Astrocyte Mitochondria in In Vitro Models of Ischemia

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There is growing evidence that preservation of mitochondrial respiratory function during cerebral ischemia–reperfusion predicts the ultimate extent of tissue injury. Because neurons are selectively vulnerable to ischemic injury, many studies have focused on neuronal mitochondrial dysfunction in ischemia. However, positron emission tomography (PET) studies in animals and humans suggest that non-neuronal cells such as astrocytes may also experience mitochondrial metabolic compromise that contributes to ischemic necrosis. Astrocytes carry out a number of functions that are critical to normal nervous system function, including uptake of neurotransmitters, regulation of pH and ion concentrations, and metabolic support of neurons. Mitochondria are important for many of these actions. We have used a cell culture model of stroke, oxygen–glucose deprivation (OGD), to study the response of astrocyte mitochondria to ischemia, and to evaluate how changes in astrocyte mitochondrial function might affect neuronal survival and recovery after ischemia.

INTRODUCTION

Over the past decade, the central role that mitochondria play in diseases of the nervous system has become increasingly clear (Beal and Al, 1992; Fiskum, 2000; Wallace, 1999). Mitochondrial functional abnormalities have been linked to genetic neurological disorders and neurodegenerative diseases (Wallace, 1999). Mitochondrial dysfunction has also been implicated in ischemic injury, in which inadequate delivery of oxygen and glucose limits mitochondrial respiration. Recent studies suggest that, in fact, the degree of mitochondrial dysfunction in cerebral ischemia may be a critical determinant of the final extent of tissue injury.

Studies on mitochondria isolated from an ischemic brain suggest that ischemia–reperfusion can cause short- and long-term alterations in mitochondrial function (Hillered *et al*., 1984; Schutz *et al*., 1973; Sims *et al*., 1986; Sims, 1991). After brief focal ischemia, isolated mitochondria demonstrate defects in State 3 (ADP-dependent) and State 4 (ADP-independent) respiration. These deficits resolve after a brief period of reperfusion. Longer periods of ischemia lead to prolonged impairment in State 3 respiration (Sims, 1991). In addition, although mitochondrial metabolism normalizes soon after ischemia–reperfusion, many studies have found a secondary decline minutes to hours later (Rehncrona *et al*., 1979; Sims and Pulsinelli, 1987; Zaidan and Sims, 1994). The cause of this delayed decline is proposed to be oxidative damage to mitochondria during reperfusion (Feng *et al*., 1998; Sims and Pulsinelli, 1987), or the effect of fatty acids released during ischemia on mitochondrial function (Hillered and Chan, 1988; Sun and Gilboe, 1994). Decreased pyruvate dehydrogenase (PDH) activity is also impaired after ischemia-reperfusion (Cardell *et al*., 1989; Zaidan and Sims, 1993).

The selective vulnerability of neurons to ischemic injury has been taken as an indication that neurons experience greater metabolic deterioration than astrocytes, which are relatively resistant to ischemia injury. Astrocytes also contain glycogen stores, which are presumed to allow them to maintain ATP production through glycolysis and mitochondrial membrane potential by reversal of the F_0F_1-ATPase. Although loss of mitochondrial membrane

potential has been documented in neurons exposed to is-chemic conditions, as will be discussed, astrocytes also exhibit early mitochondrial depolarization when exposed to oxygen–glucose deprivation (OGD).

ASTROCYTE MITOCHONDRIAL DEPOLARIZATION DURING OGD

The in vitro oxygen–glucose deprivation model used for our studies has previously been described in detail (Goldberg and Choi, 1993; Bruno et al., 1994). In this model, mixed cortical astrocytic–neuronal cocultures are exposed to oxygen–glucose deprivation (OGD) for 30–60 min. Fifty percent of neurons are irreversibly injured after 45 min of OGD, with 100% neuronal death produced by 60–70 min of OGD. In contrast, astrocytes in the same cultures are resistant to OGD-induced death, requiring 4 h for irreversible injury. Previous work with this model has found that even after 60 min of OGD, ATP levels are still at 70% of control levels. In addition, benzodiazepines and barbiturates worsen neuronal cell death through ef-fects on mitochondrial function, reproducing findings in stroke patients receiving these two classes of agents, who have a worse prognosis, and suggesting that this in vitro model mimics many of the metabolic aspects of cerebral ischemia.

Using this system, we found that exposure to 45–50 min of OGD produced a 70% decrease in mitochon-drial membrane potential (ψ_m) in astrocytes, determined by confocal imaging of tetramethylrhodamine ethyl ester fluorescence (Reichert et al., 2001). This time point cor-relates with the previously reported rise in extracellular glutamate and with the onset of irreversible injury to neu-rons observed in this model (Goldberg and Choi, 1993). In our experiments, treatment with a nitric oxide synthase inhibitor ([G]N-Arg) partially blocked the decline in mito-chondrial membrane potential (ψ_m), suggesting that ni-tric oxide (NO) or peroxynitrite were involved in loss of ψ_m. Brown and Borutaite (1999) have previously found that both NO and peroxynitrite can inhibit mitochondrial electron transport chain activity at multiple sites. We were unable to differentiate between a role for NO vs. peroxyni-trite in our model, but given the low O_2 (0.2%) in the anaer-obic chamber used for these experiments, it is unlikely that an extensive amount of peroxynitrite would be present.

Astrocyte ψ_m was also preserved during OGD by treatment with cyclosporine A, indicating that opening of the mitochondrial permeability transition pore (mtPTP) is involved in the loss of astrocyte ψ_m. Assembly of the mtPTP is triggered by several stimuli, including fatty acids, accumulation of mitochondrial calcium, and ox-idative stress—events that are reported to occur during ischemia–reperfusion injury.

In the absence of OGD, astrocytes are clearly capa-ble of using ATP to maintain ψ_m, by reversal of the F_0F_1-ATPase to support ψ_m, but why astrocytes exposed to OGD fail to support ψ_m through this mechanism is not clear. Involvement of glutamate-receptor-mediated calcium en-try and direct uncoupling by Ca^{2+} are unlikely because blocking AMPA/kainate receptors failed to protect astro-cyte ψ_m (Reichert et al., 2001). Coincubation with 1% bovine serum albumin (BSA, fatty acid free) also failed to modify ψ_m loss (Reichert and Dugan, unpublished), suggesting that fatty acid release from neurons is also not likely to be involved. Loss of ψ_m may involve glutamate, acting through the transporter, or adenine nucleotides re-leased by neurons (Fig. 2). This may add to the grow-ing body of literature describing extensive communication between neurons and astrocytes (Giaume and McCarthy, 1996; Kimelberg and Norenberg, 1989; Magistretti et al., 1993).

Recovery of astrocyte ψ_m after reintroduction of O_2 and glucose was a gradual process, requiring >1 h. There were also ultrastructural changes in mitochon-drial exposed to OGD, that persisted for a relatively pro-longed period of time suggesting that OGD causes spe-cific but reversible changes in astrocyte mitochondrial physiology beyond lack of O_2 and glucose (Fig. 1). CsA decreased the extent of morphological changes. It is pos-sible that these changes may correspond to the mitochon-drial swelling and matrix alterations reported in early post-ischemic brain using electron microscopy (Petito and Babiak, 1982).

IMPLICATIONS OF ASTROCYTE MITOCHONDRIAL DYSFUNCTION

There are a number of potential downstream effects of mitochondrial depolarization. In most cell types, such prolonged loss of ψ_m would activate apoptotic pathways and result in cell death. Depolarization might also lead to loss of intramitochondrial contents, such as ADP, leading to prolonged impairment in mitochondrial respiration and ATP production. If depolarization altered the production of reactive oxygen species, such as H_2O_2, this might result in detrimental changes in intracellular and intercellular signaling through redox-sensitive pathways, such as Ras, Erk1/2, and NFκB. We have been exploring each of these potential outcomes in turn (Fig. 2).

Initiation of the Apoptotic Cascade

Activation of the mtPTP can lead to mitochondrial depolarization and is associated in many cell types with release of cytochrome c from the inner mitochondrial
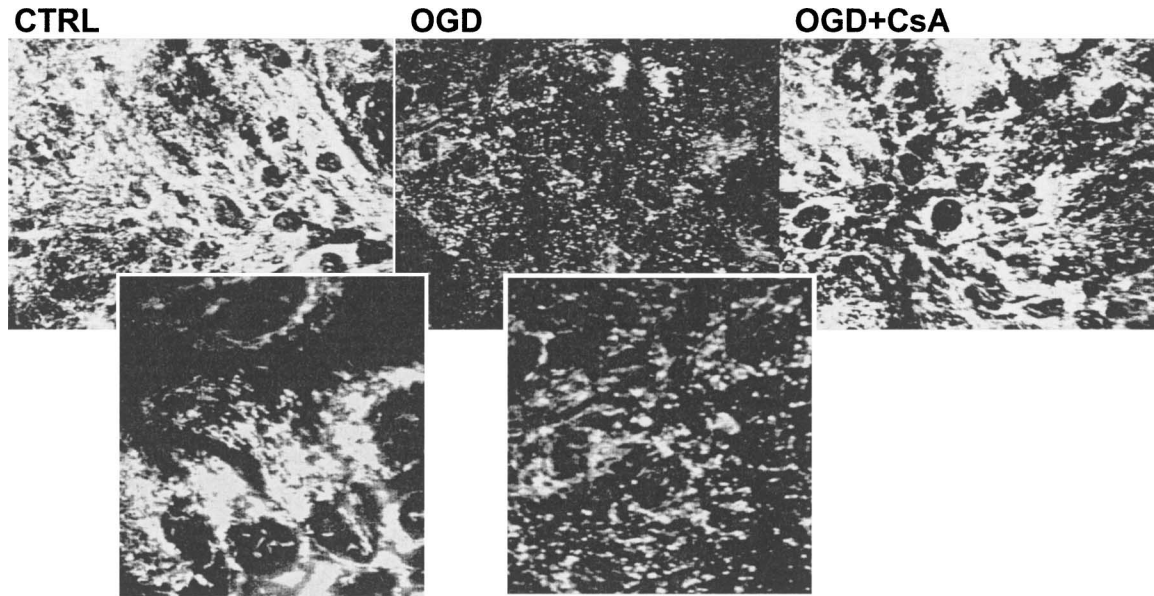
CTRL **OGD** **OGD+CsA**

Fig. 1. Fluorescence confocal images of astrocyte mitochondria after OGD. Mixed cortical cultures were loaded with TMRE (50 nM) and exposed to 50 min OGD. Panels show control, OGD, and OGD plus cyclosporine A (10 μM) conditions. Insets are at 4X magnification.

membrane (Halestrap *et al.*, 2000). The free cytochrome c may then activate caspase 9 through binding to apaf-1. Caspase 9, which resides in the mitochondrial matrix (as a pro-caspase), may be released when the barrier function of the inner membrane is lost, and can activate and work synergistically with caspase 3 to trigger downstream effectors of apoptosis (Thornberry and Lazebnik, 1998).

We observed a 20% loss of cytochrome c from mitochondria at the end of OGD that was partly blocked by CsA. However, release of cytochrome c was not accompanied by activation of either caspase-9 or caspase-3, suggesting that cytochrome c was blocked from activating the mitochondrial caspase cascade.

Decreased Energy Production and Altered Ion Homeostasis

In addition to its role in apoptosis, release of cytochrome c from mitochondria could alter mitochondrial function by hindering the efficient transfer of electrons through cytochrome aa_3 in cytochrome oxidase, enhancing upstream superoxide radical production from ubiquinone redox cycling (Wallace, 1999). Elimination of ψ_m abolishes mitochondrial Ca^{2+} uptake, and may impair many other aspects of mitochondrial metabolism, in addition to the most well known impairment of ATP production. Extensive data indicate that astrocytes are involved in a number of processes that affect neuronal survival, such as glutamate uptake, maintenance of extracellular pH and potassium, Ca^{2+} buffering, and transfer of lactate and/or

pyruvate to neurons as energy substrates (Anderson and Swanson, 2000; Forsyth, 1996; Magistretti *et al.*, 1993; Vernadakis, 1996; Walz, 2000). A number of these functions are dependent on mitochondrial membrane potential, and have been reported to be impaired early in ischemia (Benveniste *et al.*, 1984; Montgomery, 1994; Juurlink, 1997).

Altered ROS Production and Effects on Signaling

One additional effect of mitochondrial depolarization could be altered mitochondrial ROS generation. Whether ROS production would be enhanced or decreased by OGD–reperfusion is not entirely clear, and might vary with the duration of OGD, and the time after reperfusion. Mitochondria might be one source, but NADPH-depleted NOS might also contribute. Further work on the source(s) and timing of astrocyte ROS production during OGD is ongoing.

Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide radical (Dalton *et al.*, 1999; Finkel, 1999), nitric oxide, and peroxynitrite (Li *et al.*, 1998), act as signaling molecules to regulate kinase cascades, transporters, ion channels, and transcription factors. Our recent studies indicate that OGD alters activity of several signaling pathways, including MAPK (Fig. 2). To what extent changes in MAPK activity alter astrocyte and neuronal gene expression and function is an area of continuing investigation.
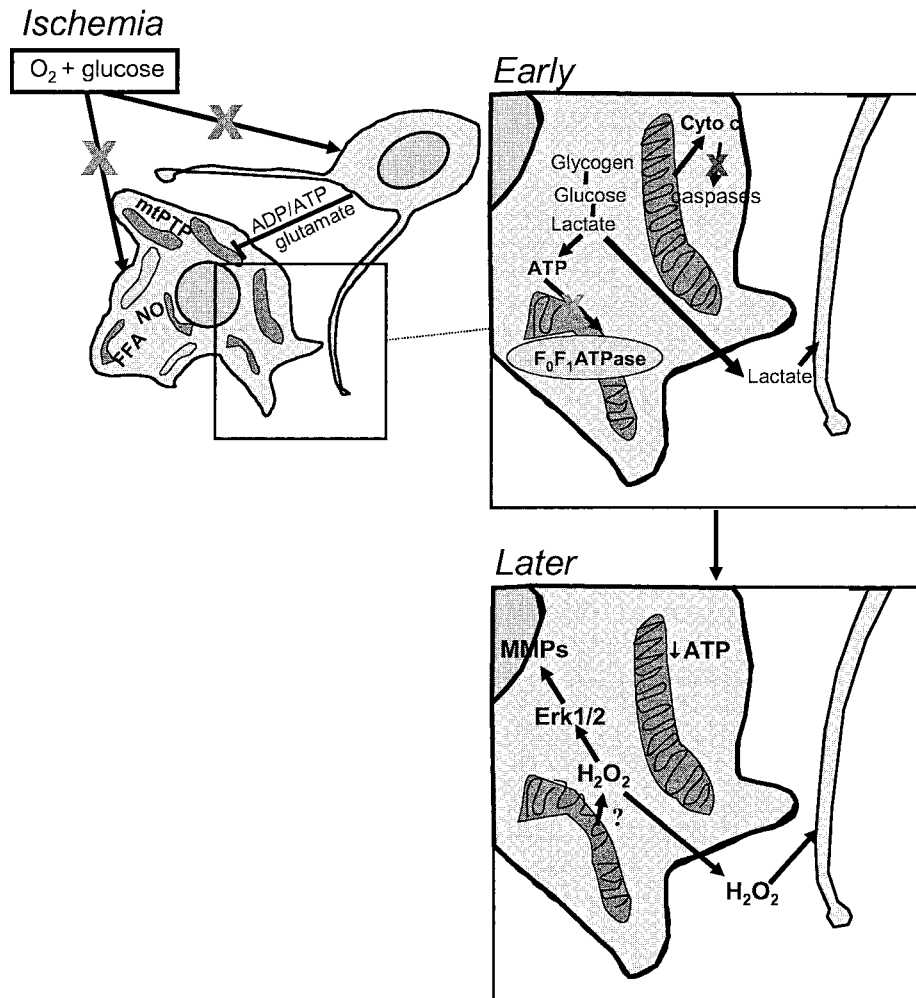
Fig. 2. Proposed sequence of astrocyte mitochondrial changes and secondary effects after OGD. (NO: nitric oxide; FFA: free fatty acid; mtPTP: mitochondrial permeability transition pore; MMP: metalloprotease) Decreased availability of O_2 and glucose is sensed by neurons and astrocytes. Astrocyte mitochondria depolarize, a process accelerated by a soluble factor from neurons (Kim-Han, unpublished), which might be glutamate through the transporter (Liao and Chen, 2003), or adenine nucleotides. Loss of ψ_m involves NO, the mtPTP (Reichert *et al.*, 2001), and possibly fatty acids (Chan *et al.*, 1988). ATP levels are preserved, but ATP is not used to support ψ_m, presumably due to blockage at the F_0F_1ATPase. This could allow shunting of lactate to metabolically compromised neurons. Cytochrome c is released but fails to activate caspases. The ratio of cytochrome c to inhibitor of apoptosis proteins (IAP)—which appears to be lower in astrocytes—might be partially responsible (Dugan and Kim-Han, unpublished). At later time points, levels of H_2O_2 in astrocytes increase, modifying redoxdependent signaling pathways, such as Erk1/2. Potential sources include the ETC or NADPH-depleted NOS. Low levels of H_2O_2 might also be released from astrocytes to alter redox-sensitive systems in neurons.

SUMMARY

Depolarization of astrocyte mitochondria during OGD might have both beneficial and harmful effects on neuronal survival. Short-term loss of ψ_m would allow astrocytes to temporarily shift use of glycogen and glucose away from aerobic metabolism to glycolysis, increasing the amount of lactate available for delivery to metabolically impaired neurons (Fig. 2). This might be tolerated for as long as astrocyte glycogen stores were available. However, prolonged loss of ψ_m in astrocytes might be expected to have injury-promoting effects during CNS ischemia. Astrocytes are involved in the normal maintenance of brain homeostasis, including several energy-dependent functions necessary for normal neuronal activity, e.g., regulation of extracellular K^+, pH, and osmolality; export of metabolic intermediates; and rapid uptake of neurotransmitters (Kimelberg and Norenberg, 1989; Magistretti *et al.*, 1993; Walz, 2000). The ability of astrocytes to maintain these functions may, in fact, be a critical determinant of neuronal survival after ischemia (Aschner *et al.*, 1999; Juurlink, 1997; Marrif and Juurlink, 1999;

Stanimirovic *et al.*, 1997). Furthermore, metabolic imaging studies have suggested that mitochondrial function in post-ischemic brain may be impaired for hours to days after the ischemic insult (Heiss *et al.*, 1997; Hoehn-Berlage, 1995; Ogasawara *et al.*, 2001; Takamatsu *et al.*, 2000; Wardlaw *et al.*, 1998). Loss of ψ_m and eventual energy failure in astrocytes might lead to an inability to provide these critical support functions during ischemia, thus exacerbating ischemic injury to neurons. Our data suggest that therapies targeted at astrocyte mitochondria might act synergistically with neuron-based strategies to provide protection to the ischemic brain.

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