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Mitochondrial Impairment in the Developing Brain after Hypoxia–Ischemia

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The pattern of cell death in the immature brain differs from that seen in the adult CNS. During normal development, more than half of the neurons are removed through apoptosis, and mediators like caspase-3 are highly upregulated. The contribution of apoptotic mechanisms in cell death appears also to be substantial in the developing brain, with a marked activation of downstream caspases and signs of DNA fragmentation. Mitochondria are important regulators of cell death through their role in energy metabolism and calcium homeostasis, and their ability to release apoptogenic proteins and to produce reactive oxygen species. We find that secondary brain injury is preceded by impairment of mitochondrial respiration, signs of membrane permeability transition, intramitochondrial accumulation of calcium, changes in the Bcl-2 family proteins, release of proapoptotic proteins (cytochrome C, apoptosis inducing factor) and downstream activation of caspase-9 and caspase-3 after hypoxia-ischemia. These data support the involvement of mitochondria-related mechanisms in perinatal brain injury.

KEY WORDS: Hypoxia-ischemia; mitochondria; caspases; *N*-methyl-D-aspartate; nitric oxide; apoptosis-inducing factor; cytochrome C; immature; membrane permeability transition; Bcl-2.

INTRODUCTION

Perinatal brain injury subsequent to birth asphyxia remains an important clinical problem. Even though we still lack effective neuroprotective strategies, considerable progress has been made in understanding the pathogenesis of neuronal damage in the immature brain (Johnston *et al*., 2002; Vannucci *et al*., 1997).

Thus, it is now well accepted that a cerebral hypoxic–ischemic (HI) event of sufficient severity to deplete tissue energy reserves (primary insult) is often followed by transient but complete restoration of glucose utilization, ATP, and phosphocreatine upon reperfusion/reoxygenation (Blumberg *et al*., 1997; Gilland *et al*., 1998a). Thereafter a secondary decrease of high energy phosphates occurs in parallel with a decrease in tissue utilization of glucose, activation of caspase-3, and DNA fragmentation (Blumberg *et al*., 1997; Wyatt *et al*., 1989; Gilland *et al*., 1998a,b; Puka-Sundvall *et al*., 2000a). Secondary energy failure develops in most brain regions 6–48 h after the insult in immature animal models.

Several studies indicate that mitochondria play an important role in adult ischemia (Fiskum *et al*., 1999), but the information about the developing brain is limited. In this brief review, I will describe critical mitochondrial events during the early recovery period and present experimental data in support of the fact that mitochondria may have a critical role in the decision of cellular fate after neonatal HI.

ALTERATIONS OF MITOCHONDRIAL MORPHOLOGY, LOCALIZATION, AND METABOLISM AFTER HI

We recently found that mitochondria (labelled with COX IV) exhibited a "fibrous" pattern of distribution throughout the soma and processes in normal neurons of the cerebral cortex in 7-day-old rats. However, already 2 h after HI, a more punctate or granular appearance of mainly juxta-nuclear COX IV staining was found (Hallin *et al*.,

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unpublished), in agreement with Northington *et al*. (2001). In another study, electron microscopy combined with the oxalate–pyroantimonate technique was used to analyze mitochondrial ultrastructure and intramitochondrial calcium accumulation after HI (Puka-Sundvall *et al*., 2000b). At 3 h and 30 min after HI, a progressive accumulation of calcium was detected in the endoplasmic reticulum, cytoplasm, nucleus, and, most markedly, in the mitochondrial matrix of neurons. Some mitochondria developed a considerable degree of swelling reaching a diameter of several micrometers at 3 h of reflow, whereas the majority of mitochondria appeared moderately affected. Chromatin condensation was observed in the nuclei of many cells with severely swollen mitochondria with calcium deposits. In conclusion, mitochondrial localization seems to change from a widespread to a more perinuclear distribution after HI, accompanied by mitochondrial swelling and accumulation of calcium in the mitochondrial matrix.

During early recovery after HI high energy phosphates in the cerebral cortex are restored as previously mentioned. During this phase, the 2-deoxyglucose (2-DG) utilization was increased, which correlated with increased levels of tissue lactate (Gilland and Hagberg, 1996) and a depression of mitochondrial respiration (Gilland *et al*., 1998a). We have also found that post-HI administration of an *N*-Methyl-D-aspartate (NMDA) receptor antagonist normalized 2-DG utilization, lactate levels, improved mitochondrial respiration and attenuated cortical brain injury (Gilland and Hagberg 1996, 1997; Gilland *et al*., 1998a,b). These data suggest that NMDA-receptor activation in the early recovery phase depresses mitochondrial respiration with a compensatory increase of anaerobic glucose cycling to lactate, which precedes development of cortical brain injury. Interestingly, a similar pattern of increased glucose use occurred in the CNS of asphyxiated infants, particularly in brain regions that were subsequently injured (Blennow *et al*., 1995). Such an increase in glucose utilization occurred in parallel with marked elevations of glutamate in the cerebrospinal fluid (Hagberg *et al*., 1993), implying that HI brain injury also in postasphyxiated infants is preceded by a phase of mitochondrial impairment related to activation of excitatory amino acid receptors.

MEMBRANE PERMEABILITY TRANSITION (MPT)

In isolated mitochondria, MPT is associated with a nonspecific permeabilization of the inner mitochondrial membrane, resulting in a dramatic swelling of the mitochondria, followed by rupture of the outer membrane (Ravagnan *et al*., 2002). Experimental studies have found evidence for MPT after ischemia in the adult brain, and MPT blockers have been shown to be potent neuroprotectants (Friberg and Wieloch, 2002). In immature rats, the above-mentioned ultrastructural changes are compatible with MPT (Puka-Sundvall *et al*., 2001). To investigate this further, [14C]2-deoxyglucose (DOG) was administered to control animals, at various time points after HI, and MPT was measured as entrapment of DOG-6-P in mitochondria (Griffiths and Halestrap, 1995). A significant increase in DOG-6-P in mitochondria indicated that MPT occurred in two phases: a primary MPT after 0–1.5 h and a secondary MPT after 6.5–8 h of reperfusion (Puka-Sundvall *et al*., 2001). We also found a loss of mitochondrial glutathione during early and late recovery (Wallin *et al*., 2000), offering additional support of a biphasic increase of mitochondrial permeability after HI. However, in contrast to the adult, the MPT blocker cyclosporine A did not affect brain injury or mitochondrial respiration in the neonatal brain.

Intrinsic Apoptotic Pathway and Caspase Activation

Studies performed on cultured cells, cell-free systems, or purified mitochondria suggest that mitochondria regulate apoptotic cell death through their capacity to release proapoptotic proteins (Ravagnan *et al*., 2002). Cytochrome C, and other apoptogenic proteins, such as apoptosis-inducing factor (AIF), endonuclease G. SMAC/Diablo, and HtrA2/Omi, can be released from the mitochondrial intermembrane space (Ravagnan *et al*., 2002). Data suggest that Bax, Bad, Bid, and other members of the Bcl-2 family are involved in the regulation of mitochondrial release of proapoptotic proteins. Cytochrome C interacts with APAF-1, ADP, and procaspase-9 to form the heptameric apoptosome, leading to activation of caspase-9, which in turn cleaves and activates pro-caspase-3. AIF, on the other hand, promotes apoptosis in a caspase-independent manner (Susin *et al*., 1999).

Many of the key elements of apoptosis have been demonstrated to be strongly upregulated in the immature brain, such as caspase-3 (Blomgren *et al*., 2001), APAF-1 (Ota *et al*., 2002), Bcl-2 (Merry *et al*., 1994), and Bax (Vekrellis *et al*., 1997). Caspase-3 is markedly activated after HI in the immature brain (Cheng *et al*., 1998; Wang *et al*., 2001; Zhu *et al*., 2000) and cells with the cleaved active form of caspase-3 colocalize with markers of DNA fragmentation in injured brain regions (Zhu *et al*., 2000). Caspase-3 inhibitors (Cheng *et al*., 1998) as well as transgenic overexpression of X-linked inhibitor of apoptosis (XIAP) (Wang *et al*., 2004) attenuate

caspase-3 activation and provide a considerable degree of neuroprotection in the neonatal setting.

It is not known if the extrinsic or intrinsic (mitochondrial) pathway is responsible for the downstream activation of caspase-3. However, assembly of the apoptosome is easily induced in homogenates from the immature (but not adult) brain (Gill *et al*., 2002), cytochrome C is released to the cytosol in response to HI (Northington *et al*., 2001; Zhu *et al*., 2003) and caspase-9 is activated (Northington *et al*., unpublished; Hallin *et al*., unpublished). In addition, other proapoptotic proteins like AIF (Zhu *et al*., 2003), SMAC/Daiblo (Wang *et al*., 2004), and HtrA2/Omi (Wang *et al*., 2004) translocate from the mitochondria to a nuclear localization, suggesting that proapoptotic proteins are indeed released during the early recovery phase after HI. We find that cells with immunohistochemical translocation of cytochrome C and AIF often exhibit signs of DNA fragmentation (detected with a hairpin probe) and nuclear condensation, and these cells are preferentially localized in regions with early loss of the neuronal marker MAP-2 (Zhu *et al*., 2003). Smac and HtrA2 translocation also occurred predominantly in injured areas, and immunostaining often occurred in cells with nuclear condensation or pyknosis (Wang *et al*., 2004). These data show an association between mitochondrial release of proapoptotic proteins and brain injury, but their direct role in the process leading to cell death remains to be clarified.

Bcl-2 FAMILY OF PROTEINS AND NEONATAL HI

There is also evidence for involvement of the Bcl-2 family of proteins. Transgenic mice overexpressing human Bcl-xL postnatally were dramatically resistant to neonatal HI- and axotomy-induced apoptosis (Parsadanian *et al*., 1998). In addition, HI induced an increase in Bax in mitochondrial-enriched cell fractions, which occurred in parallel with an increase of cytochrome C in the cytosol preceding activation of caspase-3 in the neonatal thalamus (Northington *et al*., 2001). Furthermore, HI brain injury seems to be attenuated in Bax gene-deficient mice compared to wild-type controls (Gibson *et al*., 2001). We recently found that Bax translocation to mitochondria after HI, was accompanied by an increased nuclear staining of Bcl-2 (Hallin *et al*., unpublished). Using a site-specific antibody for phosphorylation of Bcl-2 at serine-24 (PS24-Bcl-2), it was found that the number of cells positive for PS24-Bcl-2 increased during 3–24 h of reperfusion in all investigated brain areas after neonatal HI. Phosphorylation of Bcl-2 coincided with cytochrome C translocation and colocalized with, but preceded, caspase-3 activation. In summary, Bcl-2 is phosphorylated (inactivated?) and translocated to the nucleus, concomitant with increased mitochondrial Bax immunoreactivity, cytochrome C release, and activation of caspase-3. Furthermore, ceramide "preconditioning-like" protection in the neonatal setting was accompanied by upregulation of both Bcl-2 and Bcl-xL (Chen *et al*., 2001), offering additional support for involvement of Bcl-2 family proteins and mitochondria in the determination of susceptibility of the immature brain.

PARP-1 AND AIF

PARP-1 is a DNA repair enzyme that has been demonstrated to be critically involved in ischemic brain injury in the adult (Yu *et al*., 2003). Mice with PARP-1 gene disruption are resistant to ischemia (Eliasson *et al*., 1997), and PARP inhibitors provide protection (Ducrocq *et al*., 2000; Yu *et al*., 2003). We recently found that PARP-1 gene deficiency also confers protection in neonatal mice (Hagberg *et al*., 2004). PARP-1-mediated cell death has previously been explained in terms of NAD^+ consumption and mitochondrial energy failure (Eliasson *et al*., 1998). A recent study suggests that PARP-1 mediates the release of AIF from mitochondria, resulting in caspase-independent cell death, a process which could be blocked by microinjection of an antibody against AIF (Yu *et al*., 2002). In support of this hypothesis, we found that cells with increased PAR immunoreactivity after HI (indicative of activation of PARP-1) often exhibit a shift in AIF immunoreactivity from the mitochondria to the nucleus. Irrespective of which molecular mechanisms prove to be most important, it seems likely that mitochondria are important in PARP-1-mediated cell death (Yu *et al*., 2002, 2003).

NMDA-RECEPTOR ACTIVATION, NITRIC OXIDE (NO), AND MITOCHONDRIAL IMPAIRMENT

There are also other potential adverse factors in the mitochondrial environment that could be important (Fig. 1). NMDA receptor activation with increased Ca^{2+} influx, free radical formation, and induction of NO could damage mitochondrial membranes (Crow and Beckman, 1995; Nowicki *et al*., 1982; Schulz *et al*., 1995; Zaidan and Sims, 1994). As previously mentioned, administration of NMDA receptor antagonists prevents the depression mitochondrial respiration *in vivo* (Gilland and Hagberg, 1996). In addition, we have found that both NMDA receptor blockers (Puka-Sundvall, 2000c) and a combined inhibitor of inducible and neuronal NO

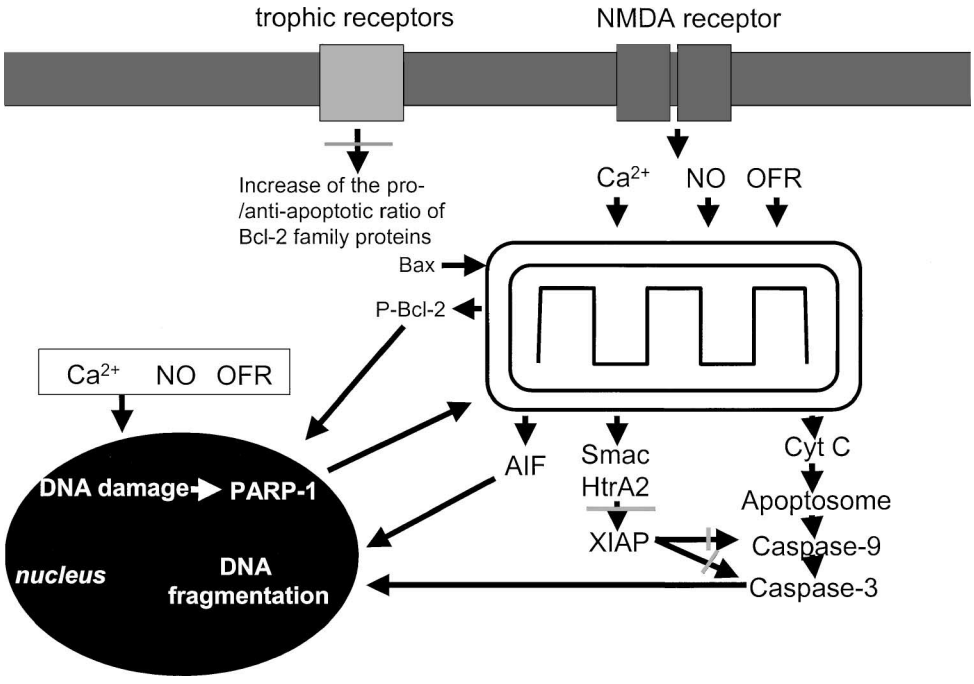

Fig. 1. Tentative role of mitochondria in cellular injury after HI in the developing CNS. AIF, Apoptosis-inducing factor; Bax, Bcl-2-associated protein X; Bcl-2, B-cell leukemia gene 2; CytC, cytochrome C; Htr/A2, high-temperature requirement serine protease A2; OFR, oxygen free radicals; Smac, second mitochondrial activator of caspase; XIAP, X-linked inhibitor of apoptosis.

synthase (2-iminobiotin) (Peeters-Scholte, 2002) inhibit activation of caspase-3, DNA fragmentation, and brain injury (Gilland and Hagberg, 1997). These data suggest indirectly that glutamate activation of NMDA receptors and production of NO may contribute to the mitochondrial release of proapoptotic proteins and subsequent activation of caspase-3.

CONCLUSION

Neonatal HI induces activation of NMDA receptors, production of NO/oxygen free radicals, and loss of trophic factor support, speculatively leading to an increase in the ratio of proapoptotic/antiapoptotic Bcl-2 family proteins. The changed environment will lead to depression of mitochondrial respiration, intramitochondrial Ca^{2+} accumulation and swelling, mitochondrial permeability transition, and release of proapoptotic proteins, resulting in caspase-dependent and caspase-nondependent cell death (Fig. 1).

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