

Received: 2004.12.23
Accepted: 2005.02.01
Published: 2005.06.15

The biology and pathology of hypoxia-ischemia: an update

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Source of support: self financing

Summary

After an hypoxic-ischemic (HI) insult, a multi-faceted complex cascade of events occurs that ultimately causes cell death and neurological damage to the central nervous system. The various cascades include, amongst others: immunological changes, such as the activation of the complement system and the generation of antibodies; increased inflammation through the actions of pro-inflammatory cytokines and chemokines; the production of reactive oxygen species leading to oxidative stress; and diminished mitochondrial function leading to the activation of apoptotic pathways and subsequent alteration in the function of neurons within the contralateral hemisphere. This review addresses the immunological aspects following HI, the role of various cytokines (both pro-inflammatory and anti-inflammatory) and chemokines after the induction of HI. In addition, the role of free radicals in producing HI-induced neurodegeneration and the contribution that mitochondrial dysfunction has in neuronal apoptotic cell death will be discussed. This review also covers the changes that the previously assumed "internal control", the contralateral hemisphere, undergoes due to HI and describes the difficulties associated with therapy intended to prevent neuronal injury associated with HI.

Key words: angiogenesis • immunity • inflammation • mitochondria

Abbreviations:

AIF – apoptosis-inducing factor, **APCs** – antigen-presenting cells, **APE/Ref-1** – apurinic/aprimidinic endonuclease, **BAF** – boc-aspartyl-(Ome)-fluoromethyl-ketone, **BDNF** – brain-derived neurotrophic factor, **CNS** – central nervous system, **CSF** – cerebrospinal fluid, **CP** – cerebral palsy, **EGCG** – epigallocatechin gallate, **G-CSF** – granulocyte colony-stimulating factor, **GM-CSF** – granulocyte-macrophage colony-stimulating factor, **HI** – hypoxia-ischemia, **HIE** – HI encephalopathy, **ICE** – interleukin-1-converting enzyme, **IEGs** – immediately early genes, **IFN** – interferon, **Ig** – immunoglobulin, **IL** – interleukin, **L-NAME** – N^o-nitro-L-arginine methyl ester, **MCP** – monocyte chemoattractant protein, **M-CSF** – macrophage colony-stimulating factor, **MIP** – macrophage inflammatory protein, **mPTP** – mitochondrial permeability transition pore, **NO** – nitric oxide, **NOS** – nitric oxide synthase, **RANTES** – regulated on activation normal T cell expressed and secreted, **rCBF** – regional cerebral blood flow, **rhIL-1ra** – recombinant human IL-1 receptor antagonist, **ROS** – reactive oxygen species, **SOD** – superoxide dismutase, **TGF** – transforming growth factor, **TNF** – tumor necrosis factor, **VEGF** – vascular endothelial growth factor.

Full-text PDF: http://www.aite-online/pdf/vol_53/no_3/7445.pdf

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INTRODUCTION

Over the past couple of decades, significant progress has been made as to the initiating factors associated with hypoxia-ischemia (HI). The imbalance between fetal and maternal blood supply ultimately leads to hypo-perfusion, altered autoregulation, and/or hypoxia (see⁶⁴ for review). Furthermore, a number of causes of ischemia have been noted, such as breaching, premature rupture of membranes, premature separation of the placenta, as well as conditions of prolonged hypoxia linked to a diminished blood supply¹⁵⁸. To date, several factors, such as hypoxia, cerebral ischemia, cerebral hemorrhage, and energy failure, have been shown to result in cerebral injury (see⁶⁶ for review). In addition, strong epidemiological evidence exists linking maternal infection with the development of neonatal brain injury (see¹¹⁷ for review).

Cerebral HI has been shown to induce acute tissue damage, resulting in functional morbidity and mortality in infants and children. HI-induced cerebral damage remains the most common neuronal complication that results in the development of debilitating conditions that include, amongst others, cerebral palsy (CP), mental retardation, and epilepsy (see^{76, 133} for reviews). In addition, HI has also been likened to the acute ischemic stroke seen in adult humans¹³². The incidence of these neurological conditions have been shown to occur in 2–4 full-term newborns per 1000 subjected to systemic asphyxia and approximately 60% of low-birth-weight and premature infants (see^{166, 169} for reviews).

An ever-expanding wealth of literature associated with HI now exists. The pathophysiological mechanisms underlying HI are becoming well established and are of growing complexity and include, amongst others, increased extracellular glutamate, increased intracellular sodium and calcium, decreased ATP production, increased reactive oxygen species (ROS) production, activation of the arachidonic acid pathway, and activation of other inflammatory mediators, to name just a few (see^{3, 11, 81, 83, 84, 167, 168, 170} for reviews). A growing body of evidence has accumulated over recent years highlighting the immune system and, more importantly, cytokines and chemokines as key mediators not just in HI^{14, 125, 136}, but also in other neurodegenerative disorders, such as Alzheimer's disease (see²⁹ for review). In addition, these mediators have been shown to be closely interrelated in a complex and often vicious positive feedback cycle, in that pro-inflammatory cytokines are known to induce ROS and *vice versa*^{1, 45, 59}. This review will, therefore, attempt to cover a number of components involved in the pathophysiology of HI, including the

immune response, the inflammatory (cytokine and chemokine) response, oxidative stress, angiogenesis, mitochondrial changes, and changes to the contralateral hemisphere.

THE IMMUNE RESPONSE IN HI

It has been known for decades that natural antibodies are present in the sera of both humans and animals following exposure to foreign material¹⁵. These natural antibodies are typically polyreactive, are usually of low affinity but have a high avidity, and in humans, mice, and rats belong to the immunoglobulin (Ig)M, IgG, and/or IgA isotypes^{5, 6}. Low levels of autoimmunity have been shown to be necessary for normal function³⁵, in that the maturation and survival of lymphocytes requires autoantigens⁶¹. In addition, evidence is accumulating suggesting that autoimmunity may play an important and complex role in lesions involving the central nervous system (CNS). The presence of autoantibodies has been shown to play an important role in the removal of dead cells, by binding to the cell surface to enhance phagocytosis, by opsonization^{12, 63, 104}.

Since the first report by Levi-Strauss and Mallat⁹⁵ in 1987 that brain cells possess the capacity to synthesize complement components, others have likewise reported that all complement components are synthesized by brain cells, these including neurons, astrocytes, microglia, and endothelial cells^{55, 156}. In addition, significant evidence now exists indicating that activation of complement may contribute to CNS injury associated with a number of neurodegenerative conditions, including ischemic stroke⁷⁸, multiple sclerosis¹⁵¹, and cerebral HI (see⁹³ for review). However, the exact role that complement plays in cerebral HI appears to be somewhat controversial, as reports have shown that complement activation can augment HI-mediated infarction³¹, whilst i.p. administration of complement inhibitors failed to decrease the HI-induced infarction size⁹⁴.

Following injury to the CNS, circulating autoantibodies have been shown to bind to dying neurons hours to days following the initial lesion to the CNS tissue¹⁵⁰. Changes in autoantibodies have also been seen in infants, whereby adult autoimmune disease has been transfected to their progeny¹³⁹. Recently it was shown that maternal autoantibodies transmitted to their progeny can trigger *de novo* neonatal pathogenic autoreactive T cell responses and T cell-mediated organ-specific autoimmune disease¹³⁹. In addition, assessment of children who suffered some form of CNS injury during childbirth showed a marked enhancement in antibody reactivity within 3 days of injury¹¹⁰. These elevated levels remained increased,

but did return to baseline levels over a period of years, and the enhancement was most typically seen for the isoform switch of IgG and IgA. Furthermore, following single or repeated bouts of hypoxia, antibodies showed a shift from the IgM to the IgG isoform, as assessed using ELISA techniques¹⁴⁹. Taken together, this information strongly suggests that cerebral HI has an autoimmune component associated with the injury.

INFLAMMATION AND HI

Substantial research now exists which implicates the immunoinflammatory system in HI-induced neuronal injury to the immature brain. Therefore, the presence of infiltrating leukocytes resulting in inflammation to the ischemic area (as with other inflammatory diseases) postulates a role for cytokines (well-known modulators of the inflammatory response) and chemokines (recruitment of immune cells) in ischemia-induced neuronal damage (see¹²¹ for review).

CYTOKINES AND HI

Cytokines appear to play a significant part in HI-induced neuronal damage (see Table 1). Pro-inflam-

matory cytokines, such as interleukin (IL)-1 and tumor necrosis factor (TNF)- α , are upregulated during the acute phase of damage (1–12 h) post-HI^{7, 42, 70, 155, 163}. In addition, IL-1 and TNF- α mRNA have been shown to increase in hypoxic cultures of villous explants from the human placenta¹⁰. By 24 h, IL-1 and TNF- α levels were the same as in controls^{7, 70, 155}, with the exception of one group which showed that TNF- α remained upregulated 24 h post-HI¹²⁴. In human asphyxiated babies and newborn infants with HI encephalopathy (HIE), cerebrospinal fluid (CSF) and plasma IL-1 β and TNF- α levels were elevated within 48 h^{46, 120, 145}. In addition, asphyxiated babies that develop CP or neurological deficits within 1 year have increased IL-1 β and TNF- α levels within 48 h of birth^{46, 115, 120}. This suggests that both IL-1 and TNF- α play an important role in the acute stages of inflammation after HI that leads to neurological deficits such as CP. Therefore, the levels of IL-1 β and TNF- α at birth may be a good predictor for the development of subsequent neurological deficits in developing infants.

Due to the important role that the initial pro-inflammatory cytokines (IL-1 and TNF- α) play in HI-induced injury, it may be beneficial to block the action of these cytokines in order to reduce the neurological deficits that follow an HI event. This mode of therapy has only been investigated in animal models and has not been tried in clinical trials to date. The recombinant human IL-1 receptor antagonist (rhIL-1ra) prevents IL-1 binding to its receptor, thus inhibiting IL-1 function. An rhIL-1ra has been developed which can be exogenously administered to block IL-1. In an animal model of HI, 100 mg/kg rhIL-1ra was administered s.c. 1 h prior to or 1 h post-HI and was neuroprotective against cell death¹⁰³. Hagberg et al.⁷⁰ administered rhIL-1ra i.c., but it was only neuroprotective when administered prior to and not post-HI. Another approach to reduce the action of IL-1 is to prevent its activation. IL-1-converting enzyme (ICE) cleaves the inactive pro-IL-1 β to active IL-1 β , so an inhibitor of this enzyme will prevent active IL-1 β formation. Unfortunately, the inhibitors of this enzyme that have been developed⁸⁹ have not been investigated in HI. However, ICE knockout mice have shown resistance to HI-induced neuronal injury, suggesting that ICE is an important mechanism that contributes to HI-induced damage⁹⁷.

IL-6 has both neurodestructive and neuroprotective effects (see⁵³ for review) and may hence play a dual role in HI-induced injury. IL-6 mRNA and protein levels are upregulated 0–24 h after HI in the rat^{42, 70, 124}, except for one group that could not detect IL-6 12 h post-HI¹⁶³. In humans, CSF, plasma, and serum

Table 1. Comparison of cytokine and chemokine levels after hypoxia-ischemia in both animals and humans

Cytokine	Chemokine
IL-1 α	↑[70] NC ^[124, 163]
IL-1 β	↑[7, 14, 42, 46, 70, 73, 115, 120, 155, 163] NC ^[120, 124, 137]
TNF- α	↑[7, 14, 42, 46, 115, 120, 124, 146, 155, 163] NC ^[120, 137, 145]
TNF- β	↑[163]
IL-2	NC ^[163] ↓[115]
IL-3	NC ^[163] ↓[115]
IL-4	NC ^[115, 163]
IL-5	NC ^[115, 163]
IL-6	↑[25, 42, 46, 70, 105, 115, 124, 137, 145] NC ^[163]
IL-7	NC ^[115]
IL-9	↑[46, 115]
IL-10	NC ^[7, 115, 137, 163]
IL-11	↑[115]
IL-13	↑[115]
IL-18	↑[73]
TGF- β	↑[88, 115, 124]
IFN- γ	NC ^[163]
GM-CSF	↑[115] NC ^[137]
M-CSF	↑[115]
G-CSF	↑[115]

NC – no change in the level of that cytokine/chemokine in HI animals/humans compared with control animals/humans; ↑/↓ – increase/decrease in the level of that cytokine/chemokine in HI animals/humans compared with control animals/humans; Gro – growth-related oncogene. The appropriate references are in parenthesis [].

IL-6 levels were upregulated in asphyxiated newborns and HIE infants within 48–90 h after birth^{25, 46, 105, 137, 145}. Furthermore, IL-6 appeared to have a positive association with HIE severity and clinical outcome of HIE, suggesting that the production of IL-6 in this setting is pro-inflammatory^{105, 137}. Similarly, IL-18 also appears to play a pro-inflammatory role after HI-induced brain damage, as IL-18 knockout mice had a 21% decrease in infarct size compared with wild-type mice after HI⁷³.

Many other cytokines are involved in the pathophysiological process that occurs after HI. IL-4 and IL-10 are T helper 2 cell-derived cytokines and are considered anti-inflammatory. However, their role in HI-induced damage is still being established. IL-4 and IL-10 were not detected 12 h post-HI in the rat¹⁶³, whereas IL-10 levels were not significantly different from control groups in both asphyxiated newborns¹³⁷ and HI rats⁷. This implies that anti-inflammatory cytokines such as IL-4 and IL-10 do not contribute to the initial inflammatory response following HI. However, exogenous administration of IL-10 (i.v.) prevented the damage seen after endotoxin administration post-HI, suggesting that IL-10 has a therapeutic effect⁴⁸. Other interleukins, such as IL-2, IL-3, and IL-5, and interferon (IFN)- γ were not detected 12 h post-HI¹⁶³ and their roles are yet to be fully elucidated in HI-induced neurodegeneration. Granulocyte-macrophage colony-stimulating factor (GM-CSF) levels were not different between asphyxiated neonates and controls³⁷. Transforming growth factor (TGF)- β , which is well known for its anti-inflammatory actions, was shown to be elevated 24 h post-HI¹²⁴. In addition, TGF- β mRNA expression is upregulated in the ipsilateral hemisphere at 1 h (severe insult) or 72 h (moderate insult) and remains elevated for at least 120 h post-HI⁸⁸. Furthermore, exogenous administration of 10 ng TGF- β i.v.c. reduced neuronal injury caused by HI^{67, 107}, suggesting that TGF- β has a neuroprotective effect.

Cerebral palsy is one of the most common and crippling motor disorders in children and produces a huge economic impact worldwide. CP has many initiators which are mainly triggered *in utero*, such as intrauterine infection and HI. By investigating the immune and inflammatory response after infection or HI, viable therapies may be produced to prevent the onset of CP. Nelson et al.¹¹⁵ undertook an investigation looking at the relationship between inflammatory mediators and CP. In this study they compared the cytokine levels of dried blood spot specimens obtained at birth from CP children and control children. CP children had higher concentrations of TNF- α , IL-1, IL-6, IL-9, IL-11, and IL-13 (they also

had higher levels of various chemokines – see section below). There was no difference in IL-4, IL-5, IL-7, and IL-10 levels between the two groups. Interestingly, CP children also had increased granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), GM-CSF, and TGF- β . Therefore, because CP children showed a similar cytokine profile as post-HI (induction of pro-inflammatory and no change in anti-inflammatory cytokines), then the prevention or block of these pro-inflammatory mediators may provide protection against the development of CP.

Irrespective of the initiating stimuli, such as HI, intrauterine infection, choriomnionitis, toxin-induced injury, and preterm labor, the resultant CNS traumas all appear to have cytokines as a final common pathway⁴⁶. The concentrations of many cytokines have been investigated in these settings, as well as in children with neurological deficits caused by these triggers, such as periventricular leukomalacia and CP^{33, 39, 40, 74, 99, 106, 109, 115, 116, 135, 140, 178–181}. All of these clinical settings appear to have increased pro-inflammatory cytokines, such as IL-1 and TNF- α , with no change in anti-inflammatory cytokines, such as IL-4 and IL-10, compared with controls. Therefore, no matter what the initial source of CNS injury in the neonate is, the inflammatory response initiated and propagated by cytokines represents a shared mode of action leading to neuronal damage.

CHEMOKINES AND HI

The role of chemokines (low-molecular-weight chemotactic cytokines) in HI-induced neuronal damage is very limited, according to the few studies investigating their effects (see Table 1). The α -chemokines (which are chemoattractants for granulocytes) include growth-related oncogene and macrophage inflammatory protein (MIP)-2 and appear to be involved in HI-mediated brain injury. MIP-2 secretion was induced after hypoxia/reoxygenation in microglial cultures¹⁷¹, while MIP-2 and gro mRNA expression was increased 0–24 h post-HI in the rat¹⁴. In addition, MIP-2 concentrations were higher in CP children compared with control children¹¹⁵, implicating the involvement of α -chemokines in neurological deficits following HI.

The β -chemokines (which are primarily chemoattractants for mononuclear cells) consist of MIP-1 α , MIP-1 β , monocyte chemoattractant protein (MCP)-1, MCP-2, and regulated on activation normal T cell expressed and secreted (RANTES). MIP-1 α and MCP-1 have been found to be elevated in the ligated hemisphere following HI for extended periods of

time that range from 6 h–14 days for MIP1 α ^{14, 32} and 1–48 h for MCP-1⁸². This suggests that MIP-1 α and MCP-1 may play a pivotal role in HI-mediated inflammatory responses. The importance of MCP-1 was revealed when ICE knockout mice (which have previously been shown to be less susceptible to HI-induced damage) attenuated the MCP-1 increase by HI, implying that MCP-1 induction is essential to produce HI-induced neuronal injury¹⁷⁷. CP children had higher β -chemokine concentrations, such as RANTES, MIP-1 α , MIP-1 β , MCP-1, and MCP-2, compared with control children¹¹⁵, indicating the necessity of β -chemokines in HI-induced neuronal damage. Conflicting evidence on the role of RANTES following HI exists, with one paper stipulating no change in mRNA expression between treatment groups at 24 h¹²⁴. In contrast, evidence from another paper showed elevated mRNA levels of RANTES at 24 h–14 days post-HI¹⁴. These results, to date, suggest that the exact role of RANTES following HI has yet to be fully elucidated.

IL-8 (the archetype chemokine) is another member of the α -chemokine family and plays a pivotal role in neutrophil accumulation and activation. In asphyxiated newborns (including those that develop CP), IL-8 concentrations in the CSF and serum were increased compared with control newborns and there was a positive correlation between the severity of HIE and the concentration of IL-8^{46, 137}. Therefore, IL-8 is crucial to the development of acute inflammation. For example, increased levels have been demonstrated following an ischemic stroke or HI. However, it seems to play little part in chronic inflammatory conditions, such as MS. In contrast, other chemokines do play an important role in chronic inflammation, as evidenced by MIP-1 α association with the relapsing-remitting MS animal model of experimental allergic encephalomyelitis⁸⁷. Therefore, the neutrophil chemoattractant function of IL-8 may in fact be important to the initial development of HI-induced neuronal damage.

ANGIOGENESIS AND HI

The process of angiogenesis begins with the proliferation and then migration of endothelial cells to the site of injury. This initiates the sprouting of new capillaries from pre-existing blood vessels (see¹⁸). Recent evidence has shown that increased angiogenesis within the infarcted tissue following cerebral ischemia is secondary to the hypoxic activation of vascular endothelial growth factor (VEGF)¹⁰². In addition, VEGF, which is the most potent angiogenic factor discovered, is now strongly implicated in neuroprotection¹⁵². It is now well established that the

process of angiogenesis is regulated, in part, by the partial pressure of tissue oxygen, in particular the pathophysiological conditions involving hypoxic stimuli. This has held true for a number of pathophysiological conditions, such as: retinopathy following premature birth², chronic exposure to normobaric hypoxia¹²³, and tumor growth^{79, 127, 144}. In addition, vascularization has been documented using positron electron tomography within the peri-infarct zone following cerebral infarction¹⁰¹. Likewise, in brain autopsies from stroke victims that had survived for extended periods of time there was an increase in cerebral blood flow and microvessel density compared with stroke victims who had died shortly after the ischemic event⁹⁰. From this, a significant correlation between blood vessel density and survival outcome was noted, suggesting that the promotion of angiogenesis in patients at risk of or suffering from a stroke may be of benefit.

VEGF has been found to be reliant on the subsequent release of nitric oxide (NO) in order to induce angiogenesis. This was demonstrated using the non-selective NO synthase (NOS) inhibitor N^o-nitro-L-arginine methyl ester (L-NAME), whereby L-NAME blocked VEGF-induced angiogenesis¹⁸⁴. Indeed, numerous studies have now shown that VEGF subsequently induces NO production^{111, 164}. In addition, *in vivo* evidence shows that supplementation with L-arginine (the precursor for NO) increases endothelial NOS activity, with a subsequent increase in angiogenesis¹¹³. Furthermore, research from our own laboratory using CD31 as a marker of angiogenesis has revealed an increase in angiogenesis in a rat model of HI. However, treatment with L-NAME resulted in a marked decrease in the angiogenic process and an associated increase in lesion size (Clarkson et al., unpublished observations). It was highlighted in a recent review article that the potential use and development of selective endothelial NOS inducers may be of significant benefit for the treatment of ischemia-induced neurodegeneration⁴¹.

OXIDATIVE STRESS

In its simplest form, oxidative stress is the balance between ROS production and the body's intrinsic scavenging capacity. The body's endogenous antioxidant enzymes include superoxide dismutase (SOD), found in cytoplasm as Cu,Zn-SOD (SOD1)¹⁷⁴ or in mitochondria as Mn-SOD (SOD2)¹⁷³. Both enzymes scavenge ROS by converting them to H₂O₂, which is later detoxified by either catalase or glutathione peroxidase to H₂O. Assessment of CNS injury in animal models of ischemic stroke has shown that overexpression of SOD1 can protect the CNS from

injury¹⁹. However, following neonatal HI the over-expression of the SOD1 gene resulted in an exacerbation of the injury³⁷. This inability to detoxify H₂O₂ in these over-expressing SOD1 animals is thought to be due to the limited capacity of the antioxidant enzyme glutathione peroxidase⁵².

It has been proposed in a recent review by Ikeda et al.⁸⁰ that the use of ROS scavengers may be a viable intervention to limit CNS injury following ischemia. It is generally accepted that antioxidants protect cells during inflammatory events by scavenging cytotoxic ROS. However, emerging evidence suggests that antioxidants may also protect cells by altering cell signaling pathways and modulating inducible enzyme systems¹⁰⁰.

It is widely accepted that ROS are key mediators of cerebral injury in a number of neurodegenerative conditions, including cerebral ischemia^{8, 20, 146}. The CNS is particularly susceptible to ROS-induced damage due to the high oxygen demands of the brain and low concentrations of endogenous antioxidants and free-radical scavengers^{9, 16, 52, 69, 143}. Over recent decades a strong relationship between cerebral ischemia and oxidative stress in humans has been documented^{22, 148}. Furthermore, antioxidants have shown potential as putative neuroprotectants for ischemia and other neurodegenerative disorders²⁴. Recent work from our laboratory has demonstrated that the two naturally occurring antioxidants, spermine and epigallocatechin gallate (EGCG), reduce CNS damage following HI by modulating the key inflammatory enzyme NOS^{27, 154}. Even though antioxidants are well known for their free-radical scavenging ability, other inflammatory mediators do modulate neurological damage. Indeed, the potent antioxidants spermine and EGCG have been shown to protect against ischemic injury due to their ability to act on a range of inflammatory pathways in addition to their ability to scavenge free radicals.

MITOCHONDRIAL CHANGES AND HI

Mitochondria are ubiquitous in their presence and are the cellular organelles responsible primarily for the production of ATP via aerobic metabolism. However, under conditions of pathophysiological stress, mitochondria become key mediators of neuronal cell damage⁴⁴ through the overproduction of ROS, abnormal Ca²⁺ homeostasis, and release of apoptotic proteins. When the oxygen supply within the cell is reduced to critical levels during neonatal HI or ischemic stroke, damage to the CNS can occur. Indeed, mitochondrial impairment has been previously documented following an ischemic episode^{27, 34, 134, 138, 147, 154}.

Previous work has shown that following HI-induced tissue damage, energy reserves follow a biphasic decrease, that is, subsequent to the initial insult, an immediate decrease in energy reserves occurs, followed by a transient restoration of glucose utilization and ATP and phosphocreatine production upon reoxygenation^{13, 58}. This is then followed by a secondary decrease in energy reserves that occurs in most brain regions 6–48 h post-insult and is accompanied by impaired glucose utilization, activation of caspase-3, and DNA fragmentation^{13, 57, 58, 130, 176}.

The opening of the mitochondrial permeability transition pore (mPTP) has been suggested to have overwhelming effects on cellular integrity. The resultant collapse in membrane potential and subsequent halting of ATP synthesis in turn may lead to cellular injury. Indeed, the initiated opening of mPTP has been implicated, in part, in apoptosis^{62, 122, 126}. Furthermore, evidence exists for the involvement of the mPTP following ischemic injury in adults with subsequent protection following treatment with mPTP blockers (see⁴⁷ for review). Following neonatal HI it has been shown that mPTP opening occurs. However, no evidence exists for its involvement in HI-mediated injury¹²⁸.

To date, relatively few studies have implicated Ca²⁺-dependent restriction endonucleases in programmed cell death following cerebral ischemia. In a model of focal ischemia, evidence shows the early activation of endonucleases is linked to DNA fragmentation^{23, 157}. In addition, caspase-activated DNase and lysosomal enzyme DNase II have been found in the hippocampal CA1 region following ischemia¹⁶¹. Likewise, apurinic/apyrimidinic endonuclease (APE/Ref-1) has also been detected⁴⁹ and treatment with the free-radical scavenger, 21-aminosteroid, significantly decreased APE/Ref-1 expression and ultimately led to a decrease in infarct size²¹. Evidence for the involvement of endonucleases following HI, however, is less conclusive. An early report by Ferrer et al.⁴³ in 1994 concluded that endonucleases may play a part in HI-mediated cell death, as they observed nuclear DNA fragmentation in juvenile rats subjected to HI. More recently, Lok and Martin⁹⁸ reported that the pro-apoptotic protein Bax precedes the activation of downstream apoptotic-effector mechanisms, caspase-3 cleavage, and endonuclease activation during excitotoxic neuronal apoptosis in the newborn rat brain.

Significant progress has been made which implicates mitochondria as being key regulators of apoptotic cell damage following HI (see^{17, 30} for reviews). A number of these key apoptotic mediators have been shown to be elevated following HI-induced

damage and include caspase-3^{77, 96, 114, 172}, APAF-1¹¹⁹, Bcl-2¹⁰⁸, and apoptosis-inducing factor (AIF)¹⁸³. The release of AIF is thought to be controlled by the anti-apoptotic protein Bcl-2, which impedes AIF release from the mitochondria¹⁵³. In addition, it has been shown that the overproduction of Bcl-2 or Bcl-xL prevents both the mitochondrial and nuclear apoptotic events³⁶.

One possible strategy to protect against HI-induced neuronal damage is to prevent the loss of mitochondrial function, or subsequently hinder the release of apoptotic mediators. Work from our laboratory investigating the protective effects of antioxidants (spermine and EGCG) has shown marked protection against HI-induced impairment of mitochondrial complex kinetics and preservation of mitochondrial integrity as demonstrated by normalization of citrate synthase and aconitase activities^{27, 154}. Numerous studies have shown a decrease in caspase-3 activity and subsequent neuronal protection following a number of different treatments that include i.v.c. injection of brain-derived neurotrophic factor (BDNF)⁷¹; i.v. injection of 2-iminobiotin, a selective neuronal and inducible NOS inhibitor¹²⁴; treatment with MK-801, a N-methyl-D-aspartate receptor antagonist¹²⁹; and post-ischemic hypothermia⁵¹. However, some studies have shown that even in the presence of decreased caspase-3 activity, preservation of CNS tissue is not apparent following treatment with boc-aspartyl-(Ome)-fluoromethyl-ketone (BAF), a multicaspase inhibitor^{85, 183}. To this end, BAF failed to inhibit the release of AIF from mitochondria, which suggested a strong involvement for AIF in HI-mediated neuronal damage¹⁸³.

CONTRALATERAL CHANGES AND HI

Traditionally, studies investigating HI-mediated neuronal damage have utilized the contralateral hemisphere as an “internal control”. This is based on observations that gross damage is most apparent ipsilateral to the insult (see, for example^{54, 159, 165}). However, remote changes contralateral to the initial insult have been documented in which loss of excitation or facilitation within an injured area renders other regions less responsive to stimuli⁴. For example, unilateral ischemia has been shown to decrease contralateral regional cerebral blood flow (rCBF) and metabolism^{38, 91, 175}. The fact that changes in rCBF can lead to changes in metabolism and lasting functional deficits⁷⁵ suggests that the contralateral hemisphere may not be a valid control in procedures of HI. Furthermore, we have shown lasting functional deficits following HI up to 90 days post-insult using extra-cellular field potential recordings from the hippocampus²⁸.

In addition to contralateral electrophysiological changes, small changes in gene expression, inflammatory markers, and neuronal density have been documented following HI in the contralateral hemisphere. The expression of immediately early genes (IEGs; c-fos and c-jun) is associated with the activation of downstream cytokine pathways, cellular injury, and cell death, primarily via apoptosis⁸⁶. A number of studies have demonstrated upregulated expression of IEGs following HI in the contralateral hemisphere and hippocampus^{56, 68, 112}. In addition to the changes in IEGs, increases in IL-1 α , IL-1 β , TNF- α , and TNF- β have likewise been documented in the contralateral hemisphere^{70, 163}. Mild neuronal loss is also well documented in the cortex and CA regions of the hippocampus contralateral to an ischemic insult^{88, 160}, and it is now evident that inflammatory processes are central to CNS injury following HI.

One mechanism that could potentially explain these contralateral changes is a phenomenon known as “slow excitotoxicity”, in which excitotoxicity may occur in the absence of any elevated levels of glutamate or other excitatory neurotransmitters^{118, 182}. Under conditions of “slow excitotoxicity” it is proposed that damage to mitochondria occurs following interference with the electron transport chain by toxins or through physical disruption and is the key element in this pathogenic process. In this, neurons become overly responsive to ambient levels of extracellular glutamate following cellular energetic impairment, which is a component attributed to mitochondria^{118, 182}. The subsequent decrease in ATP production results in a diminished exchange of sodium and potassium, in turn causing the cell membrane potential to drift into a state of depolarization. This increased state of depolarization increases the likelihood of opening voltage-sensitive sodium and calcium channels augmenting action potential firing¹³¹. This ultimately contributes to the initiation of the excitatory cascade under conditions of sub-lethal exposure to extracellular glutamate.

CURRENT AND FUTURE THERAPIES

Presently there are no neuroprotective treatments for HIE and the prevention of CP, even though many neuroprotectants have worked in animal models of HI. However, there are some promising therapies that have recently been shown to be neuroprotective against HI-induced neuronal damage in the rat and await further testing in clinical trials. The regulation of temperature has been well documented to affect the outcome of ischemia (see⁹² for review), with hyperthermia exacerbating HI-induced neuronal injury⁵⁰, while hypothermia protects against HI-induced neuronal injury⁵¹. This is presumably due to

either an increase or decrease in blood vessel dilation, respectively. Presently, hypothermic treatments such as systemic cooling or head cooling are in clinical trials for the treatment of HIE^{60, 141}. Preliminary results show that moderate HIE newborns have increased neurodevelopmental outcome with hypothermic treatment compared with normothermic controls⁶⁰. Therefore, hypothermic strategies may be a promising avenue in the prevention of brain damage after HI.

The cascade of events that occurs following HI is complex and it is extremely unlikely that any single intervention will prevent the entire cascade from being activated. However, therapies with multiple modes of action will limit the neuronal injury after HI more effectively compared with a compound with a single mode of action. Recently, in our laboratory, free-radical scavengers such as spermine²⁷ and EGCG¹⁵⁴ were not only neuroprotective against HI-induced neuronal damage (through their antioxidant action), but also modulated key inflammatory mediators such as NOS to produce neuroprotection. Other NOS inhibitors, such as 2-iminobiotin¹²⁴ and ARL-17477¹⁴², also provide neuroprotection in the animal model of HI. Therefore, anti-oxidants that have many mechanisms of action could prevent the activation of many pathways in the complex cascade of neuronal injury, but these compounds have not reached clinical trials yet. However, EGCG is in clinical trials for the treatment of various types of cancer, and its pharmacokinetic and safety profile has already been documented²⁶. Similar to stroke, HI has a narrow therapeutic window⁶⁵. With most studies aiming for a 6 h time window post-HI/stroke, it therefore seems likely that anti-inflammatory treatment offers perhaps the most promising line of therapy.

To this end, inhibitors of inflammation such as fucoidin (inhibitor of leukocyte adhesion) have

shown protection against HI-induced brain damage¹⁶². Furthermore, growth factors such as BDNF have shown protection against HI-induced damage via multiple mechanisms: activation of the extracellular signal-related protein kinase pathway⁷² and by blocking caspase-3 activation⁷¹. However, neither of these compounds, which act at multiple sites, has made it to clinical trials, but they suggest that inhibition of inflammation and apoptosis is a viable target to limit the neuronal damage after HI.

CONCLUSIONS

After an HI insult, a multi-faceted cascade of events occurs that ultimately causes cell death and neurological damage to the CNS. The various cascades include, amongst others, immunological changes, such as the activation of the complement system, increased inflammation through the action of pro-inflammatory cytokines and chemokines, the production of ROS leading to oxidative stress by lipid peroxidation and DNA fragmentation, and diminished mitochondrial function leading to the activation of apoptotic pathways and subsequent alteration to the function of neurons within the contralateral hemisphere. Due to the complex milieu induced by HI and the massive redundancy and constant changing of the inflammatory/immune response, it is extremely unlikely that any one therapy alone will be successful. Currently there are no effective treatments in the clinic to prevent neuronal injury following HI, even though some therapies produced promising results in animal models. Until the role of inflammation and other pathways of neurodegeneration have been fully established, the alleviation of symptoms of HI-induced neurodegeneration is the best we can hope to accomplish. We are still a long way from preventing HI-induced neurodegeneration, let alone preventing its onset.

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