Mechanisms of hypothermic neuroprotection

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Synopsis

Prolonged, moderate cerebral hypothermia initiated within a few hours after severe hypoxia-ischemia and continued until resolution of the acute phase of delayed cell death can reduce acute brain injury, and improve long-term behavioral recovery in term infants and in adults after cardiac arrest. Perhaps surprisingly, the specific mechanisms of hypothermic neuroprotection remain unclear, at least in part because hypothermia suppresses a broad range of potential injurious factors. In the present review we critically examine proposed mechanisms in relation to the known window of opportunity for effective protection with hypothermia. Better knowledge of the mechanisms of hypothermia is critical to help guide the rational development of future combination treatments to augment neuroprotection with hypothermia, and to identify those most likely to benefit.

Key words: Therapeutic hypothermia; fetal sheep; newborn infant; hypoxia-ischemia; neuroprotection; neonatal encephalopathy

Key Points

- Prolonged, mild hypothermia helps reduce anoxic depolarization, excitotoxicity, free radical exposure and blood brain barrier dysfunction *during* hypoxia-ischemia/reperfusion
- The 'latent' phase of recovery, before delayed deterioration after hypoxia-ischemia, represents the window of opportunity for hypothermic neuroprotection
- Key targets of delayed hypothermia in the latent phase include programmed cell death, microglial activation and abnormal excitatory receptor activity
- Hypothermia is not generally protective after the onset of the secondary mitochondrial failure, but may help reduce secondary, seizure-mediated, extension of injury
- We hypothesize that overall, mild hypothermia suppresses secondary injury processes without impairing recovery of normal brain homeostasis

Introduction

There is now compelling clinical evidence from meta-analyses of large randomized controlled trials that in term infants with moderate to severe hypoxic-ischemic encephalopathy, prolonged, moderate cerebral hypothermia initiated within a few hours after birth and continued until resolution of the acute phase of delayed cell death reduces neural injury,[1, 2] and improves neurodevelopmental outcome in the medium to long-term.[3-5] The specific mechanisms of this protection remain surprisingly unclear, in part paradoxically because a very wide range of potentially deleterious mechanisms are suppressed, making it difficult to distinguish between changes during cooling that are critically beneficial, compared with those that are indifferent or even deleterious. In the present review we will critically assess potential mechanisms of hypothermic neuroprotection in relation to the window of opportunity for cooling after severe hypoxia-ischemia (HI).

The evolution of hypoxic-ischemic injury

The central insight that underpinned development of therapeutic hypothermia was that hypoxic-ischemic (HI) injury evolves over time. We now know that although neurons may die during the actual ischemic or asphyxial event (the "primary" phase), many cells initially recover at least partially from the primary insult in a "latent" phase during which oxidative metabolism is at least partially restored despite continuing suppression of EEG activity.[6-8] After moderate to severe injury, this is typically followed by secondary deterioration, starting hours later (approximately 6 to 15 h), with delayed seizures,[9] cytotoxic edema, accumulation of excitatory amino acids (EAAs), failure of mitochondrial oxidative activity,[8, 10] and ultimately, cell death.[11] More severe

primary insults are typically associated with more severe primary damage,[12] and more rapidly developing cell death.[12, 13]

What can we learn from the window of opportunity for hypothermia?

It is not completely clear when in this process evolving cell death becomes irreversible. Empirically, neuroprotection requires that hypothermia is started during the so-called 'latent' or early recovery phase of transient restoration of cerebral oxidative metabolism, before secondary failure of oxidative metabolism, and continued until after resolution of the secondary phase.[9, 13-16] Thus, pragmatically, the window for treatment appears to close after the start of secondary energy failure, corresponding with an 'irreversible' stage in the evolution of delayed cell death.[17]

Mechanisms of action of hypothermia *during* hypoxia-ischemia

At the most fundamental level, injury requires a period of insufficient delivery of oxygen and substrates such as glucose (and lactate in the fetus) such that neurons and glia cannot maintain homeostasis. As outlined in Figure 1, the key mechanisms of primary injury and death include:

1. Anoxic depolarization. Once the neuron's supply of high-energy metabolites such as ATP can no longer be maintained during HI, the energy dependent mechanisms of intracellular homeostasis including the Na^+/K^+ ATP dependent pump begin to fail. Neuronal depolarization opens sodium and calcium channels, leading to rapid entry of these cations into cells (and potassium out). This creates an osmotic and electrochemical gradient that in turn favors further chloride and water entry leading to cell swelling (cytotoxic edema). If sufficiently severe, this may lead to acute cell lysis.[18]

Even after surprisingly prolonged and severe insults, however, many swollen neurons can still recover, at least temporarily, if the hypoxic insult is reversed or the osmotic environment is manipulated. Evidence suggests that several additional factors act to increase cell injury during and following depolarization, including:

2. extracellular accumulation of EAAs, mediated by increased release after neuronal depolarization coupled with impaired energy dependent re-uptake by astrocytes,[19] which in turn promote further receptor mediated cell swelling and intracellular calcium entry;[18]

3. generation of oxygen free radicals such as the highly toxic hydroxyl radical ($^{\bullet}OH$), leading to lipid peroxidation and DNA/RNA fragmentation;[20, 21]

4. neuronal nitric oxide synthase (nNOS) mediated release of the reactive oxygen species $NO^{\bullet}$,[22] which can damage key lipoproteins in cell membrane, organelles and mitochondria;

These damaging events are partly balanced by protective responses that help reduce cell injury, including:

1. inhibitory amino acids such as γ-aminobutyric acid that accumulate to much greater levels in the developing brain than in adult animals.[19]

2. adenosine, an inhibitory neuromodulator derived from breakdown of ATP that helps delay onset and reduces the severity of energy failure during asphyxia.[23]

Hypothermia protects the brain during severe HI by:

1. a graded reduction in cerebral metabolism of about 5% for every degree of temperature reduction,[24] which delays the onset of anoxic cell depolarization. The protective effects of intra-insult hypothermia are not simply due to reduced metabolism, since cooling substantially reduces damage for a given absolute duration of depolarization compared to normothermia.[25]

Additional factors include:

2. reduced accumulation of EAAs during intra-ischemic hypothermia in adult and newborn animals.[26, 27] This is primarily due to the delay in depolarization, although there is evidence for a reduction in the rate of release even after depolarization has occurred.[28]

3. suppression of NO and superoxide formation, presumptively due to slowing of chemical reactions, as shown in hippocampal slice cultures,[29] during ischemia and reperfusion in rodents,[30] cardiac arrest in young adult dogs,[31] and during and immediately after HI in the piglet.[27]

Cooling during reperfusion

After cerebral circulation and oxygenation are restored at end of the insult, oxidative metabolism rapidly recovers in surviving cells and cytotoxic edema resolves over approximately 30 to 60 minutes.[7, 19, 32] The key events outlined in Figure 2 include:

1. EAA levels rapidly fall in parallel with resolution of the acute cell swelling;[19]

2. the rapid restoration of tissue oxygenation is associated with a further rapid burst of NO and superoxide formation;[27]

3. breakdown of the blood brain barrier, allowing large proteins to leak out in the extracellular space. This may increase brain swelling and is associated with degradation of key regulatory proteins in the vascular basement membrane, at least in part mediated by induction of enzymes called metaloproteases.[33]

Hypothermia started immediately after reperfusion in newborn piglets appeared to accelerate this resolution as shown by reduced extracellular levels of EAAs, and reduced NO efflux in the brain.[27] Further, in adult rats, cooling after global ischemia was associated with reduced blood brain barrier (BBB) leakiness and brain edema 24 h later, provided that it was induced within 1 h after ischemia, apparently through inhibition of metalloproteinases.[33] However, metalloproteinase inhibition after HI in neonatal rats has had inconsistent effects.[34] Taken with the observation that hypothermia is neuroprotective even when delayed by more than an hour after HI,[9, 13, 15, 35] it seems unlikely that these mechanisms are critical to its beneficial effects.

Are excitotoxicity and free radicals relevant to post-insult cooling?

It is now known that:

1. both extracellular accumulation of EAAs and excess free radical production largely resolve during reperfusion after the insult and appear to have returned to normal values during the latent phase of recovery from HI;[19, 21, 27, 36]

2. *in vitro*, intra-insult hypothermia did not prevent intracellular accumulation of calcium during cardiac arrest *in vivo*,[37] or during EAA exposure *in vitro;*[38]

3. cooling initiated after wash-out of EAAs prevented neuronal degeneration *in vitro*.[38]

Thus, the ability of hypothermia to reduce release of excitotoxins does not appear to be central to its neuroprotective effects even during HI, and cannot easily account for the protective effects of *delayed* cooling. These data suggest that the critical effect of hypothermia is to block the intracellular *sequelae* of depolarization and EAA exposure.

Cell death mechanisms in the latent phase

Although the mechanisms of delayed cell loss are clearly multifactorial, there is increasing evidence that key pathways include activation of programmed cell death pathways, augmented by the inflammatory reaction and abnormal receptor activity as shown in Figure 3. Programmed cell death is activated by:

1. excessive calcium influx during and after HI,[39] promotes depolarization of the mitochondria (the 'intrinsic' pathway of apoptosis),[40] leading to permeabilization of the outer membrane of the mitochondria, with release of pro-apoptotic proteins, including cytochrome c.

2. abnormal excitatory receptor activity promoting further Ca^{2+} entry;

3. loss of trophic support from astrocytic growth factors,[41]

4. secondary inflammatory reaction to HI,[42] with release of cytokines and activation of cell surface death receptors (and thus the 'extrinsic' apoptosis pathway).[43]

Evidence that hypothermia can suppress programmed cell death

Therapeutic targets for hypothermia in the latent phase:

Programmed cell death

- Intrinsic pathway
- Extrinsic pathway

Secondary inflammation

- Microglial activation
- Microglial chemotaxis
- Cytokine release

Abnormal receptor activity

- Hyperactivity
- Receptor composition

Mitochondrial preservation

Post-insult hypothermia typically suppresses hypoxia-associated protein synthesis,[44] and multiple gene responses to ischemia, particularly genes involved in calcium homeostasis, cellular and synaptic integrity, inflammation, cell death, and apoptosis.[45] Thus it is plausible that hypothermia would help prevent 'active' forms of cell death. Although studies using morphological criteria for apoptotic cell death have had inconsistent outcomes,[44] in practice post-hypoxic cell death represents a continuum between apoptosis and necrosis, as recently reviewed.[46] Activation of caspase-3, the final 'executioner' caspase, is a reasonable, although nonspecific, marker of activation of apoptotic pathways.

In vitro, mild hypothermia directly suppressed neuronal apoptosis induced by serum deprivation, with reduced activation of caspases -3, -8, and -9 after 24 h, and reduced cytochrome c translocation, consistent with suppression of both the intrinsic and extrinsic pathways of apoptosis.[47] Further, hypothermia during focal ischemia in adult rats reduced expression of the cell death receptor Fas and activation of caspase-8, supporting a direct effect on the extrinsic pathway of apoptosis.[48]

These studies examined forms of *intra-insult* cooling. However, *in vivo,* in the near-term fetal sheep, hypothermia delayed for 90 min after ischemia markedly suppressed caspase-3 activation in white matter.[14] Similarly, in postnatal day 7 (P7) rat, an age when brain development is comparable to the late preterm human infant,[49] immediate induction of hypothermia after HI

reduced caspase-3 expression in the cortical infarct,[50] and in pre-oligodendrocytes.[51] In adult rats, after transient focal or global ischemia mild hypothermia suppressed activated caspase-3 immunoreactivity,[52, 53] upregulated the anti-apoptotic protein bcl-2, reduced expression of the proapoptotic protein p53,[54] and attenuated release of cytochrome c.[53, 55, 56] In adult minipigs, cooling after cardiac arrest reduced opening of the mitochondrial permeability pores.[57]

Finally, combined treatment with the anti-apoptotic agent, insulin-like growth factor 1, and hypothermia starting 4.5 h after cerebral ischemia in near-term fetal sheep did not show additive neuroprotection,[58] suggesting that these treatments were working in part though overlapping mechanisms.

Inflammatory second messengers

Brain injury leads to induction of the inflammatory cascade with increased release of cytokines and interleukins (IL).[59] These compounds are believed to exacerbate delayed injury, whether by direct neurotoxicity and induction of the extrinsic pathway of apoptosis or by promoting leukocyte diapedesis into the ischemic brain. For example, TNF-α and interferon-γ mediated iNOS expression were associated with mitochondrial DNA damage and apoptosis in cultured oligodendrocytes.[60]

In vitro, hypothermia inhibits microglia proliferation, chemotaxsis, and induction of pro-inflammatory cytokines, and the translocation and binding of a key inflammatory signal, nuclear factor-kappaB, and attenuated microglia neurotoxicity, during and critically, after exposure to both hypoxia and lipopolysaccharide.[61-64] In some settings, cooling may also increase release of anti-inflammatory cytokines.[65] In adult animals, hypothermia after transient focal ischemia and

brief cardiac arrest attenuated subsequent increases in cytokines such as interleukin-1β (IL-1 β) and tumor necrosis factor alpha (TNF-α).[66] Consistent with this, post-insult hypothermia suppressed activated microglia after transient ischemia or asphyxia in fetal sheep.[14, 67-69]

Intriguingly, despite potent suppression of microglia by hypothermia, it has little effect on astrocytic proliferation *in vitro*.[61] This raises the possibility that the hypothermic protection against post-ischemic neuronal damage may be, in part, the result of differential effects on glia, with suppression of microglial activation but relative sparing of restoration of the normal homeostatic environment by astrocytes.

Excitotoxicity

In contrast to their role during the primary and reperfusion phases, given that extracellular levels rapidly return to baseline values,[19, 27] the importance of EAAs *after* reperfusion is surprisingly unclear. In the temperature controlled environment of the fetal sheep, anti-excitotoxin therapy limited to the secondary phase did not reduce neuronal injury in severely injured parasagittal cortex and had only limited neuroprotective effects in other regions.[70, 71]

Nevertheless, even with normal levels of extracellular glutamate, excitotoxicity may still play an indirect role. There is evidence of pathological hyperexcitability of glutamate receptors after HI in P10 rats, with improved neuronal outcome after receptor blockade.[72] Consistent with this, in preterm fetal sheep, treatment with glutamate antagonist after asphyxia reduced neuronal loss,[73] although protection was much less than with hypothermia started at a similar time.[69] Further, in adult animals, neuronal death after ischemia has been associated with a selective, delayed change in the composition of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)

receptor, with specific down-regulation of GluR2, a subunit that limits Ca^{2+} influx. Hypothermia has been found to:

1. attenuate the post-ischemic reduction in the GluR2 subunit in adult gerbils;[74]

2. suppress excessive transient epileptiform activity in the first 6 h after asphyxia in preterm fetal sheep,[75] with a close correlation between suppression and neuroprotection.

Further studies are needed to confirm whether these mechanisms are important after hypoxic-ischemic injury in the term-equivalent brain.

Protection of the mitochondria

Mitochondrial failure is a hallmark of delayed cell death.[8] Clearly, maintaining mitochondrial function is crucial in promoting survival after HI. Post-ischemic hypothermia maintains mitochondrial respiratory activity after 2 h reperfusion in the adult gerbil,[76] and minipig,[57] and intra-ischemic hypothermia has been shown to preserve activity after 4 days recovery in neonatal rats.[77] It is unclear though whether this reflects direct protection of the mitochondria, or whether it is secondary to suppression of inflammation and programmed cell death.

Induction of growth factors

Perhaps surprisingly in view of the general tendency of hypothermia to suppress new protein synthesis, there is evidence in the adult rat that mild hypothermia after cardiac arrest is associated with augmentation of the increase in levels of growth factors such as brain-derived neurotrophic factor (BDNF) and others,[78, 79] which might help protect injured cells. Despite this,

BDNF infusion in normothermic animals was not neuroprotective.[80] Thus, induction of these growth factors does not seem to be a major mechanism of hypothermic neuroprotection.

Hypothermia in the Secondary Phase

There is compelling evidence that hypothermia started in the latent phase must be continued for 48 h or more to achieve optimal neuroprotection.[11] The precise reasons are unknown. The most likely explanation is that it is necessary to continue suppressing the programmed cell death and inflammatory pathways until normal homeostasis returns. However, it could in part reflect suppression of secondary events in this phase, including hyperperfusion, cytotoxic edema and delayed seizures (Figure 4).

Effects of hypothermia during the secondary phase

1. Possibly contributing to neuroprotection
    a. Reduced seizure burden may protect less severely injured areas of the brain by reducing anaerobic stress
2. Not contributing to neuroprotection
    a. Reduced cerebral hyperperfusion.
    b. Reduced cytotoxic oedema

Cerebral metabolism

During the latent phase cerebral blood flow and metabolism are both suppressed. This suppression is actively mediated by multiple neuroinhibitory pathways,[81] and likely helps mitigate the effects of abnormal excitatory activity. From 6 to 8 h, hyperperfusion develops

progressively, to a maximum after 36 to 48 h.[9, 15] Hypothermia suppresses the secondary hyperperfusion after ischemia in the fetal sheep,[9, 15] but late hypothermia that was not protective also effectively suppressed it.[15] Clinically, hypothermia markedly attenuated the secondary fall in the cerebral vascular resistance index, but reduced its predictive value.[82] Thus, this effect appears to be independent of neuroprotection.

Secondary cytotoxic edema

Similarly, neuroprotection with delayed cerebral cooling started 90 min after cerebral ischemia potently suppresses secondary cytotoxic edema in near-term fetal sheep.[9] However, strikingly, late induction of hypothermia (8.5 h after ischemia) also completely prevented secondary cytotoxic edema in the same paradigm, despite no significant neuroprotection.[16] These findings are highly consistent with the ability of hypothermia to reduce brain swelling after brain trauma and in other clinical settings,[83] and suggest that it is not a direct mechanism of neuroprotection.

Seizures

Intense, difficult to treat seizures are one of defining characteristics of neonatal encephalopathy.[84] Intense excitation during seizures leads to excessive local metabolic demand, which can potentially cause local neuronal death.[85] In near-term fetal sheep, treatment with MK-801, a highly potent, selective glutamate antagonist, between 6 and 24 h after cerebral ischemia prevented delayed post-ischemic seizures.[70] Despite this, there was no improvement in parasagittal neuronal loss, and only a modest improvement in less damaged regions such as the temporal lobe. These data suggest that severe seizure activity in the secondary phase can contribute to spreading of injury from the core area of damage to more mildly affected regions.

Clinically and experimentally, there is evidence of reduced seizure burden and reduced intensity of seizures during cooling.[75, 86, 87] Thus, the reduced metabolic demand associated with hypothermia in this phase might help to protect less severely injured regions from further injury.[16]

Final conclusions

The mechanisms underlying hypothermic neuroprotection are multifactorial, as summarized in table 1. Suppression of excitotoxicity, oxidative stress, inflammation, intracellular signaling and programmed cell death are all effects of hypothermia at different times. Critically, it is suppression of 'downstream' events after anoxic depolarization and excitotoxity that appear to be critical to hypothermic neuroprotection. We speculate that the differential effects of mild hypothermia to suppress programmed cell death and microglial activation without suppressing the recovery of normal homeostasis is central to long-term brain recovery. Further elucidation of these downstream pathways, particularly in the latent phase and during long-term recovery, will help us to design effective combination therapies.

Figure legends

Figure 1. Flow chart illustrating injurious events during hypoxia-ischemia and potential therapeutic targets for hypothermia. EAAs: excitatory amino acids. NO•: nitric oxide. OH•: hydroxyl free radical.

Figure 2. Flow chart illustrating potential therapeutic targets for hypothermia during reperfusion from hypoxia-ischemia (HI). NO: nitric oxide. MMPs: matrix metalloproteinases. BBB: blood brain barrier.

Figure 3. Flow chart illustrating key therapeutic targets for hypothermia during the latent phase of recovery after hypoxia-ischemia. EAA: Excitatory amino acid. GluR2: calcium impermeable subtype of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor. ER: Endoplasmic reticulum. TNFα: Tumor necrosis factor alpha. FADD: Fas-Associated protein with Death Domain. BCL-2: B-cell lymphoma 2 family of proteins. BAX: Bcl-2 associated X protein. BAK: Bcl-2 antagonist/killer. AIF: Apoptosis inducing factor.

Figure 4. Flow chart illustrating potential therapeutic targets for hypothermia, during the phase of secondary deterioration after hypoxia-ischemia.

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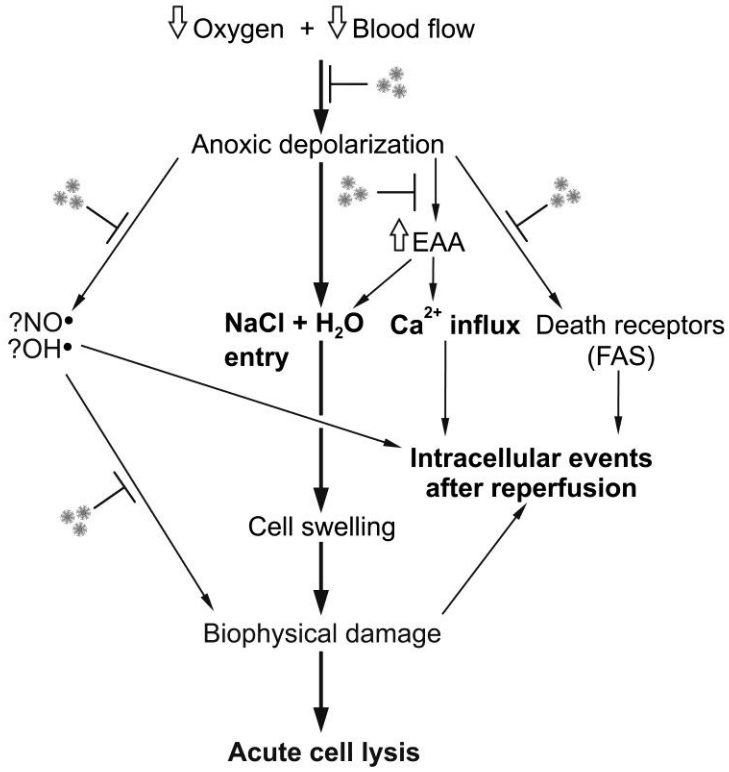
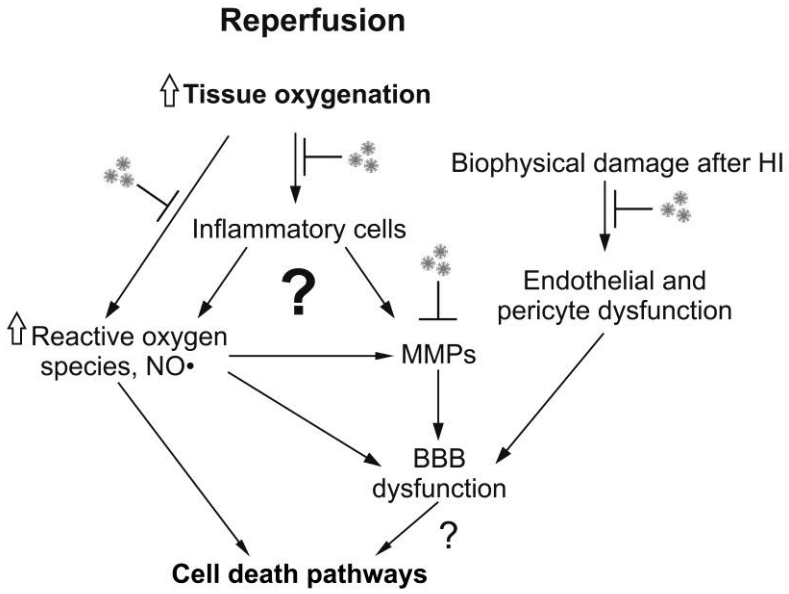
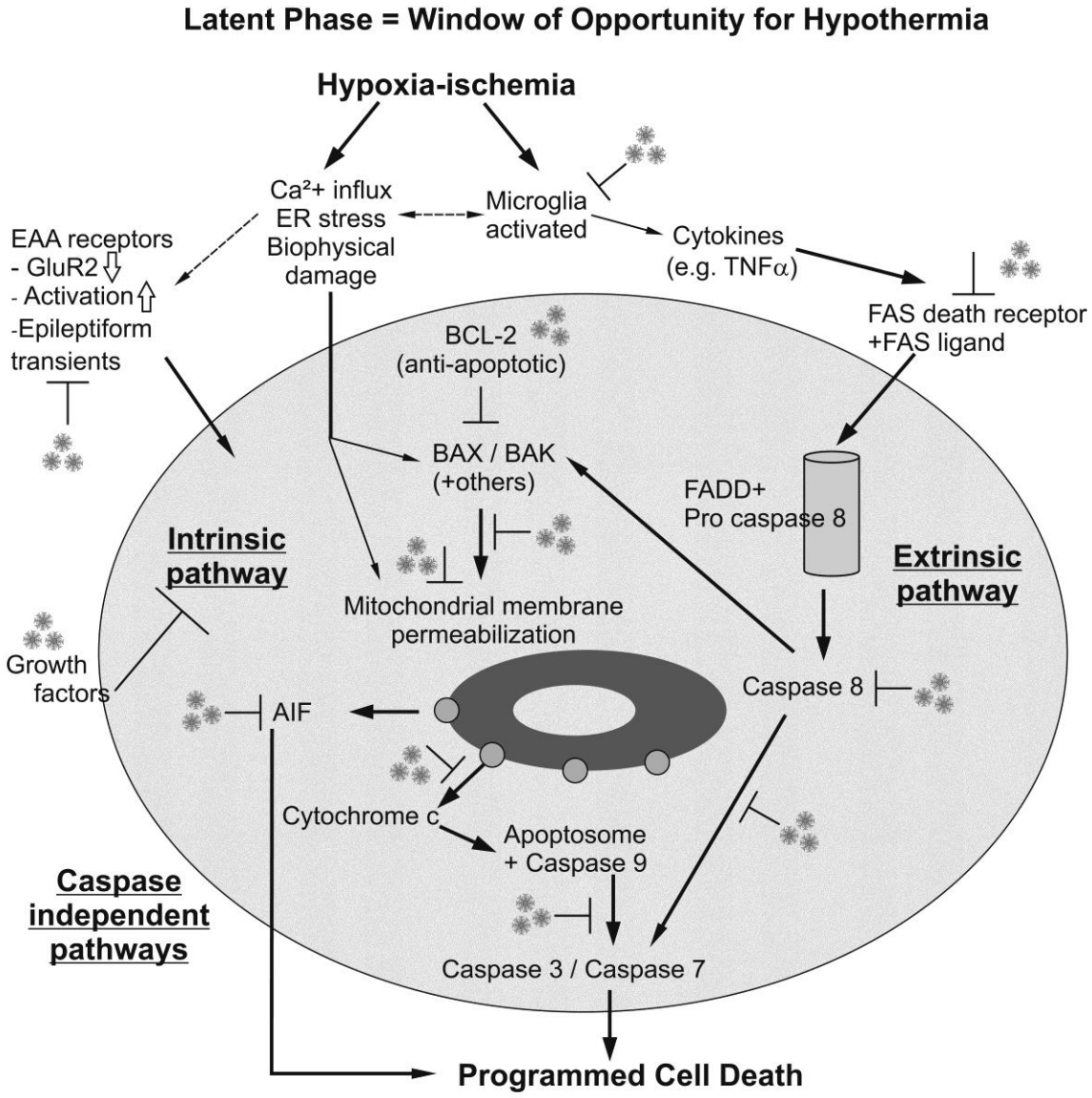
Table 1. Potential mechanisms of hypothermic neuroprotection

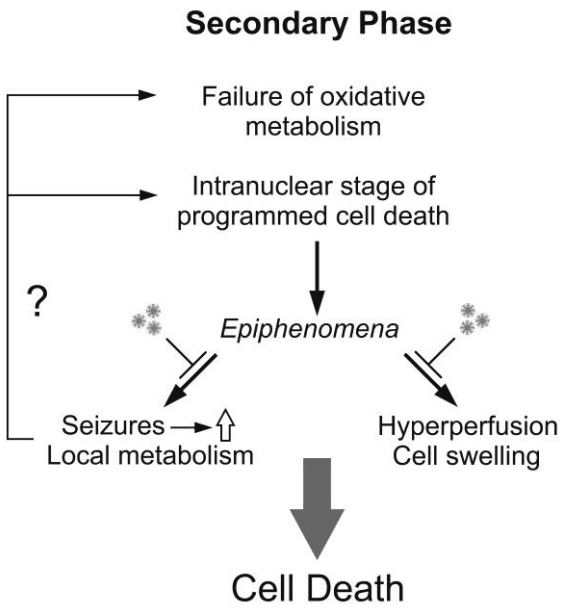
Mechanism of injury	Relevance to therapeutic hypothermia?
Anoxic depolarization	Limited. Relevant to cooling during hypoxia-ischemia such as surgery
Accumulation of EAAs / ROS	Limited. Reduced rate of release of EAAs / ROS by cooling during HI. Little evidence that it is affected by delayed cooling
Prevention of BBB breakdown	Limited. Early induction of hypothermia after ischemia can prevent BBB breakdown, however, hypothermia is neuroprotective when delayed after the apparent critical window for protecting the BBB
Programmed cell death	Strong. Hypothermia is associated with suppression of caspase-3, hypoxia-associated protein synthesis, the mitochondrial permeability transition, and components of the intrinsic and extrinsic pathways
Secondary inflammation	Strong. Mild hypothermia potently suppresses microglial activation, production of inflammatory cytokines and other neurotoxins
Abnormal glutamate receptor activation	Moderate. Hypothermia reduces adverse changes in composition of the AMPA receptor and suppresses epileptiform transients / abnormal receptor activation in the latent phase. The effect correlates with neuroprotection, but more studies needed to determine the role of these effects at term
Cerebral hyperperfusion	Unlikely. Hypothermia extends the phase of cerebral hypoperfusion and reduces hyperperfusion independently of neuroprotection
Cytotoxic edema	Unlikely. Hypothermia potently suppressed delayed cytotoxic edema, but independently of neuroprotection
Induction of growth factors	Limited. In some settings hypothermia can augment the increase in some growth factors after HI, but not clear whether this is a significant contributor to neuroprotection
Electrographic seizures	Limited. Potentially, hypothermia may reduce injury in penumbral, more mildly affected regions by reduced neural metabolism or anti-excitotoxicity effects; however, the neuroprotective effects of delayed hypothermia are much greater than anticonvulsants alone.

Primary Phase: Hypoxia-ischemia




Reperfusion
Tissue oxygenation
Biophysical damage after HI
Inflammatory cells
?
Endothelial and
pericyte dysfunction
Reactive oxygen
species, NO•
MMPs
BBB
dysfunction
?
Cell death pathways


Latent Phase = Window of Opportunity for Hypothermia
Hypoxia-ischemia
Ca²+ influx
ER stress
Biophysical damage
Microglia activated
Cytokines (e.g. TNFα)
EAA receptors
- GluR2
- Activation
-Epileptiform transients
FAS death receptor
+FAS ligand
BCL-2 (anti-apoptotic)
BAX / BAK (+others)
FADD+ Pro caspase 8
Intrinsic pathway
Extrinsic pathway
Mitochondrial membrane permeabilization
Growth factors
AIF
Caspase 8
Cytochrome c
Apoptosome + Caspase 9
Caspase independent pathways
Caspase 3 / Caspase 7
Programmed Cell Death


Secondary Phase
Failure of oxidative metabolism
Intranuclear stage of programmed cell death
?
Epiphenomena
Seizures
Local metabolism
Hyperperfusion
Cell swelling
Cell Death