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Review

Hypoxia–ischemia in the immature brain

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Summary

The immature brain has long been considered to be resistant to the damaging effects of hypoxia and hypoxia–ischemia (H/I). However, it is now appreciated that there are specific periods of increased vulnerability, which relate to the developmental stage at the time of the insult. Although much of our knowledge of the pathophysiology of cerebral H/I is based on extensive experimental studies in adult animal models, it is important to appreciate the major differences in the immature brain that impact on its response to, and recovery from, H/I. Normal maturation of the mammalian brain is characterized by periods of limitations in glucose transport capacity and increased use of alternative cerebral metabolic fuels such as lactate and ketone bodies, all of which are important during H/I and influence the development of energy failure. Cell death following H/I is mediated by glutamate excitotoxicity and oxidative stress, as well as other events that lead to delayed apoptotic death. The immature brain differs from the adult in its sensitivity to all of these processes. Finally, the ultimate outcome of H/I in the immature brain is determined by the impact on the ensuing cerebral maturation. A hypoxic–ischemic insult of insufficient severity to result in rapid cell death and infarction can lead to prolonged evolution of tissue damage.

Key words: brain injury, development, excitotoxicity, apoptosis.

Introduction

Perinatal hypoxic–ischemic brain damage is a major cause of acute mortality and chronic neurologic morbidity in infants and children. Statistics suggest an incidence of systemic asphyxia in 2–4 per 1000 full-term births and an incidence approaching 60% in low birth weight, premature newborns (Vannucci, 2000; Volpe, 1992). Between 20–50% of asphyxiated newborns with hypoxic–ischemic encephalopathy die within the newborn period, and up to 25% of the survivors will exhibit permanent neuropsychological handicaps, including mental retardation, cerebral palsy, epilepsy or learning disability. Care of the fetus and newborn infant at risk for cerebral hypoxia–ischemia is clearly a high priority in current health care and an understanding of the pathophysiology of perinatal hypoxic–ischemic brain damage is essential to the design of effective interventions. Much of our current understanding of the mechanisms of hypoxic–ischemic brain damage, as well as potential therapeutic interventions, derives from an extensive literature on experimental stroke models in the adult. Thus, it is now well appreciated that a cerebral hypoxic–ischemic event of sufficient severity to deplete tissue energy reserves, is rapidly followed by acidosis, glutamate excitotoxicity, generation of reactive oxygen species and oxidative stress, followed by prolonged periods of delayed cell death or apoptosis, and inflammation (reviewed in Dirnagl et al., 1999). However, direct application of the findings obtained in the adult brain to the newborn animal has been hampered by a paradox, in that the immature brain has generally been considered ‘resistant’ to the damaging effects of hypoxia and hypoxia–ischemia, while at the same time exhibiting periods of heightened sensitivity to injury, dependent on the specific developmental stage of the brain. Furthermore, a hypoxic or hypoxic–ischemic insult to the developing brain will impact on subsequent maturation, with long-lasting consequences for the adult brain. This short review will attempt to resolve the paradox by highlighting both the areas of apparent resistance and heightened vulnerability in the immature brain.

The majority of these experimental studies have utilized a model of unilateral hypoxic–ischemic brain damage in the immature rat (Rice et al., 1981; Vannucci and Vannucci, 1997; Vannucci et al., 1996), more recently extended to the immature mouse (Sheldon et al., 1998). This methodology consists of unilateral common carotid artery ligation followed by a period of systemic hypoxia produced by inhalation of 8% oxygen/balance nitrogen. 7-day postnatal rat pups can survive up to 2.5–3 h before significant mortality occurs. During the course

of hypoxic exposure, the pups demonstrate hypoxemia combined with hypocapnia, produced by hyperventilation; the hypocapnia compensates for the metabolic acidosis produced by lactic acidemia, and systemic pH is not different from control pups (Vannucci et al., 1995). Mean systemic blood pressure decreases by 25–30% during hypoxia, and cerebral blood flow is reduced by 40–60% of the control rate in the hemisphere ipsilateral to the ligation (Vannucci et al., 1988). Cerebral blood flow is restored to control values immediately upon return to normoxic conditions, although the period of hyperemia characteristic of reperfusion following cerebral ischemia in adult models is not observed in the immature rat model (Mujsce et al., 1990). Hypoxic–ischemic brain damage, ranging from selective neuronal death to infarction, or a combination of both, is a near universal finding in the ligated rat pups surviving a 2–3 h exposure to hypoxia. The damage is usually restricted to the hemisphere ipsilateral to the ligation and is primarily observed in the cerebral cortex, subcortical and periventricular white matter, striatum/thalamus and hippocampus. Such neuropathological damage is rarely seen in the contralateral hemisphere and never in pups rendered hypoxic without carotid artery ligation (Towfighi et al., 1995; Vannucci and Vannucci, 1997). Thus using this model, we and others have studied the effects of perinatal hypoxia–ischemia on cerebral energy metabolism, glutamate excitotoxicity, generation of reactive oxygen species and apoptotic cell death.

Energy metabolism and nutrient transport in the immature brain

The benefits and limitations during hypoxia–ischemia

Glucose is an obligate energy fuel for the adult, as well as the newborn, mammalian brain but cerebral glucose metabolism in the immature brain differs from the adult in ways that confer both resistance and vulnerability to energy failure during hypoxia–ischemia. Rates of cerebral energy metabolism are low in the immature brain and relate primarily to the level of neuronal maturation and synaptic activity at the developmental stage under study (Cremer, 1982; Nehlig and Pereira de Vasconcelos, 1993). In addition, newborn rodents and humans can readily utilize substrates other than glucose, i.e. lactate and the ketone bodies β-hydroxybutyrate and acetoacetate, to satisfy their cerebral energy requirements (Edmond et al., 1985; Nehlig and Pereira de Vasconcelos, 1993). During the first 2 postnatal weeks, suckling rodents are especially ketogenic due to the high fat content of rodent milk; ketone bodies can provide up to 60% of the cerebral energetic fuel during this time (Nehlig and Pereira de Vasconcelos, 1993). The transport of these substrates from the circulation into the brain is mediated by two families of integral membrane proteins, the facilitative glucose transporter proteins, GLUTs, and the proton-coupled monocarboxylic acid transporter proteins, MCTs (for reviews, see Dwyer et al., 2002; Price et al., 1998; Vannucci et al., 1997). Although each comprises a multi-member family of homologous proteins, GLUT1 and GLUT3, and MCT1 and MCT2, are the primary isoforms of the respective families detected in mammalian brain. Glucose transport across the blood–brain barrier (BBB) is mediated by a highly glycosylated (55 kDa) form of GLUT1; the less glycosylated 45 kDa GLUT1 is expressed in choroid plexus, ependyma, as well as all glial elements of brain, and GLUT3 is the neuronal glucose transporter (Vannucci et al., 1997). In the rat, cerebral glucose utilization is only 10% of the adult value during the first postnatal weeks, and the levels of the GLUTs are comparably low, with neuronal GLUT3 especially demonstrating an increase in expression coincident with periods of neuronal maturation and synaptogenesis (Nehlig et al., 1988; Vannucci et al., 1994). However, the ketone bodies provide a significant proportion of cerebral metabolic fuel during the first 2 weeks, and the levels of expression, especially of MCT1 in the BBB, reflect the preferential utilization. In the P1–P14 rat brain, MCT1 mRNA and protein is very high in the BBB, and is also expressed in virtually all cell types, neurons and glia, whereas MCT2 expression is primarily restricted to neuronal elements (Pierre et al., 2000; Vannucci and Simpson, 2003). Thus the immature brain is characterized by a low capacity for glucose transport and a high capacity for both ketone body and lactate transport. During hypoxia- and hypoxia–ischemia, the brain increases its reliance on anaerobic glycolysis and cerebral glucose utilization increases substantially (Vannucci et al., 1994). However, the low concentration of glucose transporters in the P7 rat brain renders cerebral glucose utilization transport-limited at this stage of development. Brain glucose levels rapidly fall to barely detectable levels, despite near normal plasma levels, and the initial phase of cerebral energy failure is routinely observed by 90 min of hypoxia–ischemia (Yager et al., 1992a). The ability to survive a longer period of H/I than the adult relates to the reduced levels of energy demand, but the inability to transport sufficient glucose across the BBB imposes a significant limitation on cerebral glucose utilization (CGU) during this insult. It has been shown that maintaining hyperglycemia during H/I in the immature rat provides significant protection (Voorhies et al., 1986), which is the opposite of the situation in the adult brain, where preischemic hyperglycemia exacerbates tissue damage. Immediately upon return to normoxia, however, brain glucose levels normally increase twofold (Vannucci, 1992), and further glucose supplementation during recovery has actually been shown to be detrimental (Sheldon et al., 1992). The other unique feature of the immature brain is the enhanced ability to transport both lactate and ketone bodies into and out of brain. Thus, elevated cerebral lactate during H/I is rapidly either cleared or utilized during the immediate recovery period, and even slightly elevated levels of circulating ketone bodies, as induced by an overnight fast or by subcutaneous injection, can provide sufficient additional fuel to provide protection during hypoxia–ischemia (Yager et al., 1992b).

Glutamate excitotoxicity and the developmental regulation of NMDA receptors

Selective vulnerability of the immature brain

Depletion of cellular energy stores that accompanies prolonged hypoxia, or hypoxia–ischemia, results in

depolarization of neurons and glia and the release of excitatory amino acids (EAA) into the extracellular space. Energy-dependent reuptake mechanisms become compromised, allowing glutamate to accumulate to excitotoxic levels, and the overactivation of *N*-methyl-D-aspartate (NMDA) receptors increases intracellular calcium levels and initiates cellular processes culminating in cell death (reviewed by Dirnagl et al., 1999).

NMDA receptor activation can be especially devastating in the immature brain. Glutamate is an important trophic factor for the immature brain and NMDA receptors mediate normal brain development and function by promoting proliferation and migration of neuronal precursors, and synaptic development and plasticity (Komuro and Rakic, 1993; McDonald and Johnston, 1990). Appropriate to these developmental functions, the composition and activity of the immature NMDA receptors differ from that observed in the adult brain, resulting in a period of enhanced sensitivity to excitotoxic insults (see Johnston, 1995, and references therein). NMDA receptors are hetero-oligomers consisting of NR1 subunits (of which there are eight possible splice variants), NR2, which has four subtypes (2A–2D), and NR3 subunits (Ciabarra et al., 1995; Moriyoshi et al., 1991; Nishi et al., 2001; Sucher et al., 1995). Whereas the NR1 subunit is essential for the formation of functional ligand-gated ion channels, the specific pharmacological and biophysical properties are determined by the component NR2 (or NR3) subunits (Cull-Candy et al., 2001; Dingledine et al., 1988; Sucher et al., 1996). During development, the expression of the NR2 subunits changes from a relatively high level of subtype 2B during the first 2 postnatal weeks in the rat, to a predominance of the 2A subunit in the adult (Gurd et al., 2002; Sheng et al., 1994; Zhong et al., 1995). This developmentally regulated alteration in the ratio of NR2A:NR2B is reflected in altered receptor properties, including increased Ca^{2+} flux on glutamate activation, which may contribute to the increased sensitivity of the neonatal brain to hypoxic–ischemic injury (Johnston, 1995).

Functional characteristics of NMDA receptors are further regulated by phosphorylation of the component subunits (Raymond et al., 1994; Swope et al., 1999). Tyrosine phosphorylation of NR2 subunits activates the receptor ion channel (Kohr et al., 1994; Wang and Salter, 1994) and can also impact on interactions with associated postsynaptic density (PSD) proteins and downstream signaling molecules (Gurd, 1997; Takagi et al., 1999). Increases in synaptic tyrosine kinase activity occur during normal cerebral maturation (Cudmore and Gurd, 1991; Gurd and Bissoon, 1990) and have been associated with developmentally related increases in tyrosine phosphorylation of the NMDA receptor (Gurd et al., 2002). Although levels of NR2A relative to P21 are low in the P7 rat, there is a higher level of basal phosphorylation, which would contribute to the increased excitability of the NMDA receptor supporting normal cerebral development at this stage but also render it more vulnerable to H/I damage (Gurd et al., 2002). Recruitment of tyrosine kinases to the post-synaptic density is an early response of the adult brain in ischemia (Cheung et al., 2001), and H/I induced changes in NR2A and NR2B are specific to the developmental stage of the brain (Gurd et al., 2002). At P7, H/I induced a selective and rapid loss of NR2A, but not NR2B, levels, although phosphorylation of the latter was increased early in reperfusion. In contrast, tyrosine phosphorylation of both NR2A and 2B subunits was increased following H/I in the P21 rat, with no decline in levels of NR2A and a delayed decrease in NR2B at 24 h of reperfusion. The relationship between subunit composition, phosphorylation changes, and NMDAR channel properties and downstream signaling is complex. However, the demonstration of such specific age-related differences in the response to H/I suggests a basis for the changing sensitivity of the developing brain to excitotoxicity at the time of the insult, and could have longer lasting effects of synaptic events involved in recovery at different ages.

Oxidative stress and hypoxic–ischemic injury in the immature brain

The connection between activation of NMDA receptors and excitotoxic cell death involves direct activation of neuronal nitric oxide synthase (nNOS) and the generation of nitric oxide, resulting in mitochondrial dysfunction and increased formation of reactive oxygen species (ROS), oxidative damage and cell death (Dugan and Choi, 1994). The age-dependent regional vulnerability to hypoxic–ischemic insults seen in the immature brain can be explained, at least in part, by the density of NMDA receptors and nNOS-positive cells (Ferriero, 2001). Destruction of nNOS-positive neurons in the neonatal rat before a hypoxic–ischemic insult diminishes the severity of the insult (Ferriero et al., 1995), and genetic deletion of nNOS is protective against H/I in neonatal mice (Ferriero et al., 1996).

Two features of the immature brain that render it especially sensitive to oxidative damage relative to the mature brain are poor antioxidant capabilities and a high concentration of free iron. Endogenous antioxidant enzymes in the brain include superoxide dismutase (SOD), which exists as Cu,Zn-SOD (SOD1) in the cytoplasm and Mn-SOD (SOD2) in the mitochondria. Both of these enzymes actively scavenge oxygen free radicals by converting them to H_2O_2, which can then be effectively detoxified by the action of catalase or glutathione peroxidase and eliminated as H_2O. Glutathione peroxidase simultaneously catalyzes the conversion of reduced glutathione to oxidized glutathione, which can then be reduced by glutathione reductase, at the expense of NADPH. Thus this regeneration system for the maintenance of antioxidant protection is dependent on the cellular energy state. Clearly, when these systems fail, as in hypoxia–ischemia, the brain suffers the consequences of oxidative damage to cellular macromolecules and death. Experimental studies designed to limit oxidative damage following stroke, including genetic overexpression of SOD1, have been shown to be protective in the adult nervous system (Chan, 1996). However, overexpression of SOD1 in the context of neonatal H/I actually exacerbated tissue damage, highlighting another significant

difference between immature and adult brains (Ditelberg et al., 1996). The reason for this, as well as the increased vulnerability of the immature brain to oxidative stress, was subsequently explained by an inability to detoxify accumulated H_2O_2, due to a limited capacity of antioxidant enzymes, especially glutathione peroxidase (Fullerton et al., 1998). Additionally, the accumulation of H_2O_2 is more damaging to the immature brain due to the higher levels of free iron in the immature, relative to the adult, nervous system, and the consequent generation of the hydroxyl radical *via* the Fenton reaction. Reducing the level of free iron with the chelator deferoxamine (DFO) has neuroprotective effects on both wild-type and SOD-overexpressing neonatal mice after H/I (Sarco et al., 2000).

Apoptotic mechanisms in the immature brain

Cell death can be classified as apoptotic or necrotic on the basis of biochemical and morphological criteria (MacManus and Linnik, 1997). Necrotic death is often the result of a severe insult and is characterized by disruption of membrane integrity, leaking of cytoplasmic contents into the extracellular space and a secondary inflammatory response. Apoptosis, on the other hand, is a highly regulated and energy requiring process whereby the cell commits suicide. Nuclear condensation and contraction is an early event, whereas membranes and organelles remain intact until the final stages. Cellular remnants bud off as apoptotic bodies, which are engulfed by neighbouring cells.

Most data suggest, however, that apoptosis as a result of hypoxia–ischemia is morphologically different from developmental apoptosis, and that many hybrid necrotic–apoptotic phenotypes are seen (Leist and Jaattela, 2001; Martin et al., 1998). Biochemically, by contrast, most studies agree that apoptotic processes are involved in hypoxia–ischemia. Indeed, key elements of apoptosis, such as caspase-3 (Blomgren et al., 2001; Cheng et al., 1998; Hu et al., 2000), APAF-1 (Ota et al., 2002), Bcl-2 (Merry et al., 1994) and Bax (Vekrellis et al., 1997) are upregulated in the immature as compared to the adult brain and could be expected to have a prominent role in pathological situations also.

Apoptosis in most mammalian cells involves a family of cysteine proteases, the caspases, which are proenzymes activated in a highly regulated proteolytic cascade leading to the downstream activation of Caspase-3, -6 or -7. Caspase-3 appears to be the key executioner in the CNS and its activation leads to cleavage of hundreds of substrates in the cell that are vital for cell survival (Cohen, 1997). These substrates include the nuclear chaperone, inhibitor of caspase-activated DNase (ICAD), which is cleaved, and caspase-activated DNase (CAD), which is induced, leading to DNA fragmentation (Enari et al., 1998). Caspase-3 can be activated either through intrinsic or extrinsic (receptor-mediated) pathways (Hengartner, 2000). Intrinsic mechanisms involve the release of cytochrome *c* and formation of the 'apoptosome' and, subsequently, caspase-9 activation. The extrinsic pathway includes the binding of the Fas-ligand to its receptor, which leads to caspase-8 cleavage and activation of caspase-3.

There are several lines of evidence that the caspase system is activated in the immature brain in response to hypoxia–ischemia. The activities of Caspase-3, -8 and -9 all increase (Blomgren et al., 2001; Cheng et al., 1998; Northington et al., 2001; Zhu et al., 2003) and their downstream substrates ICAD and poly(ADP-ribose)polymerase are cleaved (Wang et al., 2001). Furthermore, caspase-3 activity and hypoxic–ischemic brain injury can be significantly reduced either by the administration of a 'broad-spectrum' inhibitor (Cheng et al., 1998) or through transgenic upregulation of the endogenous caspase-inhibitor 'XIAP' (Wang et al., 2003, 2004).

Recent data suggest that another protein, apoptosis inducing factor (AIF), can be released from mitochondria under some conditions. AIF is an oxidoreductase with the ability to induce chromatin condensation and DNA fragmentation in a non-caspase-dependent manner (Susin et al., 1999). Poly(ADP-ribose)polymerase-dependent cell death has been shown to depend on mitochondrial release of AIF (Yu et al., 2002), and this protein was also translocated from mitochondria to the nucleus in neurons of the immature brain in the early phase of reperfusion after hypoxia–ischemia (Zhu et al., 2003). Future studies must clarify the relative importance of caspase-dependent and caspase-independent pathways in various pathological situations.

Conclusion

An asphyxial insult to the perinatal brain will result in a variable extent of permanent damage, depending on the location of the injury and the maturational state of the brain. An appreciation of the normal functional activities during cerebral maturation is vital when evaluating the impact of hypoxia–ischemia and hopefully in the design of age-appropriate interventions. Recent studies in experimental animal models discussed in this review have increased our understanding of injury to the immature brain close to term gestation. The future directions of this field will continue to unravel the distinct mechanisms of injury-induced cell death in the immature brain, especially at earlier times of development, as well as a thorough evaluation of the developmental and behavioural sequelae following injury.

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