Hypothermia protects human neurons

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Background and Aims Hypothermia provides neuroprotection after cardiac arrest, hypoxic-ischemic encephalopathy, and in animal models of ischemic stroke. However, as drug development for stroke has been beset by translational failure, we sought additional evidence that hypothermia protects human neurons against ischemic injury.

Methods Human embryonic stem cells were cultured and differentiated to provide a source of neurons expressing β III tubulin, microtubule-associated protein 2, and the Neuronal Nuclei antigen. Oxygen deprivation, oxygen-glucose deprivation, and H₂O₂-induced oxidative stress were used to induce relevant injury.

Results Hypothermia to 33°C protected these human neurons against H₂O₂-induced oxidative stress reducing lactate dehydrogenase release and Terminal deoxynucleotidyl transferase dUTP nick end labeling-staining by 53% (P ≤ 0.0001; 95% confidence interval 34–71) and 42% (P ≤ 0.0001; 95% confidence interval 27–56), respectively, after 24 h in culture. Hypothermia provided similar protection against oxygen-glucose deprivation (42%, P ≤ 0.001; 95% confidence interval 18–31 and 28%, P ≤ 0.001; 95% confidence interval 12–42) but provided no protection against oxygen deprivation alone. Protection (21%) persisted against H₂O₂-induced oxidative stress even when hypothermia was initiated six-hours after onset of injury (P ≤ 0.05; 95% confidence interval 0.57–43).

Conclusion We conclude that hypothermia protects stem cell-derived human neurons against insults relevant to stroke over a clinically relevant time frame. Protection against H₂O₂-induced injury and combined oxygen and glucose deprivation but not against oxygen deprivation alone suggests an interaction in which protection benefits from reduction in available glucose under some but not all circumstances.

Key words: brain, hypothermia, ischemic stroke, neuroprotection, stem cells, treatment

Introduction

Obstruction of an artery supplying the brain initiates a cascade of events leading ultimately to necrotic and apoptotic cell death.

With the most extreme perfusion deficits, survival is unlikely and cell death is rapid. However, in areas adjacent to the ischemic core (the ischemic penumbra), residual blood flow can preserve tissue vitality for a limited time until the obstruction resolves spontaneously or is removed by thrombolysis (1,2). After these acute events delayed neuronal death may progress for up to three-days (3). Both these phenomena (the penumbra during ischemia and delayed neural injury after ischemia) provide targets for treatment and in animal models of ischemic stroke over 500 ‘neuroprotective’ treatment strategies reported improved outcome. However, none have proved robustly effective in randomized controlled clinical trials (4). Importantly with the exception of therapeutic hypothermia (5), clinical trials of neuroprotection for stroke have largely been abandoned (http://www.clinicaltrials.gov).

The reasons for this translational failure have been hotly debated. Contamination of the preclinical data set by falsely positive results influenced by bias and lack of statistical power offer some explanation (6–8). The molecular targets of therapy may be present in rodents but not in man (9), either because of differences in genetic background (10) or because evolution of the ischemic cascade might proceed at a different pace in different species (11–13). Additionally, laboratory and clinical thrombolysis experiments show similar, and unfortunately short, windows of opportunity (2,14), which are frequently unachievable in most trials of neuroprotection (15,16). Targeting single components of the ischemic cascade might also limit our chances of success (17,18), but combinatorial pharmacotherapy therapy brings its own challenges (19).

The preclinical data set for therapeutic hypothermia is large (3353 animals) and demonstrates substantial and consistent efficacy, with only modest effects of publication bias and failure to randomize or blind (20). Hypothermia influences multiple molecular targets (21,22) and provides benefit over a wide range of times to treatment in animals (20). Clinically, evidence of a relationship between body temperature and outcome after human stroke (23–25), together with the proven neuroprotective benefit of hypothermia in adults with global ischemia after cardiac arrest (26,27) and neonates with hypoxic-ischemic encephalopathy (28,29), suggest that the targets that provide benefit in animal models of stroke will also be present in human stroke. However, the absence of clear therapeutic benefit in traumatic brain injury (30), which accrues damage similarly to stroke (31), suggests caution should be taken. A Cochrane systematic review of stroke patients treated with physical cooling devices suggests there may be a trend toward improvement. However, in most cases, treatment was initiated (8–12 h) when penumbral tissue, the main putative target, may no longer be present. Overall, the analysis supports the view that we still have insufficient data...
(32) with trials too small to allow firm conclusions to be made about the efficacy of hypothermia in stroke patients (33–35).

In this report, we show that hypothermia (33°C) protects human stem-cell derived neurons from oxidative stress induced by hydrogen peroxide and from oxygen-glucose deprivation but not hypoxia alone. Importantly, protection against oxidative stress is observed at later times (six-hours) than obtained under oxygen-glucose deprivation. The results were replicated in three independent series of experiments employing randomization and blinding of operator-dependent, outcome analyses.

**Methods**

**Human embryonic stem cell (hESC) Culture**

Experiments with these cells were carried out in accordance with the guidelines and regulations of the National Health and Medical Research Council and with the approval of the Austin Health Human Research Ethics Committee (Approval number H2008/03194) and University of Melbourne Human Research Ethics Committee (Approval number 0605017).

H9 (WA-09, WiCell, Madison, Wisconsin, USA), human embryonic stem cells (hESC), were cultured on mitomycin-C treated mouse embryonic fibroblasts (MEFs) in hESC medium consisting of high-glucose Dulbecco’s modified Eagle’s minimal essential medium (DMEM) without sodium pyruvate, supplemented with insulin/transferrin/seleum 1%, β-mercaptoethanol 0.1 mM, nonessential amino acids (NEAAs) 1%, glucose 1.6 mg/ml, penicillin 2 U/ml, streptomycin 2 mg/ml, and fetal calf serum (FCS) 20% (HyClone, Australia) or on mitomycin-C treated human foreskin fibroblasts (HFF; ATCC, CRL-2097) in KSR media consisting of DMEM/nutrient mixture F-12, supplemented with β-mercaptoethanol 0.1 mM, NEAAs 1%, glucose 2 mg/ml, penicillin 25 U/ml, streptomycin 25 μg/ml, and knockout serum replacement 20% (all from Invitrogen). All cells were cultured at 37°C in 5% CO2. Colonies were mechanically dissected every seven-days and transferred to freshly prepared MEFs or HFFs. Media was changed every second day.

**Neuronal differentiation and growth**

Neuronal differentiation was achieved using the noggin induction method described for mouse neurospheres (36) as adapted by Dottori for human neurospheres (37). The colonies were maintained (37°C, 5% CO2) in hESC medium supplemented with 500 ng/ml of Noggin (6057-NG, R&D systems, Australia) and 20 ng/ml human recombinant basic fibroblast growth factor (bFGF) (13256-029, Invitrogen). Media was changed every two- to three-days to allow neurosphere formation over two-weeks.

In order to facilitate neuronal differentiation, neurospheres were again separated into smaller pieces under a dissection microscope with three to four pieces transferred to each well of a 96-well plate [ precoated with poly-D-lysine (10 μg/ml) and mouse laminin (5 μg/ml)]. The cells were then grown for 11 days (with media changed every two-days) in NBM lacking growth factors prior to induction of injury and assessment of hypothermia.

**Injury and hypothermia induction**

On the day of experiments, the medium was changed to NBM+N2 containing a B27 preparation lacking the usual antioxidants (10889-038, Invitrogen) (NBM-AO) to eliminate their confounding effects.

Three different injury models were used to assess the effect of hypothermia on these cells. Oxygen deprivation was induced by placing the cells into a hypoxic chamber flushed with nitrogen for 20 min and maintained at the appropriate temperature (33°C or 37°C) for four-hours inside a standard 5% CO2 incubator. Culture supernatant was removed after this four-hour period and stored at 4°C until analysed for lactate dehydrogenase activity (LDH). The media was replaced with fresh NBM-AO, and the cells were incubated for a further 20 h at the appropriate temperature (33°C or 37°C) before again measuring LDH. For combined oxygen and glucose deprivation (ODG), 25 mM 2-deoxy-D-glucose was added to NBM-AO medium and equilibrated for 30 min at room temperature before the initial media change as described above. The third injury model used was oxidative stress induced by adding 50 μM of fresh H2O2 (H1009, Sigma-Aldrich, Australia) to the NBM-AO for four-hours (33°C or 37°C, 5% CO2) when LDH was measured, and NBM-AO without H2O2 was returned to the culture, which was maintained for a further 20 h before again measuring LDH. To examine whether the effects of injury continued beyond four-hours, the media containing the above stressors was removed and replaced with fresh growth factor negative NBM before resampling for LDH activity and assessment of apoptotic cell death by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining at 24 h.

To evaluate the effects of hypothermia over time from the induction of injury, incubation at 33°C was started immediately, on one-, or three-, or six-hours after induction of injury and maintained until a total of 24 h in each of the models. LDH, a marker of total cell death and TUNEL staining and a marker of DNA damage typical of apoptosis, were performed according to the kit manufacturer’s instructions (1164479001 and 11684795910, respectively, Roche, Australia). For LDH assays, three control measurements were made in addition to the test sample measurements. The cell culture medium without cells was assayed to assess background activity. Uninjured cells were assayed to detect activity due to basal cell death, and maximum possible cell death (100%) was detected by measuring LDH activity of lysed cells.
Fig. 1 β III tubulin positive human neurons after 11 days in culture with nuclei counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI).

Statistical analysis

Each experiment was repeated three times. To avoid systematic effects on individual cultures because of the arrangement of wells in the tissue culture plates, control and injury cultures were distributed across the plates at random. Complete experiments contained within each tissue culture plate were then placed alternatively at either 37°C or 33°C. To minimize the potential impact of systematic bias, before counting of TUNEL positive cells, wells were imaged and the images recorded independently before quantitation. No additional blinding was performed for the machine-read LDH assay process. Within experiments, each comparison was performed at least in triplicate and the mean of these values taken forward into group comparisons. Two-way analysis of variance was performed, followed by post hoc Dunnett's multiple comparison test with significance set at P < 0.05 using SPSS (Statistics 20, IBM, Armonk, NY, USA). All values are presented as mean ± standard error of the mean.

Results

The method of stem cell culture and neuronal differentiation employed in this study gives rise to neurons with a mature projecting phenotype (Fig. 1) that express the markers β III tubulin, microtubule-associated protein 2 (MAP2), and neural nuclei (NeuN). β III tubulin positive cells comprise 70-4% ± 1.8 of the cells present before injury (obtained by calculating the ratio of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and β III tubulin cells from 10 fields per well in three independent experiments). Dose response assays were performed to establish injury assay conditions (hypoxia, hypoxia plus glucose deprivation, and oxidative stress) that reproducibly induced 30-40% of maximal signal at four-hours to ensure that values lay within the working range of the assays used. In control cultures without injury, hypothermia had no effect on the basal level of cell death measured by LDH release at either 4 or 24 h (Figs 2-5).

H2O2-induced oxidative stress increased LDH-detected cell death 3-8-fold after four-hours. Removal of the H2O2 by replacing the culture media at four-hours dramatically slowed but did not halt the H2O2-induced injury (Fig. 2a,b). Hypothermia to 33°C reduced H2O2-induced cell death at four-hours by 45% [P ≤ 0.0001; 95% confidence interval (CI) 27.5–62.6] (after correcting for basal injury in controls) (Fig. 2a). Hypothermia effectively abolished (92% reduction) (P ≤ 0.001; 95% CI 45.3–131.3) the delayed injury that continued to accrue between removal of H2O2 at four-hours and completion of the experiment at 24 h (Fig. 2b). The net effect at 24 h was a reduction of LDH release of 53% (P ≤ 0.0001; 95% CI 34.8–71.04) (Fig. 2c). H2O2-induced oxidative stress increased TUNEL-detected apoptotic cell death 2.9-fold after 24 h, with 22% of cells present in culture killed by this mechanism upon introduction of H2O2. Hypothermia prevented 42% (P ≤ 0.0001; 95% CI 27.5–56.6) of this death (after correcting for basal injury in the control) (Fig. 2d).

Oxygen deprivation alone increased LDH-detected cell death approximately twofold after four-hours. Restoring the culture to a normal air/5% CO2 incubator and replacing the culture media at four-hours again slowed but did not completely halt the oxygen depletion induced injury (Fig. 3a,b). Hypothermia to 33°C had no discernible effect on oxygen depletion-induced cell death at either 4 or 24 h by either LDH or TUNEL assays (Fig. 3a–d).

Combined OGD caused greater injury, increasing LDH-detected cell death 3.9-fold after four-hours. This death continued at a slower rate on restoration of normal culture conditions (Fig. 4a,b). Hypothermia to 33°C reduced OGD induced cell death at four-hours by 37% (P ≤ 0.0006; 95% CI 6.8–44.8) (after correcting for basal injury in controls) (Fig. 4a). Hypothermia reduced the delayed injury that occurred between removal of OGD at four-hours and completion of the experiment at 24 h by 80% (P ≤ 0.015; 95% CI 13.8–146.8) (Fig. 4b). The net effect at 24 h was a reduction of LDH release of 42% (P ≤ 0.001; 95% CI 18.3–71.3) (Fig. 4c). TUNEL staining for DNA damage typical of apoptosis at 24 h suggested that 22% of cell death occurred by this mechanism and that hypothermia prevented 26% (P ≤ 0.001; 95% CI 12.4–52.2) of this death (after correcting for basal injury in the control) (Fig. 4d).

To confirm these observations, the H2O2 and OGD experiments were repeated as before with hypothermia throughout the period of exposure to injury (from 0 h) and with initiation of hypothermia delayed for one-, three-, or six-hours, with outcome recorded as the total % of LDH release at 24 h. As before hypothermia had no impact on cell death in control cultures but when initiated at 0, one-, three-, or six-hours protected against H2O2 toxicity by 52% (P ≤ 0.0001; 95% CI 29.8–73.5), 43% (P ≤ 0.0001; 95% CI 20.9–64.5), 34% (P ≤ 0.001; 95% CI 12.1–55.8), and 21% (P ≤ 0.05;
95% CI 0.57–43.1) (Fig. 5a), respectively, and against OGD-induced injury by 45% (P ≤ 0.0004; 95% CI 18.5–71.9), 30% (P ≤ 0.023; 95% CI 3.4–56.9), 27% (P ≤ 0.041; 95% CI 0.84–54.3), and 4% (P = 0.99; 95% CI −23.02 to 30.4) (Fig. 5b), respectively.

Discussion

This study provides the first description of protection of embryonic stem cell-derived human neurons by hypothermia. This is broadly consistent with data from ~20 publications (Table 1) that have examined the effects of hypothermia in tissues cultured from rats, mice, gerbils, guinea pigs, and three papers utilizing human teratoma lines (40–50,52,54–56). In this literature, glutamate release (40), calcium accumulation (38), and glucose utilization (45) are reduced while membrane potential (41,51) and cellular morphology are restored (49). These are consistent with reduced injury and cell death (39,44–46,48) measured by LDH release (40,45), propidium iodide uptake (46,48), 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining (54), caspase activity (44), and other immunohistochemical techniques (42,50,52,53,57).

Hypothermia significantly protected embryonic cell-derived human neurons against oxidative stress and from hypoxia when glucose concentration was reduced but was ineffective against hypoxia alone. Neuroprotective effects were seen with delays in initiation of hypothermia of up to six-hours, with the magnitude of benefit progressively decreasing as the time to hypothermia initiation increased. In our preparations protection against apoptosis was also detected, consistent with data from mouse neuronal cultures (44) and focal cerebral ischemia experiments in animals (58) showing that mild hypothermia attenuates DNA damage typical of apoptotic neuronal cell death. However, the difference between degree of injury detected at 24 h by LDH and TUNEL assays suggests cell death is also occurring in the cultures by nonapoptotic mechanisms such as necrosis.

**Research**

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<table>
<thead>
<tr>
<th>Year</th>
<th>Hypothermia temperature</th>
<th>Type of culture</th>
<th>Injury</th>
<th>Duration of injury</th>
<th>Outcome measure</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1991</td>
<td>35°C, 33°C, and 31°C</td>
<td>Gerbil hippocampal slices</td>
<td>OGD</td>
<td>Unknown</td>
<td>Intracellular Ca$^{2+}$ accumulation</td>
<td>Ca$^{2+}$ accumulation was delayed following hypothermia treatment</td>
<td>(38)</td>
</tr>
<tr>
<td>1992</td>
<td>32°C</td>
<td>Rat cerebellar granule cells and GABAergic neurons</td>
<td>OGD</td>
<td>3–6 h</td>
<td>LDH</td>
<td>Neuroprotective, reduces LDH caused cell death</td>
<td>(39)</td>
</tr>
<tr>
<td>1998</td>
<td>33°C</td>
<td>Guinea pig E40, E60, and adult slices</td>
<td>OGD</td>
<td>2 h and 12 h</td>
<td>HPLC</td>
<td>Reduced glutamate release was only observed in the immature cultures</td>
<td>(40)</td>
</tr>
<tr>
<td>1998</td>
<td>35°C, 33°C, and 27°C</td>
<td>Rat hippocampal slices</td>
<td>OGD</td>
<td>Unknown</td>
<td>Extracellular membrane potential recordings</td>
<td>27°C and 33°C the membrane potential was restored to the control conditions</td>
<td>(41)</td>
</tr>
<tr>
<td>2000</td>
<td>34°C</td>
<td>Rat hippocampal slices</td>
<td>OGD</td>
<td>10–20 min</td>
<td>Immunohistochemistry</td>
<td>Hypothermia reduced CA1 cell loss</td>
<td>(42)</td>
</tr>
<tr>
<td>2001</td>
<td>31°C</td>
<td>Guinea pig hippocampal slices</td>
<td>OGD</td>
<td>20, 30, or 40 min</td>
<td>Recovery of energy metabolism</td>
<td>When hypothermia added at 0 or 2 h postinjury induction it reduced energy metabolic rate</td>
<td>(43)</td>
</tr>
<tr>
<td>2002</td>
<td>33°C</td>
<td>Rat cortical cultures</td>
<td>OGD</td>
<td>Unknown</td>
<td>Caspase 3–8 and −9 activity</td>
<td>Hypothermia neuronal apoptotic cell death</td>
<td>(44)</td>
</tr>
<tr>
<td>2003</td>
<td>30°C and 33°C</td>
<td>Rat retina</td>
<td>OGD</td>
<td>1–2 h</td>
<td>LDH</td>
<td>Hypothermia reduced glucose utilization and lactate production</td>
<td>(45)</td>
</tr>
<tr>
<td>2004</td>
<td>31°C, 33°C, and 35°C</td>
<td>Rat hippocampal slices</td>
<td>OGD</td>
<td>60 min</td>
<td>Propidium iodide (PI)</td>
<td>Reduced PI detected cell death only at 31°C and 33°C</td>
<td>(46)</td>
</tr>
<tr>
<td>2004</td>
<td>33°C pre conditioning</td>
<td>Rat cerebellar slices</td>
<td>OGD</td>
<td>20 min</td>
<td>Immunohistochemistry</td>
<td>Hypothermic preconditioning induces acute neuroprotection</td>
<td>(47)</td>
</tr>
<tr>
<td>2005</td>
<td>35°C</td>
<td>Rat hippocampal slices</td>
<td>OGD</td>
<td>1 h</td>
<td>Propidium iodide</td>
<td>Protects all regions of hippocampus from cell death</td>
<td>(48)</td>
</tr>
<tr>
<td>2005</td>
<td>27°C or 33°C</td>
<td>Dissociated mouse hippocampal neurons</td>
<td>OGD</td>
<td>10–25 min</td>
<td>Spine shape and motility assessed by microscopy</td>
<td>Decrease in temperature reduced the spine modality, and the length of spines was reduced</td>
<td>(49)</td>
</tr>
<tr>
<td>2005</td>
<td>35°C and 31°C</td>
<td>Rat hippocampal slices</td>
<td>OGD</td>
<td>40 min</td>
<td>Immunohistochemistry</td>
<td>Decrease in temperature – increased protection in all the hippocampal regions</td>
<td>(50)</td>
</tr>
<tr>
<td>2006</td>
<td>34°C pre treatment</td>
<td>Rat hippocampal slices</td>
<td>OGD</td>
<td>20 min</td>
<td>Electrophysiology</td>
<td>Temperature lowering reduced OGD-induced cell death</td>
<td>(51)</td>
</tr>
<tr>
<td>2006</td>
<td>34°C, 30°C, or 22°C</td>
<td>Rat hippocampal neurons</td>
<td>OGD</td>
<td>Unknown</td>
<td>Expression of Bcl-2, fluorescence magnitude of intracellular Ca$^{2+}$</td>
<td>Expression of Bcl-2 was increased with decrease in temperature</td>
<td>(52)</td>
</tr>
<tr>
<td>2006</td>
<td>33°C pre conditioning for 20 min, 1 h before OGD</td>
<td>Rat cerebellar cells</td>
<td>OGD</td>
<td>20 min</td>
<td>Immunohistochemistry</td>
<td>Hypothermic preconditioning increased survival of Purkinje neurons in rat cerebellar slices after OGD</td>
<td>(53)</td>
</tr>
<tr>
<td>2009</td>
<td>33°C</td>
<td>Human NT2-N neurons</td>
<td>OGD</td>
<td>20 min</td>
<td>MTT</td>
<td>Posthypoxic hypothermia is protective in human NT2-N neurons</td>
<td>(54)</td>
</tr>
<tr>
<td>2010</td>
<td>33°C and 30°C</td>
<td>Rat cortical neurons</td>
<td>Hypoxia</td>
<td>24 h</td>
<td>Cell viability</td>
<td>Increased cell survival following hypothermia</td>
<td>(55)</td>
</tr>
</tbody>
</table>
There are a number of studies using NT2-N neurons in similar experiments with conflicting data evident. Although one study reports essentially the same result as here (54), another reports that hypothermia protects against the effects of oxygen restriction alone (59). The reasons for these differences are unclear but suggest differential expression of the metabolic or signaling machinery involved in ischemic injury. NT2-N cells are derived from the human NTERA2 embryonal carcinoma stem cell line (60) and are known to differ substantially in their broad pattern of gene expression when compared with different hESC lines (61) and therefore may not be the most appropriate models to study human disease (62).

Intriguingly, hypothermia had no effect on the basal level of cell death in the uninjured controls. The contrasting response to different injury models, lack of effect on culture dependent cell death, and different behaviors to experiments reported in other cell lines suggests interaction specifically in the processes of ischemic injury rather than cell death itself and requires further study. These differences also do not appear to be consistent with hypothermia-inducing protection simply by slowing overall metabolic activity (63) but could still be consistent with decreasing the cerebral metabolic rates of glucose and oxygen consumption (64) and slowing adenosine triphosphate (ATP) breakdown (65).

It is not clear how the time frame of protection of human neurons in vitro will be predictive of time frame in stroke. However, detection of protection against oxidative stress even after cessation of injury offers hope for the clinical setting. This may be particularly relevant if reperfusion injury (66–68) acts to lessen the potential benefit afforded by thrombolysis or thrombectomy and suggests this may be an important target for clinical trials of hypothermia. The data are certainly consistent with the recent observation that mild hypothermia reduces the deleterious side effects of tissue plasminogen activator treatment after thrombectomy and suggests this may be an important target for clinical trials of hypothermia. The data are certainly consistent with the recent observation that mild hypothermia reduces the deleterious side effects of tissue plasminogen activator treatment after thromboembolic stroke in rats (69). Different time frames of benefit via different mechanisms for a single therapeutic approach suggest the same effects are seen in induced pluripotent stem cells (iPSCs) from patients with stroke or relevant defects of oxidative metabolism such as seen in inborn errors of mitochondrial function.

**Conclusion**

This study provides the first description of protection of embryonic stem cell derived human neurons by hypothermia. If hypothermia does have a predilection for ischemic processes, this offers hope for use of hypothermia to treat ischemic stroke and spinal cord injuries where cord compression results in secondary ischemia (75). This study also provides proof of principle that human neurons derived from hESCs can be used to screen new drugs for therapeutic effect. For screening large chemical libraries, this will be more specific than screening in cell lines from other species, and more cost-effective than initial screening in animal models of stroke.

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