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Apoptotic Mechanisms in the Immature Brain: Involvement of Mitochondria

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Abstract

Brain injury after hypoxic-ischemic encephalopathy often develops with delayed appearance, opening a therapeutic window. Clinical studies in newborns show that post-hypoxic-ischemic hypothermia improves outcome. This has generated renewed interest in the molecular mechanisms of hypoxic-ischemic brain injury. In this brief review, we propose that mitochondrial permeabilization is crucial for injury to advance beyond the point of no return. We suggest that excitatory amino acids, nitric oxide, inflammation, trophic factor withdrawal, and an increased pro- versus anti-apoptotic Bcl-2 protein ratio will trigger Bax-dependent mitochondrial outer membrane permeabilization (MOMP). MOMP, in turn, elicits mitochondrial release of cytochrome C, apoptosis-inducing factor, SMAC/Diablo, and HtrA2/Omi. Cytochrome C efflux activates caspase-9/-3, leading to DNA fragmentation. Apoptosis-inducing factor interacts with cyclophilin A and induces chromatinolysis. Blockage of MOMP holds promise as a strategy for perinatal brain protection.

Pathophysiology of Neonatal Brain Injury

Perinatal encephalopathy develops after peripartum hypoxia-ischemia in 1 to 3 out of every 1000 births and remains an important problem. It is associated with a high risk of death or major neurological and neurodevelopmental abnormalities resulting from injury in the basal ganglia/thalamus and cerebral cortex.[1] The etiology behind these lesions is complex. Besides the intensity and duration of the primary/secondary insult, genetic background, gender,[2,3] stage of brain development,[4] and the activity of repair/adaptive processes[5] during the recovery phase are important. Furthermore, the presence or absence of various antecedents (eg, infection, growth retardation) are likely to be of great significance.

The development of injury can be considerably delayed after ischemic episodes (eg, global ischemia in adult gerbils and rats results in complete initial recovery of function and structural integrity but is followed by delayed selective loss of pyramidal cells in the cornu ammonis 1 of hippocampus 3 days to 4 days after the primary insult).[6,7] Experimental work in neonatal models has also demonstrated near-complete recovery after the primary insult, followed by secondary disruption of high-energy phosphates[8] and loss of glucose-metabolizing brain tissue[9] 6 hours to 48 hours later. Infants with hypoxic-ischemic encephalopathy show characteristic abnormalities in cerebral energy metabolism, which is

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frequently normal soon after birth but shows a progressive decline in [PCr]/[Pi] and increase in lactate some hours later.[10,11] Infants displaying this phenomenon develop severe neurodevelopmental impairment or die, and there is a relationship between the magnitude of the late decline in [PCr]/[Pi] and the severity of neurodevelopmental impairment.[12,13] These findings suggest that brain injury develops with a certain delay after hypoxia-ischemia, which is also supported by the fact that various treatments given only after the insult in animals[14] can reduce injury and that outcome in newborns with neonatal encephalopathy can be improved to some extent by post-hypoxic-ischemic hypothermia.[15,16] Such proof of concept that neuroprotection is feasible not only experimentally but also in the clinical setting has created renewed interest in the pathophysiology of secondary brain injury after hypoxia-ischemia, with the hope of finding novel and more efficient treatments for the future.

The mechanisms leading to secondary brain injury are still partly unknown, but neurotransmitters, including excitatory amino acids, intracellular calcium, nitric oxide/reactive oxygen species, immuno-inflammatory activation, trophic factor withdrawal, and Bcl-2 family proteins all seem to be involved.[17–19] We believe these upstream perpetrators converge on mitochondria, and at a certain level of stress mitochondrial outer membrane permeabilization (MOMP) occurs, which shifts reversible injury to irreversible cell death (Figure). In this review we summarize current evidence that supports the central role of mitochondria in neonatal brain injury.

Mitochondrial Permeabilization After Neonatal Hypoxia-Ischemia?

Electron microscopy has shown swollen “giant” mitochondria with ruptured outer membranes with large amounts of calcium in the matrix often in neuronal cells early after neonatal hypoxia-ischemia.[20] Using [14C]2-deoxyglucose entrapment in the mitochondrial matrix as an indicator of mitochondrial permeabilization,[21] we found increased mitochondrial permeability 0 hours to 1.5 hours and 6.5 hours to 8 hours after hypoxia-ischemia, indicating, at least qualitatively, that the inner mitochondrial membrane was permeabilized to some degree after the insult. However, there is stronger support for a role of the outer mitochondrial membrane in hypoxia-ischemia.

Proteins are normally localized in the intermembrane space or on the outer surface of the inner mitochondrial membrane but can translocate to the cytosol or the nucleus. For example, cytochrome C, which takes part in electron transfer in the electron transport chain, detaches from cardiolipin in the inner mitochondrial membrane[22] and translocates to the cytosol,[23] which leads to the assembly of the apoptosome[24] and activation of caspase-9[25–27] and caspase-3.[28–30] Another mitochondrial protein, apoptosis-inducing factor, is normally attached to the outer surface of the inner mitochondrial membrane. In response to poly(ADP-ribose)polymerase-1 (PARP-1)-dependent apoptotic signaling, a peptidase is activated and apoptosis-inducing factor is liberated and can, if MOMP occurs, translocate to the cytosol and nucleus.[31] Indeed, PARP-1 is activated after neonatal hypoxia-ischemia[32] and stroke,[33] and apoptosis-inducing factor translocates from the mitochondria to the nucleus in the cerebral cortex, thalamus, and hippocampus.[34] Recently, it was demonstrated that apoptosis-inducing factor interacts with cyclophilin A in the cytosol and the complex translocates to the nucleus and forms a degradasome, and chromatinolysis is initiated.[35] Also, Smac/Diablo and HtrA2/Omi localized in the mitochondrial intermembrane space translocate to the nucleus after hypoxia-ischemia.[26]

In summary, evidence is strong that MOMP occurs during reperfusion in brain regions affected by hypoxia-ischemia, but at present it is uncertain to what degree MOMP is accompanied by inner membrane depolarization and bioenergetic dysfunction after hypoxia-

ischemia. Basic research indicates that induction of MOMP results in respiratory failure due to caspase-dependent degradation of critical components of complex I in the electron transport chain.[36]

Relationship Between MOMP and Brain Injury

The extent of apoptosis-inducing factor translocation to the nucleus correlates with the morphological distribution of neuronal injury after hypoxia-ischemia,[34] and apoptosis-inducing factor deficiency confers considerable protection in mice subjected to neonatal hypoxia-ischemia.[37] Gene deletion of both PARP-1[32] and cyclophilin A [35] decreases apoptosis-inducing factor translocation to the nucleus and reduces brain injury. Caspase inhibitors have also been shown to be protective in hypoxia-ischemia[28] and the broad-spectrum caspase inhibitor Q-VD-OPh reduces injury also in apoptosis-inducing factor-deficient mice,[37] suggesting that MOMP induces activation of caspase-dependent and caspase nondependent processes, and that both contribute to brain injury.

Mechanisms of Mitochondrial Permeabilization

Most researchers agree that at least 2 alternative routes lead to mitochondrial permeabilization, an event that marks the point of no return in multiple pathways leading to cell death.[38–40] The first relies on the opening of the permeability transition pore in the inner mitochondrial membrane, and it is enhanced by cyclophilin D and desensitized by the cyclophilin D inhibitor cyclosporine A; the other requires a direct permeabilization of the outer membrane by Bax/Bak, and is considered to be cyclophilin D-independent and cyclosporine A-resistant.

In the adult brain, ischemia induces opening of the cyclophilin D-dependent mitochondrial permeability transition pore, leading to necrotic cell death.[41,42] The cyclophilin-D inhibitor cyclosporine A reduces ischemic injury[41] and cyclophilin-D gene deficiency confers marked protection in adult brain ischemia.[43] In the immature brain, however, the situation appears to be quite different. Cyclosporine A had no effect on mitochondrial respiration, inner mitochondrial permeabilization (as measured with the deoxyglucose entrapment technique), or brain injury after hypoxia-ischemia.[44] Mitochondria from 16- to 18-day-old rats exhibited greater calcium uptake capacity (in the absence of adenosine triphosphate) than adult rats and calcium-induced cytochrome C release was unaffected by cyclosporine A.[45] In agreement, the calcium retention capacity was higher in mitochondria from postnatal day 9 mice compared with mitochondria from the adult brain, and the calcium retention capacity was less cyclophilin D-dependent in the immature setting.[46] In vivo experiments demonstrated that cyclophilin D gene deficiency increased brain injury, cytochrome C translocation, and caspase activation after hypoxia-ischemia in postnatal day 9 mice, whereas injury was markedly decreased after hypoxia-ischemia at postnatal day 60.[43,46]

In the immature brain, Bax, rather than cyclophilin D, seems to be crucial for mitochondrial permeabilization. Bax (and Bak) is highly expressed in immature mitochondria[47,48] and is particularly sensitive to Bax-BH3-peptide-induced cytochrome C release in vitro.[46,47] Bax translocates from cytosol to mitochondria after neonatal hypoxia-ischemia, [23] and Bax knock-out mice are protected from hypoxic-ischemic brain injury.[49] Recently, Bax-inhibitory peptide was shown to reduce injury (by 75%) and down-stream caspase activation after hypoxia-ischemia in the immature, but not in adult, brain.[46]

In summary, cyclophilin D-dependent mitochondrial permeability transition is critical for mitochondrial permeabilization and brain injury in the adult, whereas Bax-dependent mechanisms prevail in the immature brain. The role of cyclophilin D in hypoxia-ischemia

shifts from an anti-apoptotic protein in the immature brain to a pro-necrotic mediator in the adult brain.[46]

Upstream Factors Involved in Triggering MOMP Bcl-2 Family Proteins

The above data suggest that Bax-dependent MOMP is a critical event in neonatal brain injury, and it is therefore of interest to understand which factors regulate MOMP. Although it has been long recognized that pro- and anti-apoptotic Bcl-2 family proteins regulate Bax-dependent MOMP and are critical regulators of apoptotic cell death,[22,50] their role in neonatal hypoxia-ischemia has only partly been clarified. Most pro-apoptotic multidomain (Bax, Bak, bok), BH3 only (Bad, Bim, Bid, Puma) proteins as well as anti-apoptotic Bcl-2 and Bcl-xL are highly expressed postnatally, followed by downregulation (except for Bcl-xL) with brain maturation.[47,51] Transgenic Bcl-xL overexpressing[52] as well as Bad or Bim knock-out[53] mice are all resistant to postnatal hypoxia-ischemia. Phosphorylation of Bcl-2 at serine-24 in the BH4 region (leading to inactivation of its anti-apoptotic effect) coincides with cytochrome C release after neonatal hypoxia-ischemia and precedes caspase- 3 activation. In addition, the trophic factors IGF-1, BDNF, and hexarellin, which at least partly act through increasing anti- vs. pro-apoptotic Bcl-2 family protein balance, all decrease downstream caspase activation and injury in the postnatal brain.[54–57] In summary, these studies suggest that several Bcl-2 family proteins have an important role in neonatal brain injury, probably through regulation of Bax-dependent MOMP.

Excitatory Amino Acid Receptors, Nitric Oxide, and MOMP

Excitatory amino acids are released during and after neonatal hypoxia-ischemia, activating AMPA and NMDA receptors that in turn trigger production of both reactive oxygen species and nitric oxide.[17] Furthermore, NMDA/nitric oxide activates poly(ADP-ribose) polymerase, which depletes mitochondrial NAD(H) levels and triggers mitochondrial apoptosis-inducing factor release (above). NMDA receptor antagonists improve mitochondrial respiration, attenuate caspase-3 activation, and decrease brain injury after neonatal hypoxia-ischemia.[58–60] Furthermore, the brain protection provided by the nitric oxide synthase inhibitor 2-iminobiotin was also accompanied by near-complete inhibition of caspase-3 and reduction of apoptotic cell death.[61] Taken together, these data suggest that excessive NMDA receptor activation and nitric oxide production impair mitochondrial function and contribute somehow to MOMP and subsequent activation of executional caspases and cell death.

Inflammation, Nuclear Factor Kappa B (NF-kB), and MOMP

Inflammation has previously been shown to be involved in neonatal brain injury.[14] Hence, interleukin-18,[62] caspase-1,[63] or complement C1q gene deficiency[64] or treatment with interleukin-1 receptor antagonist,[65] platelet activating factor antagonist,[66] and inducible nitric oxide synthase inhibitors[67] all reduce neonatal brain injury. Many of the proinflammatory mediators are regulated by the transcription factor nuclear factor kappa B (NF-kB). Recently; it was shown that post-hypoxia-ischemia treatment with a highly selective NF-kB inhibitor peptide (TAT-NBD) reduced brain injury by 80%.[68] Interestingly, the protection was accompanied by marked attenuation of mitochondrial accumulation of p53, mitochondrial cytochrome C release, and activation of caspase-3. The data suggest there may be a link between NF-kB-mediated inflammation and MOMP (Figure) or, alternatively, that NF-kB is directly regulating apoptosis.

In summary, we propose that many upstream events (eg, excitatory amino acids, nitric oxide, inflammation, NF-kB, trophic factor withdrawal, and Bcl-2 family proteins) previously shown to be important in neonatal brain injury all contribute to mitochondrial

stress during the post-hypoxia-ischemia phase. At a certain stress threshold, MOMP will occur, leading to irreversible caspase- and non-caspase-dependent cell death (Figure).

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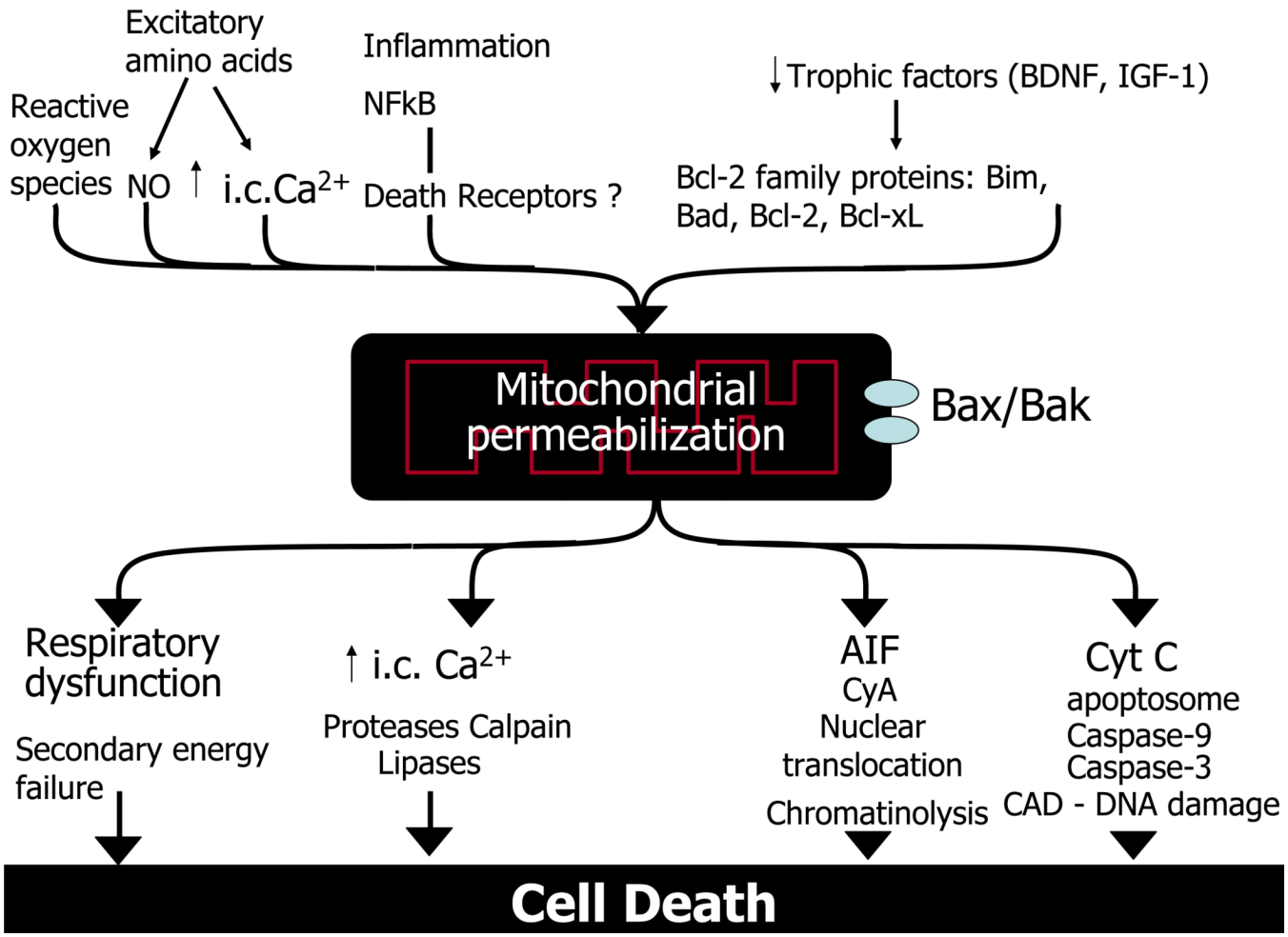

Figure.

Mitochondrial outer membrane permeabilization (MOMP). Many upstream events like excitatory amino acids, intracellular calcium (Ca^{2+}ic), nitric oxide/reactive oxygen species, inflammation, nuclear factor kappa B, trophic factor withdrawal, and Bcl-2 family proteins contribute to mitochondrial stress after neonatal brain injury. These perpetrators converge on mitochondria and together trigger MOMP, leading to irreversible caspase- and non-caspase-dependent cell injury. Abbreviations: AIF, apoptosis-inducing factor; CAD, caspase-activated Dnase; cyA, cyclophilin A; cytC, cytochrome C; BDNF, brain-derived neurotrophic factor; i.c., intracellular; IGF-1, insulin-like growth factor 1; MOMP, mitochondrial outer membrane permeabilization; NF-kB, nuclear factor kappa B.