profound hypothermia, injury from cold or re-warming may be a major limiting factor. To date, there is no evidence to indicate that longer durations and greater depths of cooling will result in better outcomes in neonates with HIE: All currently published cooling protocols limit initiation of treatment to within 6 hours of birth (Barks, 2008) and a temperature of 32°C – 34°C .

The majority of TH research has utilized animal models. Interestingly, meta-analysis of studies using TH in animal models of acute ischemic stroke revealed that TH showed greatest efficacy in studies meeting the fewest quality control checks; the efficacy of TH was lower in studies with random treatment and blinded assessment [reviewed in van der Worp *et al.* (2007)]. However, it is difficult to draw broad conclusions from this review: Studies that used briefer cooling periods also used a shorter temporal scale and those which used longer periods of TH looked at the results over a longer time period.

Due to the limitations of TH as outlined earlier, the purpose of this study was to determine how the degree, durations, and delay of hypothermia influence cell death after an insult similar to cerebral hypoxia-ischemia (oxygen and glucose deprivation [OGD]). We used an organotypic culture model of the developing rat hippocampus that is sensitive to hypothermia protection and damage (Bickler *et al.*, 2012; Warren *et al.*, 2012). A recent study in intact rats showed that when hypothermia postcerebral ischemia was delayed by 3 hours or more, it was not protective or added to injury (Sabir *et al.*, 2012), so we also examined the effects of delaying hypothermia on outcome after OGD. We examined temperatures of 32°C , 23°C , 17°C , and 4°C to span both the therapeutic and potentially injurious range of temperatures, applying hypothermia either immediately after or after a delay from simulated ischemia. Many report that a longer duration of TH is more efficacious (Colbourne and Corbett, 1994; Clark *et al.*, 2008, 2009), and we also sought to validate this concept over the range of temperatures. The main findings of the study are that while hypothermia (32°C , 23°C or 17°C) immediately after OGD protects hippocampal neurons, delaying hypothermia, applying hypothermia for greater than 8 hours, or using ultra-profound hypothermia, increases, rather than prevents, cell death in hippocampal neurons.

Materials and Methods

Preparation of hippocampal slice cultures

Hippocampal slice cultures (HSCs) were prepared with standard methods (Stoppini *et al.*, 1991) modified by our laboratory as follows: P7 Sprague-Dawley rats (Charles River Laboratories, Hollister, CA) were decapitated, and the hippocampi were quickly removed and placed in Gey's balanced salt solution containing 25 mM HEPES, 10 mM D-glucose, and 100 mM sucrose. In some cultures, 5 mM pyruvate was included in the culture media but was found to have no effect on the health of the cultures (Gregersen M, Bickler P, Gabatto P, unpublished observations 2011–2012). The hippocampi were transversely sliced 300–400 μm thick using either a Siskiyou tissue slicer (Siskiyou Design Instruments, Grants Pass, OR) or a McIlwain tissue chopper. The slices were placed on 30 mm-diameter Millipore membrane inserts (Millicell-CM; Millipore, Billerica, MA) in six-well culture trays, four to five slices per well with 1.2 mL culture media, and placed at 37°C . The media was changed the day after preparation and then, every 48–72 hours. Slice culture media for the first 5–7 days contained 75% basal medium Eagles with Earles (Invitrogen #566, Life Technologies, Grand Island, NY), 23% horse serum, 0.5% Glutamax (Gibco #35050, Life Technologies), 1.5% dH_2O , plus 36.5 mM D-glucose. After 5–7 days, media was changed to 97% Neurobasal (Gibco #21103, Life Technologies), 2% B-27 (Gibco #17504, Life Technologies), 0.5% Glutamax, 0.5% H_2O , plus 17 mM D-glucose, and 0.5 mM ascorbic acid. The media was obtained from the UCSF Cell Culture Facility.

Ischemia/hypoxia in HSCs

After 7–14 days, cultured HSCs were randomized for use in experiments. In order to perform the largest number of experiments per culture batch while maintaining statistical power, 15–20 slices were used for each treatment group. For slices receiving ischemia, warmed glucose-free Earle's basic salt solution (GF-EBSS) was bubbled with 95% N_2 /5% CO_2 for 15 minutes to remove all oxygen. Inserts were rinsed in warmed GF-EBSS, then placed in warmed, bubbled GF-EBSS, and placed in Billups-Rothenburg (BR) chambers (Billups-Rothenburg, Inc., Del Mar, CA) without the culture tray lids. Chambers were flushed with humidified 95% N_2 /5% CO_2 for 5 minutes and then placed in 37°C incubators for 1 hour. Hypoxic slices were placed in BR chambers, flushed with humidified 95% N_2 /5% CO_2 for 5 minutes, and then placed in 37°C incubators for 1 hour. After insult, slices were removed from the chambers and placed back in normal media.

Cold injury in HSCs

Slices were placed in BR chambers, flushed with humidified 95% air/5% CO_2 for 5 minutes, and placed in incubators that were set to the desired temperature for the experimental time. After treatment, slices were rewarmed by placing them back in the 37°C incubator. In a mock study, a thermocouple probe was placed in a plate with an insert and 1.2 mL culture media and treated experimentally to measure temperature changes during a typical experiment. Survival measurements and protein collection were performed 24 hours after placing the slices back at 37°C .

Assessment of cell death

The amount of cell death per hippocampal slice was measured using propidium iodide (PI) or SYTOX[®] green (Life Technologies #S7020). Both of these dyes are highly polarized and only penetrate damaged cell membranes to permanently bind to DNA. Both Sytox and PI are close predictors of cell death (Wlodkowic *et al.*, 2011). For Sytox assessment, 0.5 μ M was added to each culture well for 10 minutes. For PI assessment, 2.3 μ M was added to each culture well for 30 minutes. After dye treatment, each well was rinsed thrice with EBSS. Brightfield and fluorescent images were obtained for each slice using a SPOT Jr. digital camera (Diagnostic Instruments, Sterling Heights, MI) mounted on the side of a Nikon Diaphot microscope. For Sytox, the excitation light wavelength was 504 nm and the emission was 523 nm. For PI, the excitation light wavelength was 535 nm and the emission was 604 nm. For each of these dyes, the fluorescence intensity is a linear function of cell death. Cell death was quantified per region (particularly the pyramidal CA1 region); regions were delineated from the brightfield image, and fluorescence was quantified using Image J. Background fluorescence was eliminated by subtracting the fluorescent signal from nonslice regions of the images from the total fluorescent signal in the region of interest. Treatment group fluorescence was normalized to the control group fluorescence within each experimental set. Data were assumed to be normally distributed, and ANOVAs with Tukey-Kramer *post hoc* multiple comparison tests were performed using Prism (Graphpad Software, La Jolla, CA). Differences were considered significant at $p < 0.05$.

Results

Temperature changes during cooling and warming

Media temperature in slice culture plates during warming and cooling is shown in Figure 1. The half life of temperature

changes during cooling from 37°C to 32°C was ~10 minutes, whereas warming the chamber from 32°C to 37°C had a half life of about 20 minutes. Cooling from 37°C to 23°C was achieved with a half life of about 16 minutes, and warming from 23°C to 37 had a half life of about 30 minutes. For both these temperature excursions, the temperature changes were within one half degree of target in 40–60 minutes. The half life of cooling and rewarming between 37°C and 4°C was ~40 and 30 minutes, respectively (Warren *et al.*, 2012).

Effects of MH (32°C) on cell death after simulated ischemia

Cooling and rewarming HSCs to 32°C for periods of 6, 24, and 72 hours did not change cell death in any of the cell body regions, compared with cultures maintained at 37°C for the same periods (Fig. 2A). In Figure 2B, results of experiments in which cultures were cooled to 32°C after 1 hour of OGD are shown. OGD increased cell death by 49% (ANOVA with Tukey's test, $p < 0.05$). This increased cell death was prevented when cultures were cooled to 32°C for 6 hours immediately after this insult (ANOVA with Tukey's, $p > 0.05$; *t*-test, $p = 0.773$). However, this benefit was not seen when cultures were kept at 32°C for 24 hours before rewarming (ANOVA with Tukey's test, $p < 0.01$). In all these experiments, cell death was assessed 24 hours after rewarming to 37°C.

Effects of moderate hypothermia (23°C) on cell death after simulated ischemia

The effects of moderate hypothermia (23°C) on cell viability on uninjured HSCs depended on cell body region. In the CA1, 8 and 24 hours of hypothermia followed by rewarming did not change cell viability, but with 48 hours at 23°C, an increase in cell death was seen (ANOVA with Tukey's, $p < 0.001$). In the dentate, 24 hours at 23°C increased cell death (ANOVA with Tukey's, $p < 0.01$), as did 48 hours (Fig. 3A). When OGD was

FIG. 1. Media temperature in cultures during cooling (A and C) or warming (B and D).

FIG. 2. Effects of mild hypothermia (MH) (32°C) on cell survival in hippocampal slice cultures (HSCs). Panel (A) shows data from experiments in which cultures were cooled to 32°C for 6, 24, or 72 hours before rewarming to 37°C. All groups were similar statistically (ANOVA with Dunnett's test), with $n = 15$ –20 cultures in each group. Error bars represent standard error of mean (SEM). Images at bottom show overlay of SYTOX® fluorescence overlaid with brightfield images of representative cultures from each group. Panel (B) shows data from experiments in which MH immediately followed 1 hour oxygen and glucose deprivation (OGD) for 6 or 24 hours. Error bars represent SEM. Bars indicate statistical significance; $*0.01 < p < 0.05$, $**0.001 < p < 0.01$, and $***p < 0.001$, ANOVA with a Tukey's *post hoc* test. Representative images shown are brightfield overlaid with SYTOX fluorescence and are shown at the bottom. Color images available online at www.liebertpub.com/ther

followed by 23°C, protection from damage in CA1 was statistically significant for 8 but not for 24 or 48 hour durations of hypothermia (ANOVA with Tukey's, $p < 0.01$). The reduction in cell death compared with the OGD group was 50% (ANOVA with Tukey's, $p < 0.05$). Similarly, in the dentate region, 8 hours of 23°C hypothermia reduced OGD-induced cell death by 50%, but when longer durations of 23°C hypothermia were used, no protection was observed (ANOVA with Tukey's, $p < 0.01$) (Fig. 3B).

Effects of profound hypothermia (17°C) on cell death after simulated ischemia

Figure 4 presents results of studies in which HSCs were cooled to 17°C for 8, 24, or 48 hours after OGD treated with these periods of hypothermia alone. In Figure 4A, quantification of cell death after hypothermia alone is presented. In both the CA1 and dentate regions, 48 hours at 17°C caused about 60% increased cell death compared with controls (ANOVA with Tukey's, $p < 0.01$ and $p < 0.05$, respectively),

with 8 and 24 hours at 17°C not resulting in increased cell death in either region. When cooling to 17°C immediately followed 1 hour of OGD, a period of 8 hours at 17°C significantly reduced cell death in both CA1 and dentate (ANOVA with Tukey's, $p < 0.001$ and $p < 0.01$, respectively), but the 24 and 48 hour duration of this degree of hypothermia caused similar or somewhat greater amounts of cell death compared with OGD followed by 37°C temperatures (All ANOVA with Tukey's, 24 hours: $p > 0.05$ (NS), t -test $p = 0.302$ CA1, $p < 0.001$ dentate; 48 hours $p < 0.001$ CA1, $p < 0.001$ dentate) (Fig. 4B).

Effects of ultra-profound hypothermia (4°C) on simulated cell death after simulated ischemia

Figure 5 shows the effects of cooling and rewarming to 4°C on cell death after hypoxia. In Figure 5A, results of studies with only the intervention of hypothermia and rewarming are shown and in Figure 5B, results of cooling to 4°C immediately after an hour of hypoxia are presented. With 4°C hypothermia, a hypothermia duration of 4 hours was non-injurious, but when the

FIG. 3. Effects of moderate hypothermia (23°C) on cell survival in HSCs. Panel (A) shows data from experiments in which cultures were cooled to 23°C for 8, 24, or 48 hours before rewarming to 37°C. Error bars represent SEM. Bars indicate statistical significance; * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, and *** $p < 0.001$, ANOVA with a Tukey's *post hoc* test, with $n = 15$ –20 cultures in each group. Images at bottom show overlay of SYTOX fluorescence overlaid with brightfield images of representative cultures from each group. Panel (B) shows data from experiments in which moderate hypothermia immediately followed 1 hour OGD for 8, 24, or 48 hours. Error bars represent SEM. Bars indicate statistical significance; * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, and *** $p < 0.001$, ANOVA with a Tukey's *post hoc* test. Representative images shown are brightfield overlaid with SYTOX fluorescence and are shown at the bottom. Color images available online at www.liebertpub.com/ther

period of hypothermia was 6 or 8 hours, extensive damage was seen in both the CA1 and dentate regions of the HSCs. The increase in cell death in these regions was 200%–300% above baseline (ANOVA with Tukey's, $p < 0.05$ to $p < 0.001$). Due to the extensive cell loss caused by 4°C hypothermia and rewarming, we could not examine the effects of 4°C hypothermia on cell survival after OGD. Therefore, we examined the effects of ultra-profound hypothermia on the milder insult of 1 hour of hypoxia. In Figure 5B, data from studies in which there were 4 hours of 4°C hypothermia immediately followed 1 hour of hypoxia. We found that 4°C hypothermia after insult was not protective (ANOVA with Tukey's, $p < 0.001$).

Effects of delaying protective hypothermia on cell death in HSCs

Since TH is often delayed after cerebral injury, we examined the effects of delayed imposition of hypothermia in our

OGD hippocampal slice model. In Figure 6A, we show data from studies in which hypothermia was delayed for 4 or 24 hours after OGD before cooling to 23°C for 6 or 24 hours. Delayed 23°C hypothermia was not protective in this protocol (ANOVA with Tukey's, $p < 0.001$). Similarly, in Figure 6B, we present data from studies in which cooling to 32°C hypothermia was delayed for 24 hours after hypothermia. As with 23°C hypothermia, delayed cooling to 32°C did not reduce cell loss in the CA1, CA3, or dentate regions of the cultures (ANOVA with Tukey's, $p < 0.001$).

Discussion

We found that cooling to 17°C, 23°C, and 32°C immediately after OGD-protected hippocampal neurons was at an assessment time of 24 hours after the insult. Although outcomes were not examined at later time points, this finding of short-term protection after an ischemia-like insult is consistent with

FIG. 4. Effects of profound hypothermia (17°C) on cell survival in HSCs. Panel (A) shows data from experiments in which cultures were cooled to 17°C for 8, 24, or 48 hours before rewarming to 37°C. Error bars represent SEM. Bars indicate statistical significance; * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, and *** $p < 0.001$, ANOVA with a Tukey's *post hoc* test, with $n = 15$ – 20 cultures in each group. Images at bottom show overlay of SYTOX fluorescence overlaid with brightfield images of representative cultures from each group. Panel (B) shows data from experiments in which profound hypothermia immediately followed 1 hour OGD for 8, 24, or 48 hours. Error bars represent SEM. Bars indicate statistical significance; * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, and *** $p < 0.001$, ANOVA with a Tukey's *post hoc* test. Representative images shown are brightfield overlaid with SYTOX fluorescence and are shown at the bottom. Color images available online at www.liebertpub.com/ther

the durable protection by hypothermia that has been seen in previous *in vivo* and *in vitro* models (Krieger and Yenari, 2004; den Hertog *et al.*, 2007; van der Worp *et al.*, 2007). Preliminary studies in our laboratory (Gregersen, M, Bickler P, Gabatto, P, unpublished observations 2011–2012) indicate that protection at 24 hours is predictive of protection at 48 hours after OGD.

Hypothermia by itself with no additional insult can cause injury to developing hippocampal neurons

An important finding was that moderate (23°C) and profound (17°C) hypothermia, when applied for a prolonged duration (48 hours), caused increased cell death in the neuron regions of HSCs. At deeper hypothermic temperatures (4°C), extensive damage was seen after 8 hours. It is unsurprising that such deep temperatures would cause damage so quickly; Michenfelder noted changes in electrical activity in canines

starting at 27°C, with loss of signal occurring at ~12°C (Michenfelder and Milde, 1992). This is a new finding for moderate and profound hypothermia and is consistent with our previously published work identifying neuron-specific injury after ultra-profound hypothermia, involving cooling and rewarming to 4°C (Warren *et al.*, 2012). These findings suggest that developing rat hippocampal neurons have a clearly limited tolerance of hypothermia, with survival defined by both the temperature and duration of hypothermia exposure.

Delayed application of hypothermia can be detrimental after ischemia

HSCs were not protected against OGD when mild or moderate hypothermia was delayed. This was a surprising finding because in animal models, delayed hypothermia is often protective (Colbourne *et al.*, 2000, 2003; Krieger and

FIG. 5. Effects of ultra-profound hypothermia (4°C) on cell survival in HSCs. Panel (A) shows data from experiments in which cultures were cooled to 4°C for 4, 6, or 8 hours before rewarming to 37°C. Error bars represent SEM. Bars indicate statistical significance; * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, and *** $p < 0.001$, ANOVA with a Tukey's *post hoc* test, with $n = 15$ – 20 cultures in each group. Images at bottom show overlay of SYTOX fluorescence overlaid with brightfield images of representative cultures from each group. Panel (B) shows data from experiments in which ultra-profound hypothermia immediately followed 1 hour anoxia for 4 hours. Error bars represent SEM. Bars indicate statistical significance; $p < 0.001$, ANOVA with a Tukey's *post hoc* test. Representative images shown are bright field overlaid with propidium iodide fluorescence and are shown at the bottom. Color images available online at www.liebertpub.com/ther

Yenari, 2004; den Hertog *et al.*, 2007; van der Worp *et al.*, 2007; Zhang *et al.*, 2011; Yenari and Han, 2012).

Laboratory *in vivo* models of cerebral ischemia show that when hypothermia is not delayed (Eberspacher *et al.*, 2005), delayed by minutes (Ikonomidou *et al.*, 1989; Baker *et al.*, 1992; Colbourne *et al.*, 2000, 2003; Zhang *et al.*, 2011) or hours (Krieger and Yenari, 2004; Roelfsema *et al.*, 2004; Tsutsumi *et al.*, 2004), protection can result. However, fewer studies have looked at the permanence of hypothermic protection. When cooling is initiated during the insult, protection was demonstrated to be permanent in a rat model, but delaying the cooling until after the insult results in temporary protection only (Dietrich *et al.*, 1993). However, cooling to 32°C for 24 hours, even when delayed by 1 hour, resulted in protection of CA1 neurons (Colbourne and Corbett, 1995). Clearly, the duration of TH is of as much interest as the depth of TH.

However, in other *in vivo* animal experiments, protection was not evident when hypothermia was not started either

during or immediately after the insult (Dietrich *et al.*, 1993). Protection was still possible when started during the insult, but not when it was initiated afterward. These conflicting results may not be oppositional considering the possibility that delayed hypothermia may be protective only with milder insults. We consider that the OGD used in this study involves a moderate to severe insult and it is possible that a less severe insult would be compatible with a protective effect of delayed hypothermia as recently demonstrated in a neonatal rat model of hypoxia ischemia (Sabir *et al.*, 2012). As in the Sabir study, delayed hypothermia, or profound hypothermia, increased rather than prevented postischemic injury, which is a clinical concern.

Study limitations

The results of this study should be interpreted within the constraints of the methodology. One limitation of the study

FIG. 6. Effects of delayed hypothermia on cell survival in HSCs. Panel (A) shows data from experiments in which moderate hypothermia (23°C) was delayed for 4 or 24 hours followed by OGD. Cultures were cooled for 4 or 24 hours before rewarming to 37°C. Error bars represent SEM. Bars indicate statistical significance; * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, and *** $p < 0.001$, ANOVA with a Tukey's *post hoc* test. Representative images shown are bright field overlaid with SYTOX fluorescence and are shown at the bottom. Panel (B) shows data from experiments in which MH (32°C) was delayed for 4 or 24 hours followed by OGD. Cultures were cooled for 24 hours before rewarming to 37°C. Error bars represent SEM. Bars indicate statistical significance; * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, and *** $p < 0.001$, ANOVA with a Tukey's *post hoc* test. Representative images shown are bright field overlaid with SYTOX fluorescence and are shown at the bottom. Color images available online at www.liebertpub.com/ther

was that we used a relatively severe insult of OGD and did not investigate the outcome of hypothermia treatment after less severe insults, except in the case of using a milder insult of hypoxia for the study of ultra-profound hypothermia. It is possible that delayed hypothermia would have been of benefit after a milder insult, as described in an intact neonatal rat model of posthypoxia/ischemia application of hypothermia (Sabir *et al.*, 2012).

In electing to use HSCs, we can use a greater range of temperatures and durations than is feasible or ethical in animal studies. However, it makes comparing results with intact animal studies more difficult. Many studies have demonstrated a longer period of TH after proximal major cerebral artery occlusion (Colbourne and Corbett, 1994; Clark *et al.*, 2008, 2009), which results in a similarly severe insult to the hippocampus as OGD in HSCs. It may be that an isolated hippocampus (as in our studies) will never fare as well after an insult. For example, recently, it has been demonstrated that long-term cooling in normal adult rats is not harmful: There

were no detrimental behavioral effects or signs of brain damage in rats cooled for 21 days to 32°C (Auriat *et al.*, 2012). This study utilized focal brain cooling and looked at physiologic measures, behavior, and cellular morphology and death as outcome measures. The work presented here does not directly conflict with the results of Auriat *et al.*, as we did not note damage caused by 32°C alone, though our longest duration was 72 hours. Again, the fact that the presented research was performed in isolated hippocampi may affect the long-term viability of the neurons, as they have a limited support structure.

In addition, it is possible that our results have been clouded by the duration of survival postischemic insult. Our cell death quantification timepoints were set to 24 hours after the conclusion of TH as opposed to using a timepoint set based on the conclusion of the ischemic insult. Further experiments are warranted to fully control for the variance in postischemia time.

Our findings are based on the assumption that we accurately measure cell death. We quantified cell death with PI or

Sytox labeling, and these methods correlate well with the measurement of cell death in hippocampal cultures by histologic methods of cresyl violet staining or hematoxylin-eosin staining. A linear relationship between cell death measured with histologic techniques and the PI method has been demonstrated by others (Newell *et al.*, 1995; Laake *et al.*, 1999) and by studies in our laboratory (Bickler and Fahlman, 2009). Sytox can be substituted for propidium iodide (Wlodkowic *et al.*, 2011). This laboratory has found that Sytox provides more crisp and clear images and has gradually shifted toward its preferred use.

This study examined relatively short-term effects of hypothermia; that is, we assessed cell death 24 hours after final rewarming. This is important to consider, because *in vivo* experiments involving P7 rats by Xiong *et al.* (2009) did not reveal differences between hypothermic (32°C–33°C) and normothermic groups postischemia until 3–7 days later. This study uses an *in vitro* model, and we have not looked beyond 48 hours for longer-term changes in injury. Further, we did not investigate how rate of cooling or rewarming impacts outcome, although this was not a major determinant of injury in an earlier study of ultra-profound hypothermia in HSCs (Warren *et al.*, 2012). It is also important to note that injury from hypothermia can only be assessed after rewarming because of the current difficulty in assessing cell death in hypothermic cells (Warren *et al.*, 2012).

It is not clear as to what extent our findings apply to humans. In clinical practice, induction of TH is unlikely to occur immediately after an ischemic insult, and so, the observation that delayed hypothermia may not be protective is potentially important clinically. While we are not suggesting that this depth of hypothermia should be used clinically, we believe that these data hold important implications for therapeutic hypothermic use. Since we found that prolonged hypothermia may itself cause neuronal damage, or worsen neuronal damage under certain conditions, our studies caution that hypothermia has limitations, both in terms of intrinsic injury and in limited benefit when delayed. We deliberately used a severe insult to bring out the potential limitations of hypothermia treatment—to date, research touting the benefits of TH has been focused on ameliorating milder ischemic insults, as these are more likely to respond to TH. Further experimental studies examining these issues are warranted, because they may ultimately guide clinical applications of protective hypothermia.

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Disclosure Statement

No competing financial interests exist.

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