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SYMPOSIUM: Oxidative Stress in Neurological Disease

Oxidative Metabolism, Apoptosis and Perinatal Brain Injury

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Perinatal hypoxic-ischaemic injury (HII) is a significant cause of neurodevelopmental impairment and disability. Studies employing ^{31}P magnetic resonance spectroscopy to measure phosphorus metabolites in situ in the brains of newborn infants and animals have demonstrated that transient hypoxia-ischaemia leads to a delayed disruption in cerebral energy metabolism, the magnitude of which correlates with the subsequent neurodevelopmental impairment.

Prominent among the biochemical features of HII is the loss of cellular ATP, resulting in increased intracellular Na^+ and Ca^{2+}, and decreased intracellular K^+. These ionic imbalances, together with a breakdown in cellular defence systems following HII, can contribute to oxidative stress with a net increase in reactive oxygen species. Subsequent damage to lipids, proteins, and DNA and inactivation of key cellular enzymes leads ultimately to cell death.

Although the precise mechanisms of neuronal loss are unclear, it is now clear both apoptosis and necrosis are the significant components of cell death following HII. A number of different factors influence whether a cell will undergo apoptosis or necrosis, including the stage of development, cell type, severity of mitochondrial injury and the availability of ATP for apoptotic execution.

This review will focus on some pathological mechanisms of cell death in which there is a disruption to oxidative metabolism. The first sections will discuss the process of damage to oxidative metabolism, covering the data collected both from human infants and from animal models. Following sections will deal with the molecular mechanisms that may underlie cerebral energy failure and cell death in this form of brain injury, with a particular emphasis on the role of apoptosis and mitochondria.

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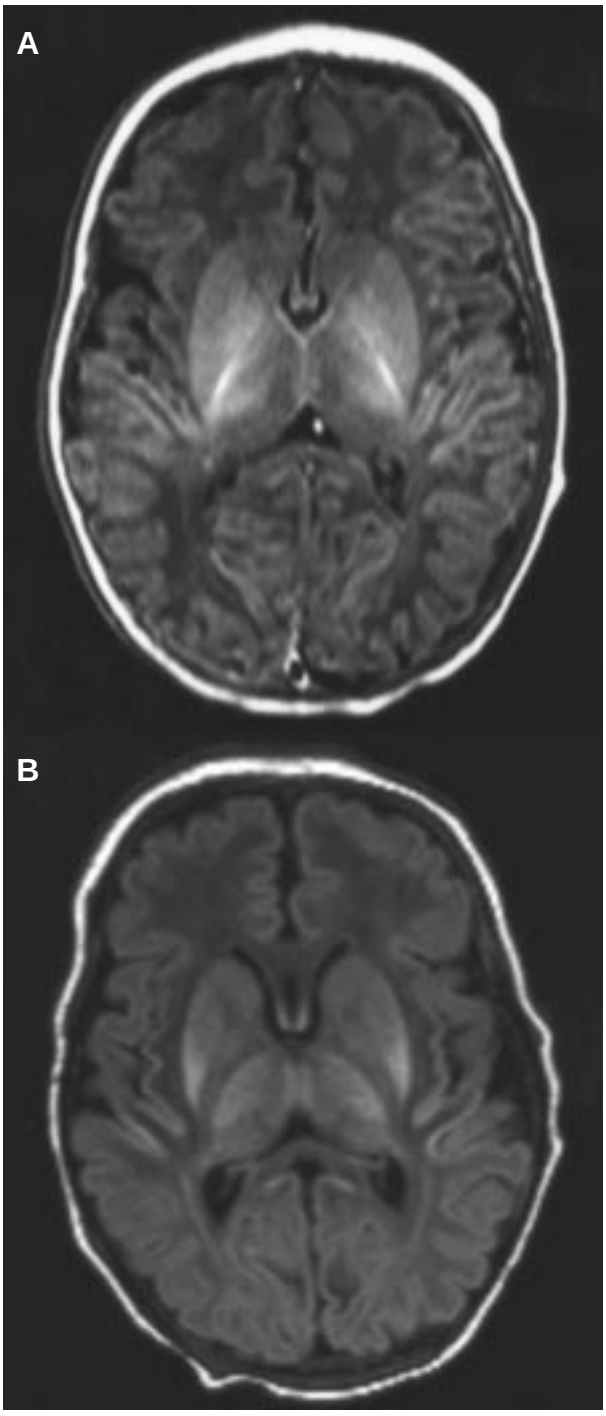
Introduction

Injury to the developing brain can lead to a variety of pathological consequences that depend not only on the type and severity of insult, but also on the maturity of the tissue. Preterm babies are conventionally described as developing a series of characteristic lesions, including (a) focal parenchymal haemorrhagic infarction due to venous infarction (Figure 1); (b) periventricular leucomalacia, often widespread in distribution and common in children who die in the newborn period; and (c) telencephalic leucoencepalopathy, which is thought by some to be common among children who survive with neurological impairment (208). In term infants different lesions are usually described. Global hypoxic-ischaemic injury, with a widespread distribution of apoptotic and necrotic cell death, is characteristic after severe birth asphyxia, and can be inferred in infants who survive from characteristic MRI appearances (178) (Figure 2). Hypoxic-ischaemic changes are also seen in many stillbirths although in these infants apoptotic death may be more obvious (35). Focal infarction is not uncommon, and may be under-diagnosed in survivors unless sophisticated techniques such as diffusion weighed imaging are employed (29).

It has been conventional to view the majority of these forms of brain damage as the result of impaired oxidative phosphorylation by hypoxia and/or ischaemia. Research has thus focused upon clinical measures of cerebral perfusion and oxygenation. Equally a large number of animal experiments have investigated the mechanisms of cerebral HII in immature animals.

However, recently there has been an increasing body of evidence that casts doubt on the primary or unique role of hypoxia and ischaemia in injury to the developing brain, particularly in preterm infants. Although it is clear that severe restriction of cerebral blood flow will eventually damage the brain, this mechanism does not always seem to be the primary event in clinical practice. Measurements of cerebral blood flow in preterm infants have demonstrated extremely low rates of blood flow in normal infants (54), and clinical studies examining the correlates of proven brain damage frequently found that haemodynamic variables such as blood pressure were less predictive of injury than indices of infection.



Recent work has emphasised the role of both intrauterine and neonatal infection in preterm brain injury. For example, meningitis caused by group B streptococci in infant rats can lead to both necrosis and apoptosis in brain cells (101). Moreover, there are close relations between placental inflammation, increased pro-inflammatory cytokines in blood or amniotic fluid and cerebral injury (224, 225). While no study has excluded the possibility that inflammation executes its deleterious effect through a haemodynamic mechanism, there are compelling reasons to consider inflammatory and immune mechanisms as aetiological factors in preterm brain injury.

A similar re-evaluation is being undertaken concerning birth asphyxia in the term infant. Studies have shown that cerebral palsy is often associated with measures such as maternal fever when there may be little clinical evidence of impaired intrauterine gas exchange (55), and there is now considerable controversy about the primary importance of hypoxia-asphyxia. A part of this uncertainty is created by the very imprecise measures available to clinicians to detect intrauterine hypoxaemia or cerebral ischaemia (144).

Against this background, precise data on cerebral oxidative metabolism are needed for any discussion of oxidative injury in the developing brain to be meaningful. Fortunately this can often be obtained in infants using techniques such as magnetic resonance spectroscopy (MRS). Such studies have defined at least a subgroup of infants in whom a characteristic process of damage to oxidative metabolism can be clearly defined.

This review will concentrate on such infants, and on pathological mechanisms of cell death in which there is

Figure 1. (Left) Panel **A**: Magnetic resonance image of the brain of an infant born at 26 weeks gestational age. This coronal section shows normal appearances, with characteristic bands in the white matter, representing groups of migrating glial cells. Panel **B**: Magnetic resonance image of the brain of an infant born at 25 weeks gestational age and suffering extensive intraventricular haemorrhage and haemorrhagic parenchymal infarction. Fluid levels are apparent in the ventricles and infarction is seen as a fan shaped region. A low signal intensity is seen in white matter on the left, where the normal white matter band structure is destroyed. Images courtesy of Prof. G. Bydder, Imperial College School of Medicine.

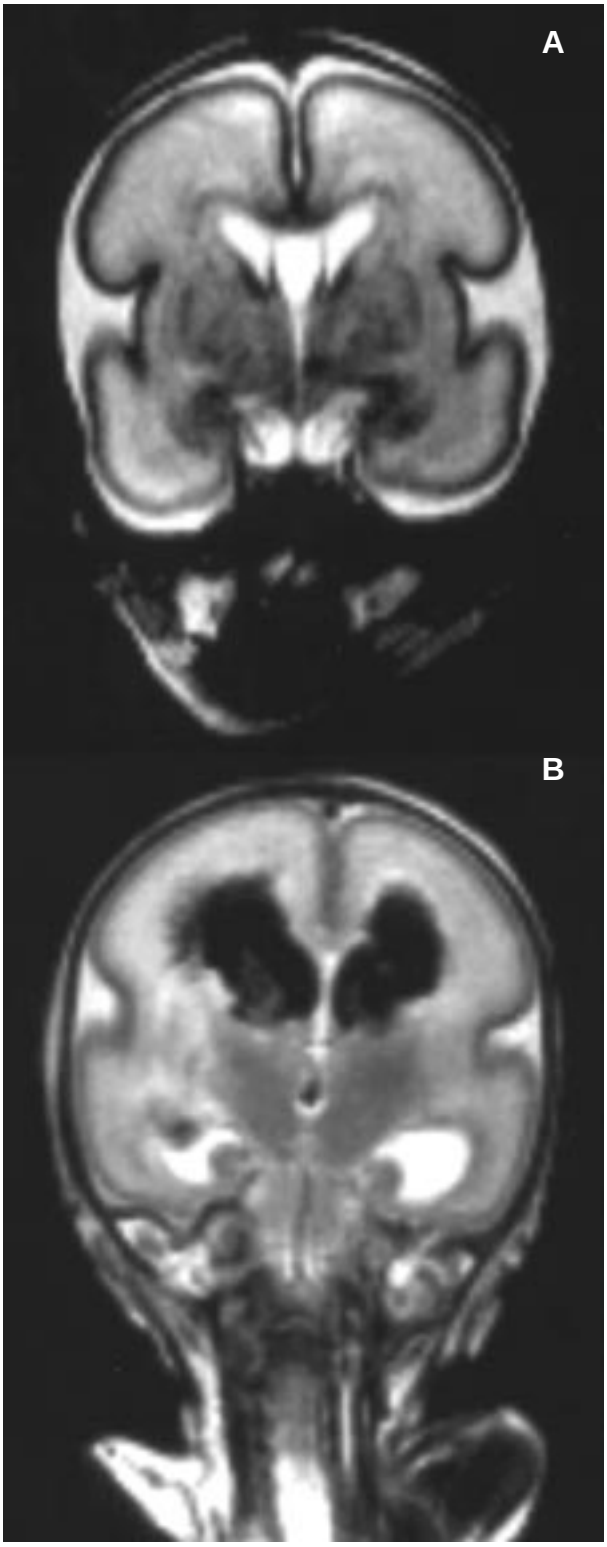


Figure 2. (Right) Panel **A**: Magnetic resonance image of the brain of an infant at term showing normal grey white matter differentiation, normal basal ganglia and thalami and high signal from myelin in the posterior limb of the internal capsule. Panel **B**: Magnetic resonance image of the brain of an infant born at term but suffering severe hypoxic-ischaemic injury, and showing characteristic widespread damage. There is abnormal high signal intensity within the lateral lentiform nucleus and the thalamus, with reduced signal intensity from myelin in the posterior limb of the internal capsule. The cortex is highlighted in the insula with low signal subcortical white matter adjacent to it. Images courtesy of Prof. G. Bydder, Imperial College School of Medicine.

a disruption to oxidative metabolism and/or damage to cellular mechanisms by oxidative stress. The first sections will give the evidence of the process of damage to oxidative metabolism, covering the data collected both from human infants and from animal models. Following sections will deal with the molecular mechanisms that may underlie cerebral energy failure and cell death in this form of brain injury, with a particular emphasis on the role of apoptosis and mitochondria. Some important pathological mechanisms, such as the role of excitotoxins and inflammatory cytokines are largely excluded for reasons of space and because they are covered in depth elsewhere.

Impairment of oxidative metabolism in perinatal brain injury

Hypoxic-ischaemic injury in infants born at term. ^{31}P MRS permits detection and quantification of intracellular pH (pHi), and of the cerebral concentrations of adenosine triphosphate (ATP) and other metabolites such as phosphocreatine (PCr) and inorganic phosphate (Pi). When ATP generation is impaired energy flux is maintained by the breakdown of PCr while Pi increases (see below), so that a decline in the ratio (PCr)/(Pi) is a valuable indicator of impaired energy metabolism, even in the presence of normal or near normal concentrations of ATP. ^{31}P MRS thus gives a non-invasive measure of the adequacy of cerebral oxidative metabolism. This can be extended by use of ^{1}H MRS which allows observation of lactate within brain tissue, as well as metabolites such as n-acetyl aspartate which is thought to be largely restricted to neurons and therefore may give a measure of neuronal loss. The practicalities of MRS mean that the majority of data have been collected from mature infants, and much less is known about the cerebral metabolic events associated with injury to the preterm brain (21).

In the 1980's Reynolds and his colleagues at University College London used MRS to demonstrate

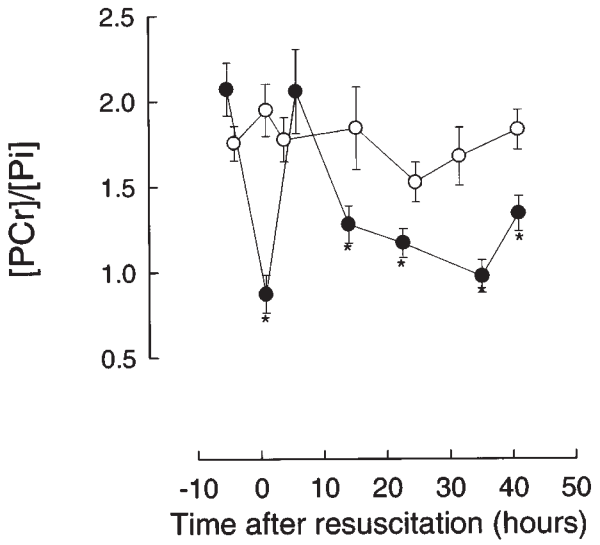


Figure 3. Magnetic resonance spectroscopy data showing secondary impairment of energy metabolism following unilateral carotid artery occlusion and reduced inspired oxygen concentration for 90 minutes in 14 day old rat pups (closed circles), compared to sham operated controls (open circles). The cerebral concentration ratio of phosphocreatine to inorganic phosphate ((PCr)/(Pi)) is observed as a measure of cerebral phosphorylation potential. The effect of hypoxia-ischaemia is seen, with a reduction in (PCr)/(Pi) that recovers after resuscitation, but then declines again after 10 hours. The data are presented as means ± SEM. *P = 0.05. Reproduced from ref. 14.

that term infants with clinical evidence of perinatal hypoxia-ischaemia showed characteristic abnormalities in cerebral energy metabolism. In particular they showed that these infants typically had normal cerebral energy metabolism soon after birth, but some hours later a progressive decline in (PCr)/(Pi) occurred. Infants displaying this phenomenon developed severe neurodevelopmental impairment or died (74).

These findings suggested the concept of 'secondary energy failure' which has been developed and extended by several groups (60, 74, 129). Delayed declines in (PCr)/(Pi) beginning some 8-12 hours after birth asphyxia have been confirmed and quantitated in a number of studies, and a comparable increase in cerebral lactate concentration (measured as the ratio lactate/total creatine) has been described (60). Interestingly it was found that these delayed disruptions of cerebral energy metabolism were associated with a normal or increased in pHi, in clear contrast to acute hypoxia-ischaemia when pHi characteristically falls (6, 75).

The relevance of delayed disruption to cerebral oxidative metabolism is demonstrated by the close relationship between the magnitude of the late decline in (PCr)/(Pi) and the severity of neurodevelopmental impairment one and four years later. Further, infants with significant 'secondary energy failure' show reduced head growth velocity, implying abnormal brain growth (175, 176).

Hypoxic-ischaemic brain injury in immature animals. The phenomenon of secondary energy failure after hypoxia-ischaemia has been examined further in global hypoxia-ischaemia in the newborn piglet (117, 162), and in focal stroke in rat pups (14, 151, 216, 222). The results of both MRS and biochemical studies in these models have broadly supported the working hypothesis that hypoxia-ischaemia causes two distinct periods of abnormal cerebral energy metabolism. During hypoxia-ischaemia there is a primary energy failure when intracerebral (PCr)/(Pi) falls, lactate increases, and pHi becomes acidic. Eventually ATP declines, however, even if this falls to undetectable levels the reprovision of substrate usually causes the metabolites to return to normal values within an hour or two. Some hours later the period of secondary energy failure begins during which (PCr)/(Pi) again declines (Figure 3), lactate increases but pHi becomes alkaline. There is a dose response relationship between the severity of the hypoxic-ischaemic insult and the magnitude of the secondary changes in cerebral energy metabolism. Equally, the more severe the cerebral metabolic impairment, the more severe the histological injury.

Studies of fetal sheep have provided further evidence for a biphasic model of cerebral injury (215). Using cerebral impedance as a reflection of the cells' ability to maintain normal membrane ion transport, it was shown that global total ischaemia led to an increase in impedance during the insult that returned towards normal after reperfusion. However a characteristic large and prolonged second increase in impedance began some hours later, which was accompanied by the development of electrocortical seizures and an increase in the concentrations of excitatory amino acids and nitric oxide metabolites in CSF (197).

Cerebral haemodynamics during delayed cerebral injury. Although simultaneous measurements of cerebral metabolism and haemodynamics have not been made, the delayed changes in cerebral energy metabolism occur at a time when cerebral blood flow and volume are increased, and it is unlikely that delayed hypoperfusion adequately explains the fall in high energy phosphates (127, 168, 219). However, cerebral perfusion is not completely normal during secondary energy

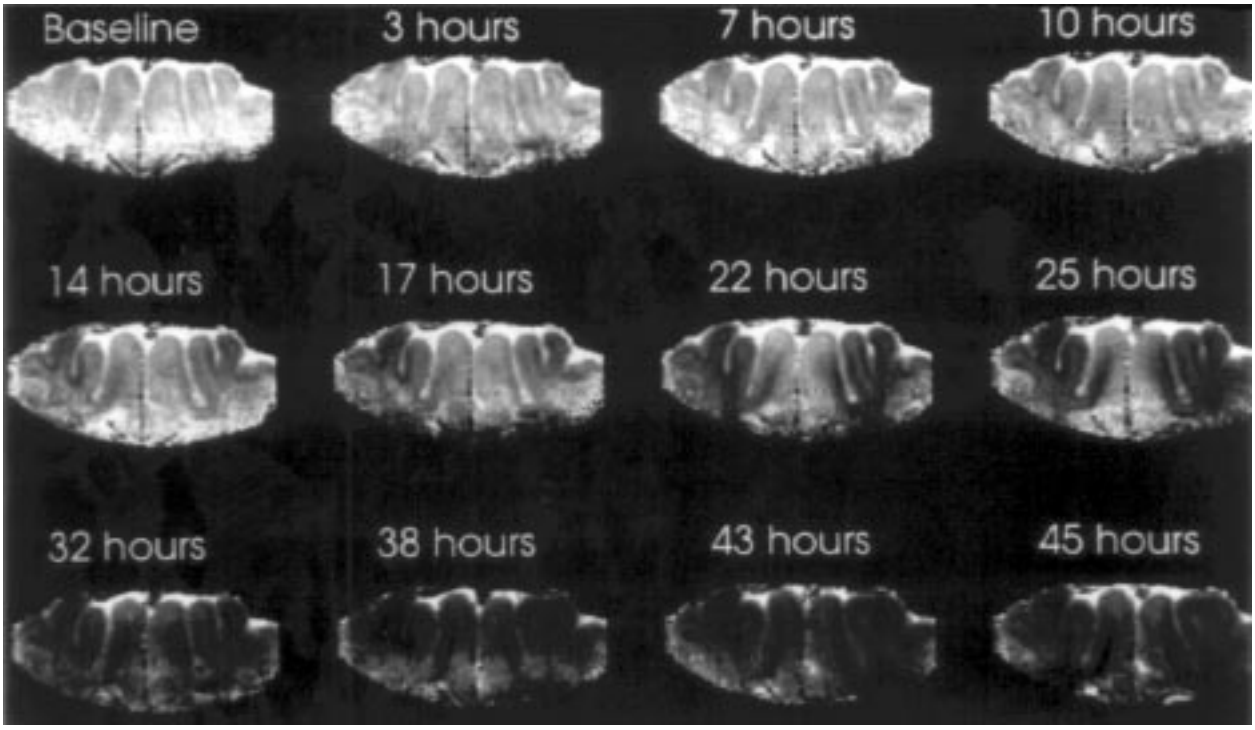


Figure 4. Magnetic resonance images showing apparent diffusion coefficient in a newborn piglet following hypoxia-ischaemia induced by transient bilateral carotid artery occlusion and reduced inspired oxygen concentration. The images show cerebral cortex, with darker areas representing brain tissue with abnormal apparent diffusion coefficient, indicative of abnormal cellular function. The images are taken at specified times after resuscitation from hypoxia-ischaemia and show the progression of change during the delayed phase of injury. Initially the tissue is apparently normal, but abnormalities appear first in the lateral cortex, and then progress to involve the more central regions. The data are presented as means ± SEM. *P = 0.05. Reproduced with permission from ref. 201.

failure, as loss of both the normal response to changes in arterial carbon dioxide tension, and blood pressure autoregulation have been reported (168, 220). Studies of fetal sheep using near infrared spectroscopy demonstrated that delayed increases in cerebral impedance are accompanied by an increase in cerebral perfusion which is partially mediated by nitric oxide, but also by other changes in the optical properties of the brain that may be attributed to a decline in the concentration of oxidised cytochrome aa3 (126, 127).

Problems with the current concepts of delayed impairment to energy metabolism after hypoxia-ischaemia. The concept of biphasic cerebral damage has been useful in focusing attention on the progressive nature of oxidative damage in the developing brain, emphasising the importance of delayed cell death and mechanisms that might explain this, such as apoptosis. However, recent data have shown it to be an oversimplification.

First, diffusion weighted MRI studies in piglets have shown that although changes in the apparent diffusion coefficient of brain (an index of the restriction of movement of water, and thus of membrane integrity) closely parallel changes in (PCr)/(Pi) during secondary energy failure, the changes are not uniform through the brain. Rather, abnormal diffusion begins laterally and then progresses towards the parasaggital and central regions (201) (Figure 4). Clearly a change in global (PCr)/(Pi) obscures considerable complexity of regional response.

Second, MRI of infants who develop secondary energy failure after birth asphyxia confirms that damage is distributed heterogenously throughout the brain. Damage to the basal ganglia and deep structures of the brain seem to predict adverse neurological outcome most accurately (178). Localised MRS measurements also show that metabolite concentration abnormalities differ in different regions of the brain; for example, lactate seems to be higher in the thalamus than the occipital region (160). Clearly, the concept of secondary energy failure needs to be adjusted to take account of regional effects.

Third, it is now clear that abnormal cerebral energy metabolism continues for much longer than was previ-

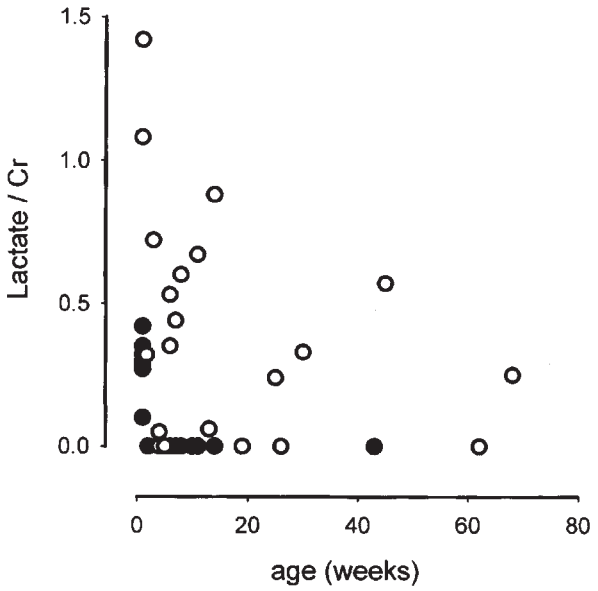


Figure 5. Magnetic resonance spectroscopy data from term infants suffering or having suffered birth asphyxia, showing that lactate (expressed as the ratio of lactate to total creatine (Lac/Cr)) was detectable soon after the suspected insult, but that it rapidly became undetectable in infants with normal neurodevelopmental outcome (filled circles) while it could be detected for a prolonged period in infants with neurological damage (open circles). Reproduced from ref. 61.

ously known. In children who are neurologically normal following birth asphyxia, cerebral lactate concentrations rapidly fall. However in those who develop neurodevelopmental impairment lactate can be detected in the brain for many months (Figure 5) (61). This extreme prolongation of 'secondary energy failure' has been noted in adult stroke. Although the mechanism is unknown, it may be due to altered redox potential in damaged cells, prolonged mitochondrial inhibition or perhaps phagocyte infiltration. It is interesting to note that in adult rats moderate hypoxia-ischaemia is followed by a very prolonged period of increased apoptotic death in affected areas (33).

Nevertheless, the idea of delayed energy failure remains of value, and this can be seen in studies of neural rescue therapies. There is a very close relation between the severity of secondary energy failure and the amount of cell death after hypoxia-ischaemia (14, 137, 228). Treatments that reduce the magnitude of secondary energy failure also reduce the amount of cell death and improve histological outcome. For example, moderate hypothermia applied for 12 hours after a hypoxic-ischaemic insult in newborn piglets prevented the delayed decline in (PCr)/(Pi) and (ATP), and significantly reduced the number of cells undergoing apoptosis (200, 34). On the other hand, several treatments that are ineffective at reducing cell death also have no effect on the delayed phase of injury (126, 161).

Cerebral injury to the preterm brain. Damage to the more immature brain is an increasingly important problem for two reasons. First, increasing numbers of very immature infants are delivered and survive after intensive care. Abnormalities can be detected by cerebral imaging in a large number of these infants and the rate of neurological impairment is relatively high. Secondly, as it becomes clear that birth asphyxia accounts for probably no more than 10% of all cases of cerebral palsy, there is increasing interest in the mechanisms by which the brain might be damaged early in development in utero.

Less is known about the cerebral metabolic events associated with preterm brain injury. It is clear that cerebral metabolism is different in the less mature brain. Cerebral blood flow and oxygen consumption are lower, and cerebral lactate can be detected in healthy preterm infants. There is some evidence that secondary energy failure may also be a useful concept in some preterm brain injury: infants with echodensities on cerebral ultrasonography had lower (PCr)/(Pi) similar to term infants suffering asphyxia (59). However, studies in fetal sheep have shown that the delayed phase of injury is less marked at earlier gestations, and suggest that the more immature brain may suffer a more rapidly evolving injury in response to hypoperfusion (171). It would therefore be premature to suggest that a central role can be proven for hypoxia-ischaemia with typical secondary energy failure in the preterm brain especially considering the growing evidence that preterm white matter injury has an inflammatory component (226, 227).

Biochemical mechanisms of cerebral energy failure

The biochemistry of cerebral energy failure during hypoxia-ischaemia can be distinguished from those occurring after restoration of substrate supply.

Biochemical events during hypoxia-ischaemia. Hypoxia-ischaemia leads to inadequate supplies of glucose and oxygen and thus reduces ATP generation. Although ATP synthesis from oxidative phosphorylation is curtailed, cytosolic ATP utilisation continues. Initially the breakdown of PCr by the creatine kinase reaction is driven by the accumulation of ADP and H^+ and maintains ATP levels. As such the fall in PCr precedes that in ATP as the cells attempt to maintain optimal levels of

ATP (30, 111, 118). However, PCr stores are soon depleted (37) and lactate is generated by anaerobic glycolysis (44). Unlike oxidative phosphorylation that produces 36 moles of ATP per mole of glucose consumed, glycolysis only produces 2 moles of ATP per mole of glucose. Initially anaerobic glycolysis maintains ATP levels at about 90% of control values as long as glucose is available (37), but ultimately the total adenine nucleotide pool (ATP+ADP+AMP) decreases. These perturbations have been well characterised in experimental models of perinatal hypoxic-ischaemic injury (205).

The loss of cellular ATP during hypoxia-ischaemia severely compromises those metabolic processes that require energy. The Na^+/K^+ ATPase is curtailed and cell membrane depolarisation occurs, accompanied by increased intracellular Na^+ and Ca^{2+}, and decreased intracellular K^+ (62, 63, 145). Movement of Na^+ and Cl^- into cell is accompanied by osmotically obligated water leading to oedema formation and with cell membrane depolarisation, calcium enters neurons due to the opening of voltage-sensitive Ca^{2+} channels, and AMPA and NMDA receptors. In addition to influx from the extracellular compartment, the release of Ca^{2+} from intracellular stores may increase cytosolic (Ca^{2+}). Reduced energy dependent pumping of Ca^{2+} into stores further exacerbates the situation. Elevation of intracellular Ca^{2+} leads to the activation of many Ca^{2+} sensitive systems some of which have adverse effects on the cell.

Biochemical changes after hypoxia-ischaemia. Soon after transient hypoxia-ischaemia oxidative phosphorylation is partially resumed and ATP is resynthesised. The initial restoration in cerebral concentrations of ATP and other adenylate compounds may be complete or incomplete, and may take several hours (14, 38, 117). PCr levels may be normalised at this time, but a persisting intracellular acidosis can shift the creatine kinase equilibrium in the direction of PCr hydrolysis (186). Despite marked acidosis during ischaemia, pHi is rapidly restored after resuscitation (14, 117). This recovery may reflect re-established H^+ extrusion from cells with recovery of ATP-dependent ion exchanges across the plasma membrane.

Tissue glucose, previously depleted during hypoxia-ischaemia, increases above pre-ischaemic levels, and high levels of lactate decline (160, 214, 221) indicating that an inhibition of glycolytic flux proximal to the formation of pyruvate, with lactate serving as the principal substrate for the TCA cycle (204). The inhibition of glycolytic flux is completely reversed by 4h of recovery, at which lactate, pyruvate and a-ketoglutarate have recovered to normal levels (151).

During recovery from HII, the redox state of the cytoplasmic compartment in the immature rat brain remains persistently reduced (low NAD^+/NADH ratio), while the redox state of the mitochondria is normalised by one hour of recovery (221). This suggests that tissue is permanently damaged by the hypoxic-ischaemic event so that either the transfer of reducing equivalents (NADH) from the cytosol across the mitochondrial membrane is inhibited (185) or reducing equivalents generated within mitochondria are consumed but with inadequate production of energy equivalents (ATP). The data thus suggest that even during the apparent recovery soon after reperfusion there are persistent alterations in mitochondrial function.

Some 8 to 12 hours post hypoxia-ischaemia, the delayed phase of reduced phosphorylation potential and increased tissue lactate begins (205). In the second phase normal or slightly alkaline pHi together with increased lactate concentration is consistent with a relatively reduced intracellular redox state and a high NADH/NAD^+ ratio (186). The precise cause of the delayed impairment in energy metabolism is unknown, but the combination of high lactate concentration, increased pHi and reduced (PCr)/(Pi) is consistent with a change in the cytosolic redox state that persists for many weeks in infants who develop neurodevelopmental impairment after birth asphyxia (172).

Modes of cell death following oxidative injury

During cerebral energy failure, cells vulnerable to hypoxia-ischaemia begin to die. Cell death can occur by different routes, each with characteristic morphological criteria as an end point.

Cell death is conventionally classified as apoptotic or necrotic. In necrosis, cell death is triggered by an overwhelming external insult damaging cellular organelles such as mitochondria resulting in the loss of membrane integrity and leaking of cytoplasmic contents into the extracellular matrix. In contrast, cells dying by apoptosis carry out a well conserved and highly regulated genetic programme of cell death. They do not lose membrane integrity and the organelles remain largely intact. In the final stages, cell fragments are 'shrink-wrapped' in the contracting plasma membrane and bud off as apoptotic bodies which are subsequently phagocytosed by healthy neighbouring cells. Since the apoptotic response to damage largely circumvents inflammatory responses this mode of cell death would be particularly beneficial following injury to the brain which has only a limited

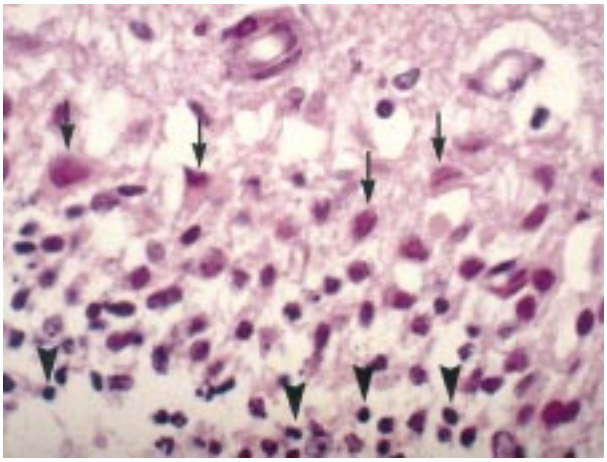


Figure 6. Histopathological appearance of cells in the dentate gyrus of the hippocampus of a newborn piglet 48 hours after severe hypoxic-ischaemic injury, showing that apoptosis predominates in the small neurons of the inner layer of the dentate fascia (arrowheads), while the larger cells in the outer layer are necrotic (arrows). Haematoxylin and eosin staining. Magnification = X 750.

capacity for repair and regeneration.

Apoptosis is a biochemically and genetically programmed cell death that is distinct from necrosis because it requires time, energy and, in some cases, new gene transcription and translation. However, as discussed later, the distinction between apoptosis and necrosis is not always clear cut and, in many instances, these two modes of cell death can be regarded as a continuum of a single cell fate following injury.

Apoptosis and necrosis following hypoxia-ischaemia. Both apoptotic and necrotic cells are found in tissue after hypoxia-ischaemia (Figure 6). A large body of literature indicates that necrosis can occur during hypoxia-ischaemia or immediately following resuscitation when blood flow is very low. This acute lack of oxygen can result in a rapid reduction in cerebral energy production, membrane pump failure, severe ionic imbalances and necrotic cell death (119). These acute early changes may be particularly associated with an increase in excitotoxic amino acid levels and free radical production, the damaging effects of which can be blocked by specific receptor antagonists and scavengers (163).

Apoptotic cells have been reported following hypoxic-ischaemic injury in both the immature (12, 45, 137) and adult brain (108, 121). The protein synthesis inhibitor cycloheximide has been shown to reduce neuronal loss following cerebral ischaemia, suggesting that an active physiological process is involved (51), although it is possible that the neuroprotective effects of cycloheximide may be mediated, at least in part, by a reduction in glutamate release (112). Administration of the anti-apoptotic growth factor insulin-like growth factor-1 (IGF-1) after the primary insult ameliorates delayed injury, while glutamate receptor blockade is only partially protective (50, 196). Further evidence for apoptosis resulting from hypoxia comes from *in vitro* studies. (17, 173).

Apoptosis has been shown to be involved in human perinatal brain injury. Infants who die after intrauterine injury, either with or without evidence of hypoxia-ischaemia, have a significant number of cells in the brain with the morphologic characteristics of apoptosis (Figure 7) (35, 183).

Relation between apoptotic and necrotic death in cerebral injury. It might be convenient to consider immediate cell death following hypoxia-ischaemia as necrotic and delayed cell death as apoptotic. However, published data do not entirely support such a simplifying concept. In experimental porcine hypoxia-ischaemia apoptosis and necrosis occurred in adjacent populations of neurons and glial cells in the cingulate sulcus while quantitative analysis of cell death showed that the numbers of both apoptotic and necrotic cells were linearly related to the severity of injury (228). Human infants who have suffered secondary energy failure have a preponderance of necrotic cells, while those dying *in utero* show a higher proportion of apoptotic cells (Figure 7) (35).

These differences may arise from the fact that some necrotic cells represent the secondary degradation of apoptotic cells. In this context, primary cell necrosis, sometimes called oncosis, can be regarded as an acute response to severe injury and is the common pattern of change in cerebral infarction. Secondary cell necrosis can follow either oncosis or apoptosis (123). Following either apoptotic or oncotic death, cells undergo similar changes that include mitochondrial swelling, karyolysis, and disruption of plasma membranes (202). Cell necrosis should be distinguished from tissue necrosis, in which a large number of cells undergo necrosis together, which may be primary, secondary or both.

Thus oxygen-glucose deprivation may induce first apoptosis then secondary necrosis in cerebellar granule cells. (86). Similarly, primary apoptosis precedes secondary necrosis following the injection of excitatory amino acid receptor agonists into the adult rat brain (46), and in rat dorsal root ganglion cell cultures treated

with oxidised low density lipoprotein (159).

Studies with cultured neurons subjected to oxygen deprivation have confirmed that necrosis and apoptosis can occur in a single cell population (206). In one such study, the administration of Trolox, which interferes with lipid peroxidation, prevented necrosis but allowed neurons to undergo apoptosis (28). In a separate study, the induction of apoptosis in target cells by cytotoxic T lymphocytes was blocked by inhibitors of transcription or translation; instead, the target cells underwent necrosis (231). Thus it may be possible to switch the fate of damaged cells from necrosis to apoptosis.

These observations suggest that both the apoptotic and necrotic routes to death are available to cells for an extended period, and may proceed in parallel. Indeed, we have found that in both global (84, 136) models of cerebral HII, as many as 10% of the dead cells display morphological characteristics of both apoptosis and necrosis (Figure 8). Similar observations have been reported following glutamate treatment of a mouse hippocampal cell line (195). Factors that lead a cell to apoptosis or necrosis are discussed below.

Molecular evidence of apoptosis following brain injury

A number of molecular signals associated with apoptotic death are induced by impaired oxidative phosphorylation and oxidative injury.

Caspases and poly (ADP ribose) polymerase cleavage. The nuclear enzyme, poly (ADP ribose) polymerase (PARP) provides one of the most apparent links between oxidative stress, impaired energy metabolism and cell damage. Free radicals such as nitric oxide and peroxynitrite can damage DNA, leading to activation of PARP, which catalyses attachment of ADP ribose units from NAD to nuclear proteins. However, excessive activation of PARP can deplete NAD^+ and ATP (which is consumed in NAD regeneration) leading to an increase in the NADH/NAD^+ ratio and eventually cell death by energy depletion (15). PARP is induced by hypoxia-ischaemia, and genetic disruption of PARP provides profound protection against both glutamate-mediated excitotoxic insults *in vitro* and major decreases in infarct volume after reversible middle cerebral artery occlusion (39). These results provide compelling evidence for a primary involvement of PARP activation in neuronal damage.

Genetic deletion of PARP or PARP inhibition by 3-aminobenzamide reduced infarct size after transient cerebral ischaemia (193), but did not reduce the density of apoptotic cells (41). The susceptibility of primary neurons towards apoptosis is unaffected in PARP-/- mice, suggesting that PARP activation is not necessary for apoptosis (103, 212) Thus in cerebral ischaemia,

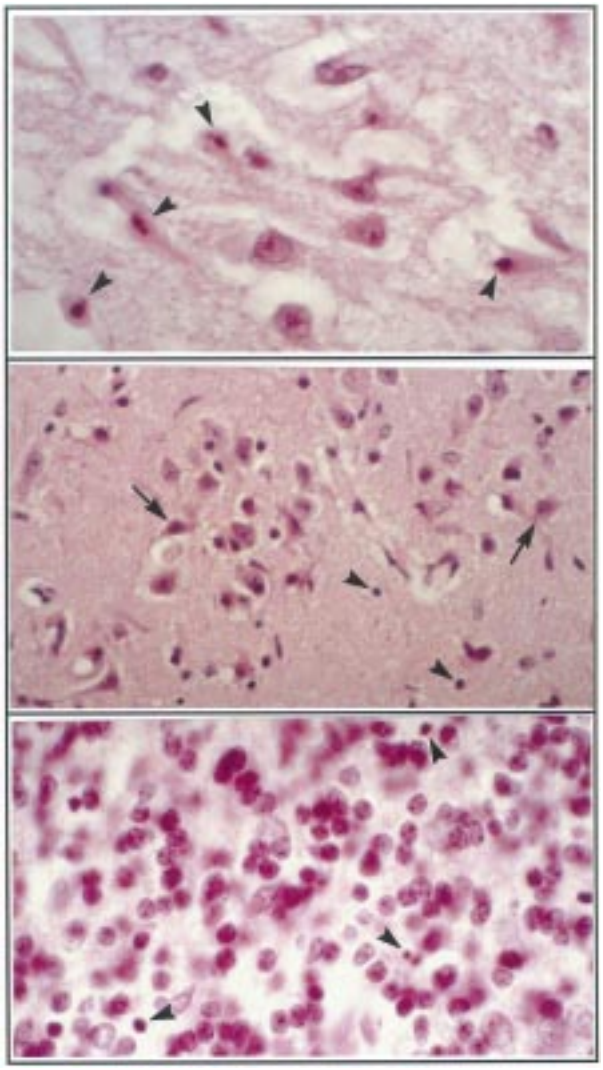


Figure 7. Histopathological appearance of cells in the hippocampus (top panel) and internal granule layer of the cerebellum (bottom panel) of a single human infant dying after birth asphyxia and the subiculum (middle panel) of a separate human infant suffering sudden unexpected intrauterine death. Apoptotic nuclei can be seen within eosinophilic cytoplasm of pyramidal neurons, consistent with secondary necrosis in the hippocampus (arrowheads, top panel), while in the cerebellum only classical apoptotic granule neurons are detected (arrowheads, bottom panel). In the subiculum (middle panel), both apoptotic (arrowheads) and necrotic cells (arrows) can be seen in the same field, indicating that both modes of cell death occur following sudden intrauterine death. Haematoxylin and eosin staining. Magnification = X 750 (top and bottom panels) or X 375 (middle panel).

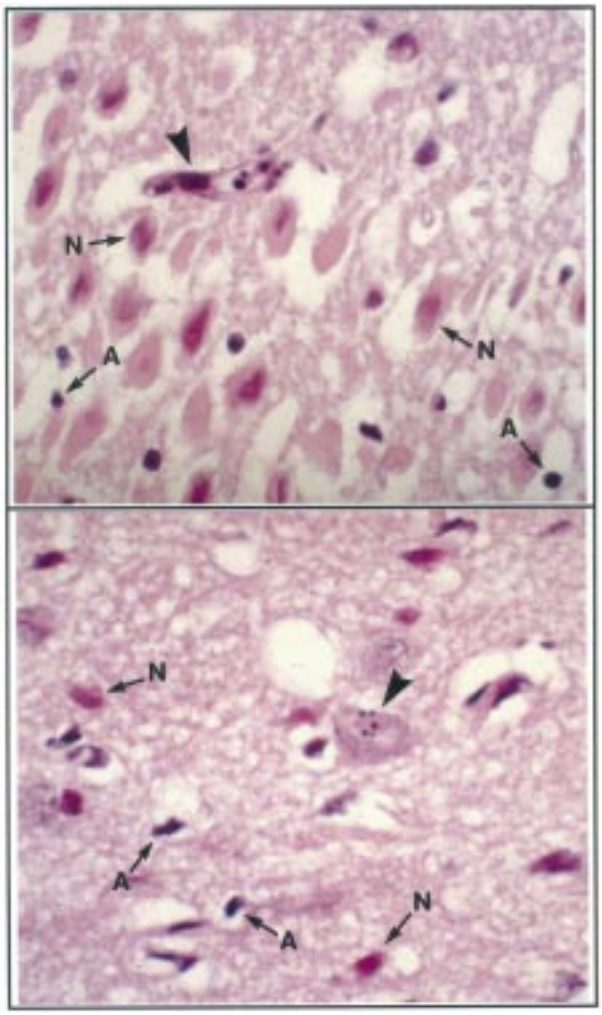


Figure 8. Histopathological appearance of cells in the hippocampus (top panel) or basal ganglia (bottom panel) of a newborn piglet 48 hours after severe hypoxic-ischaemic injury. In both brain regions adjacent apoptotic (A) and necrotic (N) cells can be seen. In the hippocampus, a pyknotic, basophilic nucleus can be seen inside a cell with eosinophilic cytoplasm (arrowhead). Similarly, in the basal ganglia a karyorrhectic nucleus can be seen inside a cell with swollen cytoplasm (arrowhead). These are both examples of cells with the morphological characteristics of both apoptosis and constitute about 10% of all dying cells following cerebral HII. Haematoxylin and eosin staining. Magnification = X 750.

PARP may contribute to cell death by NAD^+ depletion and primary energy failure without direct involvement in apoptotic responses.

However, PARP is involved in apoptosis in another way. Specific proteolytic cleavage of PARP by caspase 3 is an important event in at least one pathway to apoptosis (100), possibly as a mechanism to maintain the ATP needed for successful completion of the apoptotic programme. Both caspase activity (143) and PARP cleavage (84) have been shown to increase immediately following ischaemic injury *in vivo* followed several hours later by morphological features of apoptosis.

Caspase inhibition can reduce neuronal loss following oxygen-glucose deprivation of cortical neurons (52) and following HII *in vivo* (42, 114). However some results suggest that while caspase inhibition may prevent the cellular manifestations of apoptosis, it does not prevent cell death (135). The precise roles of PARP cleavage and caspase activity in cell death after hypoxia-ischaemia need to be defined further.

Gene expression and activation. Apoptotic death is often preceded by the induction and activation of a considerable variety of genes, including immediate early genes (32, 96, 141), zinc finger genes, heat-shock proteins (HSPs), β-amyloid precursor protein (7, 104) and nuclear factor-kappa B (NF-kappa B) (26).

A number of these genes have been shown to possess either pro- or anti-apoptotic functions (120). For example, NF-kB (27) and HSPs (166) are thought to be protective against oxidative stress, while c-Jun, which is phosphorylated by stress activated protein kinases (SAPKs), is strongly implicated in triggering neuronal apoptosis (32).

In vivo studies of ischaemic injury to the heart, kidney, liver and brain have shown that several SAPKs are activated by injury (77, 140, 209, 210, 211). However, it remains to be determined whether these kinases are pro- or anti-apoptotic. Early activation of p38 SAPK has been shown to protect cells from apoptotic death following tumour necrosis factor (TNF) treatment (177), while studies of CD40 and B-Cell receptor signalling have shown that Jun N-terminal kinase (JNK) and P38 activation do not precede apoptosis triggered by the engagement of these receptors (191). Conversely, the JNK inhibitor CEP-1347 is able to rescue motoneurons from apoptosis following the withdrawal of neurotrophic factors (128), while the p38 inhibitor SB203580 is able to prevent glutamate-induced apoptosis in cerebellar granule neurons (92). In the context of oxidative brain injury, the anti-apoptotic protein Bcl-2 is a substrate for JNK but not p38 (134). Although this phosphorylation is specific, the function is not clear, since the protective effects of Bcl-2 appear to be independent of this post-translational modification.

Cytokines and receptors. The activation of SAPKs as one of the earliest signals downstream of death receptor activation provides evidence that intercellular signalling

may be involved in triggering cell death during tissue injury. Receptors for pro-apoptotic signals including TNF and interleukin-1β (IL-1β) are expressed acutely in the injured brain and may contribute to the progression of neuronal damage following hypoxia-ischaemia. (19, 89, 187, 192). A recombinant human IL-1β receptor antagonist markedly protects against focal cerebral ischaemia in the rat (113). It is not yet clear if the pro-apoptotic receptor Fas (142) is expressed after perinatal brain injury, but Fas mRNA expression increases in neuronal cells following global cerebral ischaemia to the adult mouse brain (133), and oxidative stress is a potent inducer of Fas ligand expression (207).

Further observations implicate endogenous inflammatory cytokines in HII. Mice lacking NF-kB are more susceptible to TNF-induced damage (10). On the other hand, TNF null mice are more susceptible to HII (20), while intracerebroventricular injection of recombinant IL-6 significantly reduces ischaemic brain damage, suggesting that the large increases in TNF and IL-6 following cerebral ischaemia are endogenous neuroprotective responses (115).

Factors determining the choice between apoptosis and necrosis

A number of different factors influence whether a cell will undergo apoptosis or necrosis, including development, cell type, severity of injury and availability of ATP.

Developmental age, differentiation and cell type. A general principle of development in multicellular organisms is that excess numbers of cells are made, and then those surplus to requirement are selected to undergo programmed cell death by apoptosis during the maturation of functional organs (83). In the developing nervous system more than 50% of neurons are lost during development due to limiting trophic support from target tissues that they innervate (148).

The stage of cell development may be a particular determinant of apoptosis versus necrosis. For example, cerebellar Purkinje cells differentiate early in brain development. In the porcine model of HII, these cells are very sensitive indicators of HII but only undergo necrosis, never apoptosis. In contrast cerebellar granule cells (that continue to divide and migrate after birth) undergo apoptosis on a large scale following HII (228). Similarly, following excitotoxic injury to the newborn rat striatum, neuronal death occurs by apoptosis, while in the adult the same insult produces rapid cytoplasmic disintegration, consistent with necrosis (167).

Model	Insult	Reference
PC12 cells (rat pheochromocytoma)	6- hydroxydopamine	(146)
neonatal rat cerebrocortical slices	NMDA excitotoxicity	(66)
embryonic hippocampal rat neurons	lysophosphatidic acid	(76)
AR4-2J cells (rat pancreatic acinar line)	menadione	(180)
INS-1 (mouse pancreatic beta cell line)	streptozotocin	(179)
rat cortical neurons	peroxynitrite	(16)
mastocytoma cell line	heat	(64)
SK-N-MC (dopaminergic cell line)	respiratory chain complex complex I inhibitors	(65)
Molt-4 (human T cell line)	gamma irradiation	(3)

Table 1. Examples of apoptosis or necrosis determined by the severity of injury.

The mode of cell death may also depend on the degree of differentiation of the cell. Dividing progenitor cells are particularly sensitive to apoptotic stimuli, since the cell cycle machinery is intimately linked to apoptosis (174). This is illustrated by the observation that axotomy can lead to apoptotic death in some neurons and proliferation in others (69).

A number of separate studies have also indicated that specific cell types may be particularly sensitised to either necrosis or apoptosis (48, 91, 190).

Severity of injury. The same cell type can be triggered to undergo apoptosis following mild injury but necrosis if the damage is severe (169). This has been demonstrated in a number of *in vitro* systems (Table 1).

It is not clear precisely how the severity of injury dictates apoptosis or necrosis. One possibility is that the degree of damage to cell organelles influences the mode of cell death. Mitochondria are particularly sensitive to hypoxic injury and play a central role in both apoptosis and necrosis. For example, inhibitors of complex I trigger apoptosis at low concentrations and necrosis at high concentrations in neuronal cell lines in culture (65). Indeed, Kroemer and colleagues have suggested that the severity of mitochondrial damage is the most important deciding factor between these two modes of cell death (99); in this scenario, a small increase in mitochondrial membrane permeability can result in the controlled release of apoptogenic factors through the outer membrane, while severe mitochondrial damage releases a flood of Ca^{2+} and reactive oxygen species into the cytosol, leading to the disruption of plasma membrane integrity and cell necrosis. The importance of oxidative damage in this process is underlined by the finding that complex I inhibitor-dependent apoptosis is decreased by pretreatment with free radical scavengers (184) although

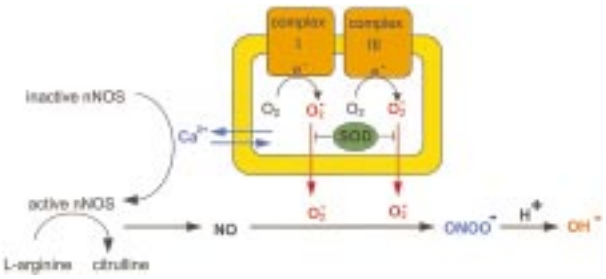


Figure 9. Schematic diagram of reactive oxygen species production during ischaemia. Superoxide (O_2^-) is formed from mitochondrial metabolism due to excessive Ca^{2+} cycling across the membrane. Excessive production of O_2^- overwhelms antioxidant defences, including superoxide dismutase (SOD), and ROS leak out into the cytosol. Nitric oxide (NO) from Ca^{2+} activated nitric oxide synthase (NOS) reacts with O_2^- to from peroxynitrite ($ONOO^-$), which decomposes further on protonation to the highly toxic hydroxyl radical (OH^-).

it remains to be determined whether free radical accumulation results directly from the block in the mitochondrial respiratory chain or indirectly from the apoptotic process itself.

Availability of ATP. Apoptosis requires energy (88) while complete ATP depletion causes necrosis; thus ATP availability is likely to be a significant determinant of apoptotic or necrotic death. If cells sustain a lethal insult they will be able to execute the apoptotic programme if ATP levels are sufficient, while in situations of limiting ATP the same degree of cellular injury can only result in necrosis. Consistent with this possibility, glutamate excitotoxicity has been shown to induce either necrosis or apoptosis depending on mitochondrial function. During and shortly after exposure to glutamate, neurons that died by necrosis had reduced mitochondrial membrane potential ($\Delta_{\psi m}$) and swollen nuclei. In contrast, neurons that recovered $\Delta_{\psi m}$ and ATP levels subsequently underwent apoptosis (4). Consumption of ATP by cell repair enzymes may thus also influence the mode of cell death. Inhibition of PARP prevents necrosis and triggers apoptosis in cells exposed to hydrogen peroxide (156, 213).

Perhaps the most direct demonstration of the importance of ATP is in cultured mouse proximal tubular cells, where ATP depletion itself caused apoptosis or necrosis depending on the levels: cells subjected to antimycin to deplete ATP to below 15% of controls died of necrosis, while cells with ATP levels between 25% and 70% of controls all died by apoptosis (107). Extending these observations, it is also possible to alter the mode of cell death by manipulating cellular ATP levels (36, 102).

Preserving ATP levels can thus reduce the proportion of necrotic cells following HII and increase the relative amount of apoptosis. Choi and colleagues demonstrated that following severe ischaemia, primary cell necrosis is reduced by the addition of the N-methyl-D-aspartate antagonist, MK-801. Residual neurons underwent a form of cell death with the hallmarks of apoptosis (56). On the other hand, protection from apoptosis can condemn cells to a necrotic death. Thus, cells treated with agents that reduce $\Delta_{\psi m}$ alone underwent apoptosis, whereas those kept in identical conditions in the presence of the caspase inhibitor, Z-VADfmk, died from necrosis (71).

Oxidative stress in injury to the newborn brain.

The newborn infant, particularly the preterm infant, is thought to be particularly prone to tissue damage from oxidative stress because of reduced total antioxidant capacity, and several studies have looked at the possibility of reducing morbidity by ameliorating the effects of oxidative stress in the newborn (181).

Oxidative stress results from an imbalance between the production of free radicals and the ability of the cell to combat them. Under normal conditions free radicals and reactive oxygen species (ROS) are generated during mitochondrial respiratory metabolism and are efficiently neutralised by cellular antioxidant defences (58). Mitochondrial oxidative metabolism, nitric oxide, phospholipid metabolism, and proteolytic pathways are all potential sources of intracellular free radicals and ROS. During and after hypoxia-ischaemia, a breakdown in cellular defence systems contributes to oxidative stress, with a net increase in ROS, subsequent damage to lipids, proteins, and DNA, inactivation of key cellular functions and ultimately cell death (218).

The majority of ROS formed within the cell arises as a by-product of oxygen metabolism by mitochondria. Superoxide (O_2^-) is formed during the operation of complex I and complex II in the mitochondrial electron transport chain, and at least a proportion is formed as a result of the semiquinone state of ubiquinone donating electrons to molecular oxygen. Mitochondria contain superoxide dismutase (SOD) an antioxidant enzyme that removes ROS from neurons in the brain by converting superoxide to H_2O_2, which is then catalysed to H_2O and O_2 by glutathione peroxidase and catalase.

Accumulation of ROS and oxidative stress is initiated early during hypoxia-ischaemia due to the dramatic increase in cytosolic Ca^{2+} concentration, which leads to a disturbance of mitochondrial Ca^{2+} handling (Figure 9).

Normal Ca^{2+} cycling across the inner mitochondrial membrane serves to regulate mitochondrial enzymes such as pyruvate dehydrogenase and α-oxyglutarate dehydrogenase, and requires little energy. However, when intracellular Ca^{2+} increases over the set point for net calcium accumulation or when the Ca^{2+} release pathway is stimulated by pro-oxidants, cycling may become excessive and lead to increased ROS production, loss of mitochondrial membrane potential, general leakiness of the inner mitochondrial membrane, inhibition of ATP synthesis, mitochondrial damage and cell death.

The reperfusion/reoxygenation phase immediately following hypoxia-ischaemia contributes significantly to cerebral injury. During reperfusion of the previously ischaemic brain, ROS and calcium-induced production of nitric oxide, both produced in excess with reoxygenation, can react to form highly cytotoxic oxidants. Free radicals are also produced from other sources including endothelial cell xanthine oxidase, activation of nitric oxide synthase, free fatty acid and prostaglandin metabolism, activated neutrophils and macrophages.

Mechanisms of free radical damage in the developing brain

Xanthine oxidase. Under aerobic conditions, hypoxanthine is converted to xanthine and uric acid by xanthine dehydrogenase. However, during HI, the rise in intracellular Ca^{2+} activates proteases which convert xanthine dehydrogenase to xanthine oxidase. The latter uses molecular oxygen rather than NAD^+ upon reperfusion of the tissue producing superoxide and H_2O_2. Xanthine oxidase is concentrated within endothelial cells lining the cerebral microvasculature, thus targeting the blood brain barrier for oxidative attack (13, 199)

Inhibition of xanthine oxidase with allopurinol has been shown to be neuroprotective in adult animal models of ischaemic brain injury (132). In the immature rat pretreatment with allopurinol preserved cerebral energy metabolism during hypoxia-ischaemia, and resulted in a reduction in brain oedema and in infarct volume (152, 216). However, high doses of allopurinol needed to be administered to produce these neuroprotective effects. Thus allopurinol protection could occur through mechanisms other than xanthine oxidase inhibition, such as blocking the release of neutrophil lysosomal enzymes, scavenging OH^- and chelation of transition metals.

Superoxide Dismutase. The superoxide radical is considered relatively inactive, and only exerts its cytotoxic effects when converted to more reactive oxygen species. The apoptotic death of cultured sympathetic neurons deprived of nerve growth factor (NGF) is delayed by injecting CuZn/SOD, but only if administered before the peak of ROS production (53). Furthermore, neuronal cells derived from transgenic mice that overexpress mitochondrial MnSOD, are more resistant to apoptosis induced by Fe^{2+}, amyloid β-peptide and NO generating compounds (94). Glutathione peroxidase activity levels were increased in cells overexpressing MnSOD, suggesting a compensatory response to increased H_2O_2 levels.

The situation in the developing brain may be different from that in adults: Hydrogen peroxide is selectively toxic to immature neurons *in vitro* (139). Seven day old transgenic mice overexpressing CuZn/SOD had increased infarct volume after hypoxia-ischaemia, suggesting that H_2O_2 plays a role in perinatal HII (31). No differences were observed in glutathione levels in transgenic mice compared to the wild type, consistent with an absence of H_2O_2 breakdown. In contrast, similar experiments conducted on adult mice from the same transgenic strain found no difference in infarct volume after ischaemia (22), suggesting that the enzymes responsible for the pathogenesis of brain injury may be differentially expressed in the perinatal and adult brain. Consistent with this, it has been shown that antioxidant enzymes, including SOD and glutathione peroxidase, are present at lower levels in perinatal animals (82, 194).

Nitric Oxide. Another source of toxic free radical species associated with hypoxic-ischaemic injury is nitric oxide (NO) (9). NO is produced by at least three isoforms of the enzyme nitric oxide synthase (NOS), two of which are constitutive (neuronal NOS (nNOS) and endothelial NOS (eNOS)), and an inducible form (iNOS) found mainly in phagocytic immune cells.

In endothelial cells, astrocytes and neurons, NO is rapidly generated by the constitutive enzymes in response to an increase in intracellular calcium. A late but sustained increase in NO levels may occur after hypoxia-ischaemia due to expression of iNOS (a calcium-independent isoform) within macrophages, astrocytes and invading inflammatory cells. However this may be dependent upon maturity and development as we have recently found very little calcium independent NOS activity in 14 day old rats subjected to hypoxia-ischaemia (2).

The detrimental role of NO has been demonstrated in mutant mice deficient in nNOS where infarct volumes and neurological deficits were significantly lower than

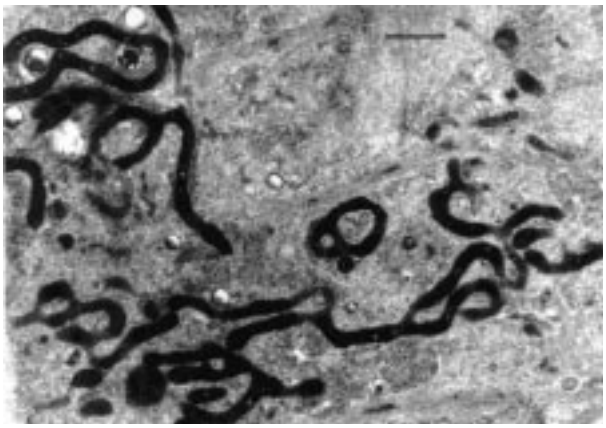


Figure 10. Electron micrograph showing mitochondrial changes in murine Swiss 3T3 fibroblasts following a two hour treatment with the NO^+ donor, sodium nitroprusside. The cytoplasm is organelle rich, but the most notable features are the mitochondria, which are uncommonly long, sinuous and electron dense. Magnification = x 13 750 (Bar represents 1 μm). Reproduced from ref. (95).

Neuroprotective Strategy	Treatment	Reference
Xanthine oxidase inhibition	Allopurinol	(80, 153)
Xanthine oxidase inhibition	Oxypurinol	(150)
Reactive oxygen species scavenging	Lazaroids	(8)
Metal chelation	Deferoxamine	(155)
Nitric oxide synthase inhibition	L-NAME	(5, 154)
Nitric oxide synthase inhibition	aminoguanidine	(70)
Nitric oxide synthase inhibition	7-nitroindazole	(70)

Table 2. Antioxidant strategies against ischaemic injury in the neonatal rat.

in controls following middle cerebral artery occlusion in adults (79). In neonatal nNOS deficient mice, a similar protective effect was observed following HII, with a reduction in hippocampal and cortical damage compared to the wild type (47).

Specific inhibitors of the nNOS (7-nitroindazole) and iNOS (aminoguanidine) provide selective protection against NO-mediated brain injury (70, 81). However, the use of non-selective inhibitors of NOS, such as L-NAME, has produced conflicting data, with some studies showing protection and others reporting increased cerebral injury. Significantly, L-NAME exerts no protection against secondary energy failure after hypoxia-ischaemia and fails to improve histological outcome (125, 126).

Nitric oxide has many potential targets to initiate neurotoxic cascades. A predominant mechanism by which NO may kill neurons is through the reaction of NO with O_2^- to generate peroxynitrite ($OONO^-$) which is directly cytotoxic. Peroxynitrite, a potent oxidising agent, induces calcium efflux from mammalian mitochondria, which can be blocked by Cyclosporin A, suggesting that peroxynitrite induces the mitochondrial permeability transition (149). Peroxynitrite has been implicated in cortical neuronal apoptosis and as an inducer of apoptosis in several cell lines. On the other hand, the peroxynitrite scavenger uric acid is protective, suggesting a central role for $OONO^-$ in oxidative stress induced apoptosis (94). At the molecular level, $OONO^-$ can nitrate or hydroxylate protein tyrosine residues, oxidise thiols or decompose to NO_2^- and OH^-. An important pathway of $OONO^-$ cytotoxicity may be $OONO^-$-mediated DNA damage and subsequent activation of PARP (229). NO is also a potent inhibitor of mitochondrial function (25).

Different NO group donors can trigger entirely different biological effects. Other redox related forms of the NO group, NO^+ and NO^- are also important in neuronal survival or destruction (109). For example, while NO reacts with O_2^- to form $ONOO^-$, which is neurotoxic, NO^+ can react with a redox modulatory site on the NMDA receptor to downregulate its activity, resulting in neuroprotection from excitotoxic insults. These effects may be cell- and insult-specific, since in mouse fibroblasts we have found that NO^+ is more toxic than NO (95) and is particularly damaging to mitochondria (Figure 10). Moreover, low concentrations of both NO and NO^+ can protect against neuronal apoptosis following NGF withdrawal (93).

Oxidative stress and apoptosis. Oxidative stress is considered to a major mediator of apoptosis in several cellular systems including neurons. Stimuli that cause oxidative stress, including culture in high oxygen (43), exposure to β-amyloid (11, 49, 116) and transient hypoxia-ischaemia (108, 122, 137, 147) all trigger apoptosis. Both H_2O_2 and NO can induce apoptosis when applied exogenously and are produced in toxic concentrations by cells that are undergoing apoptosis by other physiological stimuli.

Oxidative signals for apoptosis can originate both extracellularly and intracellularly and can also be generated by reduced concentrations of antioxidants. Indeed, a number of cerebroprotective strategies are directed at specifically reducing oxidative stress (Table 2). Thus, glutathione depletion in immature embryonic cortical neurons leads to oxidative stress and cell death by apoptosis that can be prevented by antioxidants (170). The

use of Cyclosporin A, an inhibitor of pro-oxidant-induced Ca^{2+} release from mitochondria, has demonstrated a role for mitochondrial alterations in apoptotic processes. Cyclosporin A can protect against loss of cell viability induced by pro-oxidants or by NO, can favourably alter mitochondrial function after ischaemia (57) and protect neuronal cells against apoptosis induced by Fe^{2+}, amyloid β-peptide or NO generating compounds (94).

Mitochondria and oxidative injury

While mitochondrial failure and falling ATP levels might be either the trigger or the consequence of death, it has been understood for many years that mitochondrial failure can cause necrosis, while direct inhibition of mitochondrial metabolism can also trigger apoptosis (65, 217). Precise mechanisms are beginning to be elucidated (99, 138) and much current interest is focused upon the mitochondrial membrane permeability transition.

The mitochondrial permeability transition pore. One of the possible effects of free radical damage on mitochondria was first put forward in a hypothesis by Sulachev (188) and confirmed later by direct experimentation. The hypothesis suggested that the mitochondrial megachannel or permeability transition (PT) pore is involved in preventing ROS accumulation. High concentrations of ROS in the mitochondria would trigger opening of the pore, allowing release of free radicals into the cytosol. The resulting decrease in mitochondrial ROS would subsequently allow pore closure. Conversely, persistent free radical accumulation in mitochondria would prevent pore closure and eventually lead to the release of the apoptosis-inducing proteins. In this way, cells producing excess amounts of free radicals would be eliminated by apoptosis. In favour of this hypothesis, it is clear that the mitochondrial PT pore is activated by ROS (24).

The opening of the megachannel can also be triggered independently of ROS, although the result in all cases is a sudden increase in the permeability of the inner mitochondrial membrane to small molecules (98). While transient opening of the PT pore may be involved in calcium homeostasis or free radical release, sustained PT causes uncoupling of the respiratory chain enzymes, failure of ATP generation and the release of specific apoptosis-initiating proteins such as cytochrome-c into the cytosol (see below).

Although the exact composition of the PT pore is not known, experiments using specific inhibitors of mitochondrial protein function have identified key components. For example, bongkrekic acid, a ligand of the mitochondrial adenine nucleotide translocator (ANT), abolishes mitochondrial permeability transition and inhibits p53-dependent thymocyte apoptosis induced by DNA damage (124). Similarly, the protective effects of Cyclosporin A implicate cyclophilin D as a key component of the PT pore. Other members include cytosolic hexokinase, porin (the voltage-dependent anion channel), creatine kinase and at least one member of the Bcl-2 family of proteins (99).

Bcl-2 family members and mitochondria. Bcl-2 is a mitochondrial membrane protein that blocks the apoptotic death of many cell types (72). The precise mechanism of Bcl-2 protection is unclear. There is some evidence that Bcl-2 regulates an antioxidant pathway at sites of free radical generation (67, 68, 73, 87). However, new data indicating that Bcl-2 family members prevent the mitochondrial permeability transition (203) suggest that the inhibition of free radical generation may be a secondary effect.

Consistent with this role, cytochrome-c release can be initiated by the pro-apoptotic protein Bax (189) and blocked by Bcl-2 (223). Furthermore, Bcl-XL can physically interact with another mitochondrial derived protein Apaf-1, to inhibit its association (and subsequent activation) of caspase-9 (78, 157). On the other hand in some cell types Bcl-2 cannot prevent or delay the decrease of the cellular ATP level subsequent to metabolic inhibition, suggesting that it blocks apoptosis at a point downstream of the collapse of cellular energy homeostasis (131).

Although the precise mechanism of Bcl-2 protection is a matter of some debate, it has been shown to protect neurons from apoptosis following cerebral ischaemia. Overexpression of the human Bcl-2 protein under the control of neuron-specific promoters reduced neuronal loss following permanent middle cerebral artery occlusion (130).

Mitochondrial permeability transition and cell death. In intact cells, an early consequence of mitochondrial permeability transition is a reduction in mitochondrial membrane potential, a process that can be measured using specific fluorogenic dyes (164). At later time points a disruption of outer membrane integrity leads to the release of proteins which are involved in apoptotic execution (165). The finding that supernatants from mitochondria undergoing the permeability transition can cause apoptotic changes in isolated nuclei, sup-

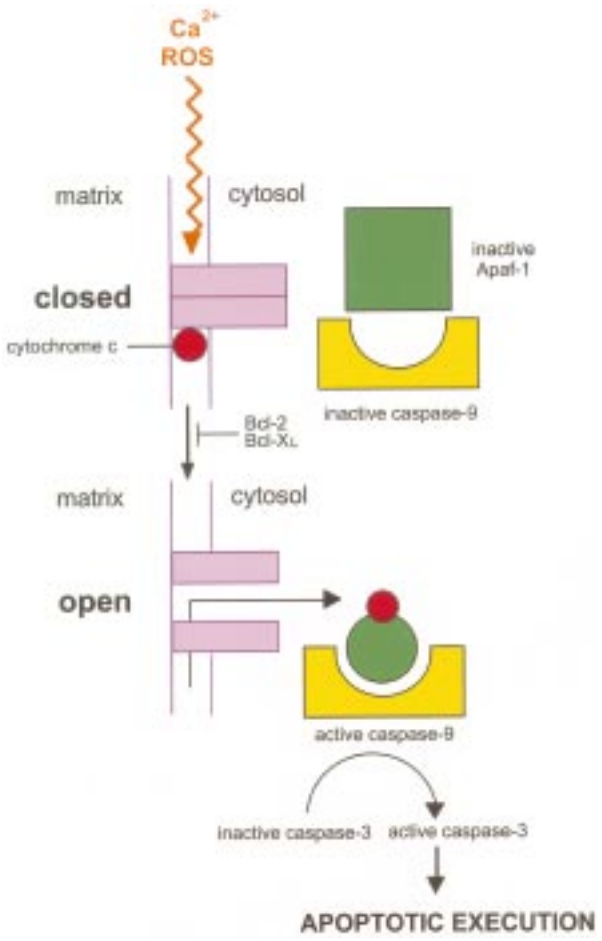


Figure 11. Schematic diagram of cytochrome c-dependent apoptosis. Accumulation of calcium ions (Ca^{2+}) and reactive oxygen species (ROS) in the mitochondria trigger the opening of the permeability transition pore (shown in pink), leading to the release of apoptogenic proteins including cytochrome c (red circle). Pore opening is inhibited by the anti-apoptotic Bcl-2 proteins. Once in the cytosol cytochrome c can form a complex with Apaf-1 and caspase-9. This results in the activation of caspase-9 which, in turn, proteolytically activates caspase-3. Active caspase-3 then triggers apoptotic execution by activating downstream caspases and endonucleases.

ports this proposal (182).

Much of the information regarding the identity of mitochondrial factors involved in apoptosis has been obtained from cell-free systems established to reproduce the apoptotic programme in vitro (40). In one such model, apoptosis could be initiated by addition of dATP but also required cytochrome c. Intact cells undergoing apoptosis showed a translocation of cytochrome c (which normally functions as an electron carrier in the respiratory chain) from mitochondria to the cytosol (110) which in turn results in the activation of specific caspases (18), proteases that are specifically involved in apoptotic execution. Among these enzymes, caspase-3 is thought to be pivotal in apoptotic execution, since cells that lack detectable levels of this enzyme fail to undergo cytochrome c dependent apoptosis (105). While caspase inhibitors substantially reduce mitochondrial membrane potential and cell death, they do not prevent the passage of cytochrome c from mitochondria to the cytosol, which must occur upstream of caspase activation.

Along with cytochrome c and dATP, Apaf-1 was been identified as an important protein that participates in the activation of caspase-3 (230). Apaf-1 shares significant homology with *Caenorhabditis elegans* CED-4, a protein that is required for apoptosis in the nematode. A third protein factor, Apaf-3, is also required for caspase-3 activation *in vitro*. Apaf-3 was identified as a member of the caspase family, caspase-9 (106). Genetic studies suggest that caspase-9 is the most upstream member of the apoptotic protease cascade and the formation of an Apaf-1/caspase-9 complex activates caspase-3 in a cytochrome c and dATP dependent manner (Figure 11).

The effects of cytochrome c translocation to the cytosol are paradoxical. While ATP is required for apoptotic execution, the mitochondrial cytochrome c deficit will eventually result in shutdown of the respiratory chain and consequently ATP synthesis will cease. Thus, Fas-driven apoptosis in Jurkat cells results in the inactivation of cytochrome c with cessation of oxygen consumption (1). However the converse is not always true; we have recently found that apoptosis induced by branched chain amino acids is preceded by a fall in oxygen consumption without cytochrome c release (85). Other reports have confirmed that apoptosis can proceed in the absence of cytosolic translocation of cytochrome c (23, 198).

Nevertheless, cytochrome-c release is an important link between mitochondria and apoptosis in at least some systems. Consistent with this concept of the mitochondria as a controller of apoptosis, mitochondrial changes precede the activation of cytosolic enzymes involved in apoptotic execution and inhibitors of the permeability transition pore can prevent apoptosis (71).

Conclusions

The developing brain is particularly vulnerable to injury following oxidative stress. Historically, necrosis was considered to be the predominant form of cell death in oxidative brain injury. However, it is now clear that both apoptotic and necrotic cell death are important components of neuronal loss. Moreover, the distinction

between apoptosis and necrosis is becoming less clear. The mode of cell death is determined by a number of cellular factors including developmental status, the severity of injury and mitochondrial function. The complexity of the events involved in oxidative damage to the brain underlines the need for therapeutic approaches that will combat multiple mechanisms of damage, and interrupt both apoptotic and necrotic processes. (90, 97, 158).

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