Mechanisms of hypothermic neuroprotection

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Abstract

There is now compelling clinical evidence that 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 subsequent neuronal loss and improve behavioral recovery in term infants and 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 from it.

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1. Introduction

There is now compelling clinical evidence that 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 subsequent neuronal loss and improve behavioral recovery in term infants and adults after cardiac arrest.1–3 The specific mechanisms of this protection remain unclear. In part, paradoxically this reflects the range of potentially deleterious mechanisms that are suppressed, making it difficult to distinguish between changes during cooling that are critical to benefit, indifferent or perhaps even deleterious – but balanced by other, beneficial, effects. In some ways this may seem to be moot given that hypothermia has proved itself in clinical practice. However, the protective effects of hypothermia are incomplete, and many children continue to die of neural injury or survive with handicap with current hypothermia protocols.3 Better knowledge of the key therapeutic targets of cooling will help to rationally improve protection using hypothermia, and will be central to developing innovative combination therapies to augment the protective effects of hypothermia alone.4 In the present review we will critically assess the multiple effects of hypothermia in relation to the known window of opportunity to start cooling after severe hypoxia–ischemia.5

2. Hypoxic–ischemic (HI) injury evolves

The seminal insight that underpinned development of therapeutic hypothermia was that hypoxic–ischemic encephalopathy (HIE) is not a single ‘event’ but is rather an evolving process.6 We now know that although neurons may die during the actual ischemic or asphyxial event (the ‘primary’ phase, as shown schematically in Fig. 1), many neurons initially recover at least partially from the primary insult in a ‘latent’ phase, only to die many hours or even days later (secondary or delayed cell death).

During the primary phase of hypoxia–ischemia there is a profound reduction in oxygen availability and metabolism, leading to depletion of high energy metabolites, progressive depolarization of cells, allowing entry of salt and water causing cytotoxic edema (cell swelling), excessive entry of calcium and conversely efflux of potassium out of the cell, following their concentration gradients. There is marked extracellular accumulation of excitatory amino acids (EAAs) due to both failure of reuptake by astroglia and excessive depolarization-mediated release. After cerebral circulation and oxygenation are restored at end of the insult (reperfusion phase), the hypoxia-induced impairments of cerebral oxidative metabolism, cytotoxic edema and accumulation of EAAs resolve at least partially over approximately 30–60 min. Infants who do not show any recovery of oxidative metabolism have a consistently adverse outcome.7

The ‘latent’ phase that may follow reperfusion is characteristically ‘quiet’, even though many important events are initiated here, as will be discussed next. Electroencephalogram (EEG) activity is markedly suppressed. Brain blood flow having initially normalized...
after reperfusion shows a delayed onset of hypoperfusion, which may last for many hours. This hypoperfusion is associated with reduced oxygen consumption, but normal high energy metabolite levels on magnetic resonance spectroscopy. Approximately 6 h or as long 15 h after birth in some studies, the latent period may be followed by a secondary deterioration that extends over many days. At term equivalent, this secondary deterioration is often marked by delayed seizures, secondary cytotoxic edema (cell swelling), accumulation of excitotoxins, failure of cerebral mitochondrial activity, and ultimately, cell death.

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

Although we do not know exactly when the series of events leading to final cell death and dissolution becomes irreversible, there is overwhelming evidence that the early recovery (latent) phase, before the start of the secondary deterioration, represents the effective window of opportunity for initiation of post-insult cooling. For example, in the near-term fetal sheep, moderate hypothermia induced 90 min after reperfusion from a severe hypoxic–ischemic insult, for 34 min, improved outcome even when the absolute duration of depolarization is not severely reduced.22,34 Cooling during hypoxia–ischemia, for example, is partially protective through delaying the onset of anoxic cell depolarization. However, the protective effects of hypothermia even in this phase are not simply due to reduced metabolism, since mild cooling disproportionately improves outcome even when the absolute duration of depolarization is controlled.33,34 Cooling potentially reduces post-depolarization release during hypoxia–ischemia of many toxins including EAs in both adults25 and newborns.26

The increase in oxygen levels during reperfusion is associated with a transient damaging burst of oxygen free radicals, leading to peroxidation of structural cell membrane lipids.27 There is some evidence that a reduction in oxygen metabolism by cooling during this brief phase can suppress the oxygen free radical burst and lipid peroxidation from studies in the young adult dog28 and gerbil.29,30 During reperfusion, excitotoxin levels fall and the acute cell swelling resolves within a hour or so.31 It is likely that this resolution may be accelerated by cooling; microdialysis measurements over a period of 5 h showed that hypothermia started immediately after hypoxia–ischemia in newborn piglets was associated with reduced levels of excitatory amino acids, and reduced nitric oxide (NO) efflux in the brain.26

However, given that these mechanisms rapidly resolve during reperfusion before the latent phase of recovery from the end of hypoxia prevented cortical infarction, whereas 6 h of cooling had an intermediate effect.13 Similarly, in anesthetized piglets exposed to either hypoxia with bilateral carotid ligation or to hypoxia with hypotension, either 12 h of mild whole body hypothermia (35 °C) or 24 h of head cooling with mild systemic hypothermia started immediately after hypoxia prevented delayed energy failure,14 reduced neuronal loss,15,16 and suppressed post-hypoxic seizures.16

Starting cooling as soon as possible after resuscitation is essential but does not appear to be sufficient. At present it is typically impractical to start cooling immediately, because the majority of infants requiring resuscitation or with metabolic acidosis on cord blood do not go on to develop HIE.17 By the time that encephalopathy can be reliably diagnosed, significant time, often hours, may have passed. The experimental literature strongly suggests that when hypothermia is delayed a relatively prolonged interval of cooling is required for significant protection. As previously noted, mild hypothermia (2–4 °C reduction in temperature) for a few hours was partially protective when started immediately after hypoxia–ischemia in piglets and neonatal rats, but not when cooling was delayed just 15–45 min after the primary insult.5 These results are consistent with the finding in adult gerbils that, when the delay after cerebral ischemia before initiating a 24 h period of cooling was increased from 1 to 4 h, neuroprotection in the CA1 region of the hippocampus after six months’ recovery fell from 70% to 12%.18 This chronic loss could be prevented by extending the duration of moderate (32–34 °C) hypothermia to ≥48 h, even when the start of cooling was delayed until 6 h after reperfusion.19,20

To summarize the implications of timing: whatever the critical mechanisms are, they must arise in the early recovery (latent phase), although optimally, cooling should be continued until the secondary events are resolving.

4. Potential mechanisms of hypothermic protection

4.1. Metabolic inhibition in the primary phase and reperfusion

The best-known feature of hypothermia is the associated graded reduction in cerebral metabolism of about 5% for every degree of temperature reduction.21,22 Cooling during hypoxia–ischemia, for example, is partially protective through delaying the onset of anoxic cell depolarization. However, the protecive effects of hypothermia even in this phase are not simply due to reduced metabolism, since mild cooling disproportionately improves outcome even when the absolute duration of depolarization is controlled.33,34 Cooling potently reduces post-depolarization release during hypoxia–ischemia of many toxins including EAs in both adults25 and newborns.26

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hypoxia–ischemia, typically within 30–60 min, they cannot readily account for the protective effects of delayed, post-insult cooling.

4.2. Cerebral blood flow and metabolism in the latent phase

Following reperfusion, cerebral blood flow typically recovers transiently, followed by secondary hypoperfusion. Hypoperfusion is consistently seen during the latent phase; indeed one of the markers of the end of the latent phase is a transition to hyperperfusion. The duration of hypoperfusion in the latent phase is broadly proportional to the severity of the insult. The significance of this apparently low perfusion has been controversial, since prolonged cerebral hypoperfusion after perinatal hypoxia–ischemia is associated with an adverse clinical outcome. However, there is now increasing evidence that it does not reflect ‘poor’ perfusion, but rather is actively mediated by suppressed cerebral metabolism and is associated with increased, not decreased, tissue oxygen levels.

In the fetal sheep, delayed post-cerebral hypothermia was associated with a marked extension of the phase of secondary hypoperfusion, to nearly 24 h after the insult. This prolongation of reduced blood flow was associated with improved neural outcome. Hypothermia started at both 1.5 h and 5.5 h after ischemia also prevented the later development of hyperperfusion during the secondary phase. Post-insult hyperperfusion is strongly associated with injury, and appears to reflect a true luxury ‘perfusion’, i.e. excess perfusion contrasting with progressive failure of oxidative metabolism in the phase of secondary deterioration.

Mitochondrial failure is a hallmark of the phase of secondary deterioration. Clearly, maintaining mitochondrial function is crucial in promoting survival after hypoxia–ischemia. Post-ischemic hypothermia maintains mitochondrial respiratory activity after 2 h reperfusion in the adult gerbil. Hypothermia suppressed reactive oxygen species-mediated mitochondrial damage and preserved mitochondrial membrane potential in cultured myocytes. Currently there are no data directly evaluating mitochondrial function in vivo during therapeutic hypothermia following hypoxia–ischemia. However, prolonged cooling in the piglet and in rodents preserves cerebral high-energy metabolite production, and thus strongly suggests that mitochondrial function was preserved by hypothermia.

Interestingly, despite these effects on cerebral metabolism and perfusion, and the reduction in neuronal loss, post-insult hypothermia did not significantly reduce the rate of electrophoretic seizures. By contrast with the lack of effect on total numbers of events, in at least one study in preterm fetal sheep cooling markedly reduced mean post-asphyxial seizure amplitude. It is likely that cooling may have ameliorated in part the excessive local metabolic demand associated with seizure activity, which in turn has been linked with local neuronal death in some settings. Nevertheless, a prolonged period of hypothermia delayed until just after the onset of post-ischemic seizures was not associated with a significant reduction in neuronal loss. Furthermore, hypothermia delayed until this point (8.5 h after reperfusion) was able to abolish secondary cytotoxic edema — a major feature of delayed energy failure, despite not affecting neuronal loss. Consistent with this finding, infusion of MK-801, a highly potent, selective glutamate antagonist, between 6 and 24 h prevented delayed post-ischemic seizures and completely suppressed the fetal EEG. Despite this there was no improvement in parasagittal neuronal loss scores, although there was a small reduction in less severely affected regions. These data suggest that alterations of delayed overt electrographic seizure activity in isolation are unlikely to be a major therapeutic target in infants with HIE, and cannot be critical to hypothermic neuroprotection.

4.3. Intracellular pathways in the latent phase

The effects of hypothermia on pathways distal to cell membrane ion channels are likely to be more important than direct suppression of toxins in the latent phase. For example, hypothermia did not prevent intracellular accumulation of calcium during cardiac arrest in vivo, or during extracellular glutamate exposure in vitro. Moreover, in the same in vitro study, cooling initiated after the excitotoxins had been washed out effectively prevented neuronal degeneration. Thus, the ability of hypothermia to reduce release of excitotoxins or to reduce hypoxia–ischemia–related calcium overload do not appear to be central to its neuroprotective effects. Rather these data suggest that the critical effect of hypothermia is to block the intracellular sequelae of excitotoxin exposure. Finally, we should note that cooling can prevent intracellular ion and water entry and the consequent osmotic cell swelling even if the ATP-dependent Na⁺/K⁺ pump is inhibited by ouabain. This mechanism is likely to be relevant to its ability to attenuate secondary cytotoxic edema, but as discussed above, not to protection.

Key mechanisms implicated in the latent phase include programmed cell death, post-ischemic inflammation, and abnormal excitatory receptor activity.

4.4. Programmed cell death

There is increasing evidence to suggest that hypothermia may have a particular role in suppressing the evolution of programmed post-ischemic cell death, often referred to as apoptosis by analogy with developmental cell death. Post-HI apoptosis can be triggered by the mechanisms discussed above, including glutamate receptor excitotoxicity and consequent intracellular calcium accumulation, inflammation, and oxidative stress. The intracytoplasmic stage of apoptosis involves alterations in the ratio of various intracellular factors such as Bcl-2, which inhibits apoptosis, and Bax which promotes apoptosis, and activation of cysteine proteases (caspases). The final, irreversible execution phase of apoptosis is intranuclear, involving endonuclease-mediated DNA fragmentation. By contrast, necrosis was suggested to reflect biophysical damage to the cell (cell membrane instability, ion shifts etc.), particularly lysis in the primary phase. Both patterns are clearly described in infants dying after perinatal asphyxia.

Recent clinical studies have shown that apoptosis is a major contributor to post-asphyxial cell death in the developing brain. Studies using morphological criteria have had mixed outcomes. In the piglet, hypothermia started after severe hypoxia–ischemia was reported to reduce apoptotic cell death, but not necrotic cell death, with similar results reported after injury in rats. However, in the adult rat, delayed post-ischemic cell death prevented by hypothermia had a necrotic appearance on detailed electron microscopic criteria, consistent with findings of a maturation-related reduction in caspase-3 expression after hypoxia–ischemia in the rat.

In practice, there is now evidence that post-hypoxic cell death typically includes elements of both apoptotic and necrotic processes, with one or the other being most prominent depending on factors such as maturity and the severity of the primary insult. Many mechanisms, including mitochondrial calcium overload and activation of components of the caspase pathway, seem to contribute to both apoptotic and necrotic cell death. Although multiple pathways are likely to be involved in such
post-ischemic apoptosis, caspase-3 is reported to play a crucial role as the final ‘executioner’ caspase.56 Protection with post-ischemic hypothermia in the near-term fetal sheep was closely linked with suppression of activated caspase-3.67

These data are consistent with in-vitro studies of hypothermia after severe hypoxia in developing rat neurons. Strikingly, in that model hypoxic preconditioning activated a program that stimulated the expression of anti-apoptotic proteins such as Bcl-2 and HSP-70, whereas hypothermia did not trigger these active processes, but rather depressed cell activity and abolished hypoxia-associated protein synthesis, thus suppressing apoptotic pathways.68 69

4.5. Inflammatory second messengers

Brain injury leads to induction of the inflammatory cascade with increased release of cytokines and interleukins (IL).60 These compounds are believed to exacerbate delayed injury, whether by direct neurotoxicity and induction of apoptosis through the so-called extrinsic pathways linked to cell death receptors,70 or by promoting leukocyte diapedesis into the ischemic brain. Experimentally, cooling potently suppresses multiple aspects of the inflammatory reaction.5 For example, in vitro, hypothermia inhibits proliferation, superoxide and NO production by cultured microglia,71 and in adult rats hypothermia suppresses the post-IL-1β2 and accumulation of polymorphonuclear leukocytes.73 Similarly, post-ischemic hypothermia can suppress microglial activation following transient ischemia in the fetal sheep.67 The broad reduction in inflammatory signaling may offer significant mitochondrial protection. Cytokine-mediated inducible nitric oxide synthase (iNOS) expression increases NO levels which compete with molecular oxygen at its binding site on cytochrome oxidase,74 potently suppressing oxidative metabolism and thus reducing ATP levels.75 Tumour necrosis factor (TNF)-α also has this effect by inhibiting complex-I of the electron transport chain.76 Furthermore, TNF-α and interferon-γ–mediated iNOS expression was associated with mitochondrial DNA damage and apoptosis in cultured oligodendrocytes.77

4.6. Hypothermia and excitotoxicity in the latent phase?

Classically, cell death due to abnormal glutamate receptor activation (excitotoxicity) has been related to pathologically elevated levels of extracellular glutamate, as occurs in grey matter during hypoxia–ischemia,49 although there is evidence that this does not occur in white matter.78 Following reperfusion, extracellular glutamate in grey matter rapidly returns to control values,31 and thus we might predict that excitotoxicity should not be important after reperfusion. Supporting this, in an elegant study in the adult rat, Nurse and Corbett have shown that the apparent neuroprotective effect of NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), a glutamate antagonist, administered from 1 h after mild ischemia, was actually mediated by mild endogenous hypothermia for several days, developing an hour after drug administration. Similar neuroprotection was induced by mimicking this profile of cooling over a period of 24 h, while conversely NBQX neuroprotection was abolished by warming.79

More recent data, however, show that pathological hyperexcitability of glutamate receptors can occur for many hours following hypoxia–ischemia in preterm fetal sheep and that receptor blockade can suppress this activity and improve neuronal outcome.60 Although overall ECG activity is profoundly suppressed for many hours after asphyxia in preterm fetal sheep, regardless of whether injury later developed or not, transient epileptiform activity was only seen in the early recovery phase after a profound insult that was associated with severe injury.81 Further, the frequency of transients was correlated with the severity of neuronal loss in the striatum and hippocampus82,83 (e.g. see Figure 3 in Bennet et al.84). These events occur in the early recovery phase before secondary failure of mitochondrial function, and are abolished by N-methyl-D-aspartate receptor blockade with MK-801,85 raising the possibility that these early events may be an in-vivo analogue of glutamate receptor hyperactivity and directly contribute to injury.86

Clearly these events could simply be a manifestation of developing injury. However, a possible causal relationship is supported by a combination of observations: that suppression of EEG transients with a glutamate receptor antagonist reduces cell loss,65 and conversely that increased EEG transient activity during blockade of inhibitory z₂-adrenergic receptor activity was associated with increased neuronal loss.82 Supporting this interpretation, neuroprotection with post-asphyxial moderate cerebral hypothermia in the preterm fetal sheep was associated with a marked reduction in numbers of epileptiform transients in the first 6 h after asphyxia and reduced amplitude but not numbers of delayed seizures.77 Thus, this abnormal glutamate-receptor-associated activity in the early recovery phase may be an important therapeutic target for hypothermia. Continuous unfiltered EEG recordings from infants with HIE in the first few hours of life are now needed to validate whether similar events occur during clinical encephalopathy.

5. Conclusions

Suppression of cerebral metabolism has historically been regarded as the primary mechanism of protection. It is now clear that the mechanisms underlying hypothermic neuroprotection are multifactorial. Key potential mechanisms in the latent phase that have been shown to be suppressed by hypothermia include programmed cell death (‘apoptosis’), inflammation and the extrinsic cell death pathway, and abnormal receptor activity. It is likely that it is the intracytoplasmic, ‘downstream’ effects of these phenomena that are critical to protection. Further elucidation of the contribution of each of these pathways in the latent phase and at what level the cascades become irreversible by hypothermia are key to further improving neuroprotective strategies in the future.

Practice points

- The specific mechanisms of hypothermic neuroprotection are not established; whatever the critical mechanisms are, they must arise in the early recovery (latent phase).
- Mechanisms that have been implicated in the latent phase include suppression of programmed cell death (‘apoptosis’), inflammation and the extrinsic cell death pathway, and abnormal receptor activity.
- Although hypothermia reduces cerebral metabolism, markedly suppresses cytotoxic edema, and modulates seizure intensity (and thus likely metabolic demands and excitotoxin release), none of these mechanisms appears to be independently protective.
- Suppression of free radical production and of excitotoxin release may contribute to protection when cooling is started during hypoxia–ischemia or reperfusion, but is unlikely to be important for the typical clinical setting of delayed cooling started well after resuscitation.
Research directions

- Disentangling the multiple pathways that are modulated by hypothermia is vital to provide a strong basis for finding additive treatments to further improve neuroprotection.
- Hypothermia appears to act at multiple levels of the cell death cascade. It would be of considerable assistance in developing new protective regimes to know at which level of the intracellular cascade hypothermia can no longer prevent progression to cell death. At present this can only be assessed by empirically treating at different times.

Conflict of interest statement
None declared.

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