Mitochondrial Glutathione: A Modulator of Brain Cell Death

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The small fraction of glutathione in mitochondria in nonneural tissues is an important contributor to cell survival under some conditions. However, there has been only limited characterization of the properties and function of mitochondrial glutathione in cells from the brain. In astrocytes in culture, highly selective depletion of this glutathione pool does not affect cell viability, at least in the first 24 h, but does greatly increase susceptibility to exposure to nitric oxide or peroxynitrite. In vivo, a selective partial loss of glutathione develops during focal cerebral ischemia and persists during reperfusion. The timing and distribution of glutathione loss shows an apparent association with the likelihood that tissue infarction will subsequently develop. Furthermore, infarct volume is greatly decreased by intracerebroventricular infusion of glutathione monoethylester, a compound that can increase mitochondrial glutathione. Together these recent findings indicate that alterations in mitochondrial glutathione are likely to contribute to the severity of tissue damage in stroke and possibly other neurological disorders. Thus, this antioxidant pool provides a potentially useful target for therapeutic intervention.

INTRODUCTION

Glutathione in cells is a major antioxidant acting both directly to remove reactive oxygen species and as a substrate for several peroxidases (Dringen, 2000). This tripeptide is also involved in other reactions including the conjugation of foreign molecules catalyzed by glutathione S-transferases. Cellular glutathione is found in two separate but interacting pools located in the cytoplasm and mitochondria. The cytoplasmic pool typically accounts for 85% or more of the total glutathione in cells (Griffith and Meister, 1985; Lash *et al*., 1998; Meister, 1995). Most, if not all, of the synthesis of glutathione occurs in the cytoplasm (Griffith and Meister, 1985; Lash *et al*., 1998; Martensson *et al*., 1990). Thus, the long-term maintenance of mitochondrial glutathione depends on transport from this site.

MITOCHONDRIAL GLUTATHIONE AND CELL VIABILITY

In many cells, glutathione in the mitochondria is much more important than the larger cytoplasmic pool in maintaining cell viability and limiting damage to various potentially toxic treatments. This key contribution of mitochondrial glutathione in preserving cell viability was first proposed by Meredith and Reed (1982, 1983) on the basis of the greatly increased death of hepatocytes when both glutathione pools were experimentally depleted compared to cells with losses of cytoplasmic glutathione alone. Subsequent studies identified similar responses in other types of cells and further showed that depletion of glutathione from the mitochondria was associated with much greater dysfunction and loss of viability in cells challenged with a range of oxidative stresses and other insults (Colell *et al*., 1998; Colell *et al*., 2001; Dhanboora and Babson, 1992; Fernandez-Checa *et al*., 1991; Shan *et al*., 1993; Wullner *et al*., 1999). In many

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of the relevant investigations, the deleterious response to mitochondrial glutathione depletion was demonstrated on a background of cytoplasmic glutathione loss making it difficult to unequivocally evaluate the contribution of the mitochondrial pool alone. Selective partial depletion of mitochondrial glutathione has been achieved without accompanying changes in the cytoplasmic pool in only a few studies. Such cells also exhibited substantially decreased viability in response to substances including *t*-butylhydroperoxide (Fernandez-Checa *et al*., 1991) and tumor necrosis factor (Colell *et al*., 1998; Colell *et al*., 2001) providing more direct evidence of the essential role of mitochondrial glutathione in preserving cell function.

These findings point to the potential for mitochondrial glutathione changes to contribute to tissue dysfunction and damage in disease states. Partial losses of mitochondrial glutathione develop in vivo under some conditions that include long-term ethanol feeding (Fernandez-Checa *et al*., 1991) and liver ischemia with reperfusion (Grattagliano *et al*., 1999). Glutathione loss induced in the liver by ethanol feeding is restricted to the mitochondrial pool. It results from a decreased glutathione uptake into mitochondria due to changes in the fluidity of the mitochondrial membranes (Coll *et al*., 2003; Colell *et al*., 1998; Colell *et al*., 2001; Fernandez-Checa *et al*., 1991). This partial loss of glutathione increases the susceptibility of the liver cells to several potentially toxic treatments (Colell *et al*., 1998; Colell *et al*., 2001; Fernandez-Checa *et al*., 1991; Zhao *et al*., 2002).

INTERACTIONS OF MITOCHONDRIAL AND CYTOPLASMIC GLUTATHIONE

The mechanisms of glutathione uptake into mitochondria and the control of this process have mostly been investigated in organelles isolated from the liver and kidney. The relevance of the findings to other tissues including the brain remains to be established. Gluathione carries a net negative charge. Its transport into mitochondria involves movement against the charge gradient across the inner mitochondrial membrane and therefore requires either energy or exchange with another anion. In kidney mitochondria, the 2-oxoglutarate and dicarboxylate transporters appear to be major contributors to glutathione uptake (Chen and Lash, 1998; Lash *et al*., 2002). Transport into liver mitochondria shows some similarities but also significant differences in properties (Coll *et al*., 2003; Martensson *et al*., 1990; Meister, 1995), suggesting tissue specific characteristics. The 2-oxoglutarate transporter again seems to be involved (Coll *et al*., 2003).

Although the mitochondria have an ongoing reliance on the cytoplasm for the supply of glutathione, these two pools can be separately modulated under some conditions. The glutathione synthesis inhibitor, buthionine sulfoximine, has been found in many different types of cells to substantially deplete cytoplasmic glutathione while having little initial impact on the mitochondrial pool (Martensson *et al*., 1989; Meister, 1995). Indeed, treatment with this compound in vivo typically causes major losses of the cytoplasmic pool within a few hours in a range of tissues but only produces slow depletion of mitochondrial glutathione over many days or weeks. Partial selective losses of the mitochondrial glutathione pool also develop in response to some treatments of cells in vitro (Colell *et al*., 1998; Colell *et al*., 2001; Shan *et al*., 1993) and in disease models in vivo (Anderson and Sims, 2002; Fernandez-Checa *et al*., 1991; Wallin *et al*., 2000). Thus, under the conditions examined in these studies, the transport processes do not replenish glutathione content in the mitochondria even though cytoplasmic glutathione is preserved.

MITOCHONDRIAL GLUTATHIONE IN CELLS FROM BRAIN

Despite the evidence for the important roles of mitochondrial glutathione in nonneural cells, surprisingly little attention has been given to the properties and functions of this antioxidant pool in cells from the central nervous system. Cerebellar granule neurons in culture exhibit marked functional deterioration and die in response to complete loss of both the mitochondrial and cytoplasmic glutathione but not with cytoplasmic glutathione loss alone (Wullner *et al*., 1999). Thus, these neurons exhibit a similar dependence on mitochondrial glutathione to other cell types. Complete depletion of glutathione in motor neurons (Rizzardini *et al*., 2003) and in astrocytes in culture (Huang and Philbert, 1996) also promotes cell dysfunction and death, although these studies did not relate changes in cell vulnerability to the responses of the individual glutathione pools.

We have recently established conditions based on ethacrynic acid treatment of cortical astrocytes in culture that result in complete loss of mitochondrial glutathione while leaving the cytoplasmic pool essentially unchanged. Ethacrynic acid has been widely used to produce total glutathione depletion in various cells, including neurons and astrocytes in culture. This compound is conjugated with glutathione in both the cytoplasm and mitochondria in reactions catalysed by some glutathione S-transferases. In neurons and astrocytes (Huang and Philbert, 1996;

Wullner *et al.*, 1999), but not in some nonneural cells (Meredith and Reed, 1982), ethacrynic acid produces more rapid depletion of the glutathione in the mitochondria than in the cytoplasm. This differential selectivity has not previously been exploited experimentally. Indeed, as far as we are aware, there are no previous reports for any cell type in which comparable selectivity of mitochondrial glutathione depletion has been achieved. Thus, this preparation provides a valuable tool for assessing the function of mitochondrial glutathione in a major population of cells derived from the brain.

Astrocytes with depleted mitochondrial glutathione showed no change in viability compared with nondepleted cells when incubated under normal conditions for 24 h. However, the glutathione-depleted astrocytes were much more susceptible to exposure to the peroxynitrite donor, 3-morpholinosydnonimine (Sin-1) and to nitric oxide (Muyderman *et al.*, 2004; Muyderman *et al.*, submitted). The cells treated with Sin-1 exhibited earlier and larger changes in cell function compared with astrocytes with preserved mitochondrial glutathione and also showed a substantial increase in cell death based on lactate dehydrogenase release and propidium iodide staining at both 3 and 24 h after treatment.

Interestingly, the glutathione content of the mitochondria recovered only partially over several hours following ethacrynic acid treatment despite the ongoing availability of glutathione in the cytoplasm. Thus, these cells showed much less rapid restoration of glutathione content than expected based on studies of isolated mitochondria from other tissues. This finding suggests either that the glutathione transport exhibits different properties in these cells or it has been modified as a result of the ethacrynic acid treatment. A slow recovery of partially depleted mitochondrial glutathione despite a preserved cytoplasmic pool has also been seen following treatment of COS cells with 4-hydroxynonenal (Raza *et al.*, 2002). This compound is also conjugated in a reaction catalyzed by glutathione S-transferase raising the possibility that the products of such reactions might interfere with glutathione uptake into the mitochondria.

Mitochondrial glutathione in the astrocytes treated with ethacrynic acid could be fully replenished using glutathione monoethylester as a precursor. Importantly, such restoration of the mitochondrial glutathione blocked the increased cell death resulting from exposure to Sin-1, clearly implicating the glutathione loss (and not some other consequence of ethacrynic acid treatment) as the basis for the greater vulnerability of these cells. The cell loss was also blocked by incubations with cyclosporin A, suggesting a role for induction of the mitochondrial permeability transition in the increased susceptibility of glutathione-depleted astrocytes. The oxidation of specific protein sulfhydryls promotes induction of the permeability transition (Kowaltowski *et al.*, 2001), providing a likely link between glutathione depletion and this deleterious mitochondrial change.

TISSUE DAMAGE IN STROKE: A ROLE FOR MITOCHONDRIAL GLUTATHIONE DEPLETION?

In keeping with the relatively limited investigations of the role of mitochondrial glutathione in the central nervous system, there have been very few attempts to address whether mitochondrial glutathione is altered in neurological disorders. Transient selective depletion of glutathione in the mitochondria develops as an early change in a model of neonatal hypoxia-ischemia (Wallin *et al.*, 2000). We have also identified decreases in this antioxidant pool in a model of stroke in adult rats and provided evidence suggesting that this change could contribute to the tissue damage that develops in this major neurological disease. These changes and their possible implications for the pathology associated with stroke are briefly reviewed below.

Stroke in humans most commonly arises from blockage of an intracerebral artery. Although this occlusion is usually long-lasting or permanent, temporary occlusion is also seen in a subgroup of patients, particularly with recent increased use of thrombolytic agents to reverse the arterial blockage. The focal cerebral ischemia resulting from arterial occlusion typically produces immediate functional impairment. Unless the occlusion is reversed within the first hour or so, changes are initiated that lead over many hours to tissue infarction due to the death of all cell types within parts of the perfusion territory of the affected vessel. The location and volume of infarcted tissue are important determinants of the long-term symptoms of stroke. Thus, there has been considerable effort in recent years to elucidate the molecular events contributing to the ischemia-induced cell loss with a view to identifying therapeutic targets to limit tissue damage and improve outcome (Lipton, 1999; Sims and Anderson, 2002; Zheng *et al.*, 2003). These studies have provided evidence that interactions of multiple deleterious changes are required for cell death, with the exact mechanism being greatly influenced by factors including the severity of the ischemia and whether arterial occlusion is permanent or temporary.

Changes in mitochondrial function are one of the factors likely to contribute to ischemic cell death under at least some conditions (Sims and Anderson, 2002). The most direct mitochondrial effects arise from the reduced

delivery of oxygen to the tissue. The resultant impairment of the electron transport chain contributes to marked disruption of ATP and related metabolites in the most severely ischemic "core" or "focal" tissue within the perfusion territory of the affected vessel and also to lesser changes in surrounding perifocal tissue that is subjected to more moderate reductions in blood flow (Folbergrova et al., 1992, 1995). Many of these metabolite changes substantially recover on reperfusion even in tissue destined to become infarcted but lactate often remains elevated, suggesting ongoing mitochondrial impairment. Cell susceptibility may be further compromised by direct decreases in mitochondrial capacity for respiratory function which develop during ischemia and reperfusion (Anderson and Sims, 1999; Kuroda et al., 1996; Nakai et al., 1997). Increased production of nitric oxide and its derivatives has been strongly implicated as a contributor to tissue damage in stroke (Eliasson et al., 1999). One deleterious effect of these substances could result from their ability to inhibit the electron transport chain (Radi et al., 2002). More direct support for a mitochondrial contribution to cell death is provided by the substantial protection achieved using a mitochondrial potassium channel opener in both permanent and temporary ischemia (Liu et al., 2002; Shimuzu et al., 2002) and also by inhibitors of the mitochondrial permeability transition administered near the time of reperfusion in temporary ischemia (Matsumoto et al., 1999; Yoshimoto and Siesjö, 1999).

We have identified a partial loss of glutathione in mitochondria isolated from ischemic brain regions in a rat model of stroke (Anderson and Sims, 2002). This change persisted for at least several hours of reperfusion. The glutathione losses during ischemia were not accompanied by changes in total tissue glutathione and were only seen with ischemic periods sufficient to induce subsequent infarction. Indeed, the time at which mitochondrial glutathione loss was first detected during ischemia in focal tissue from the striatum and cortex and in cortical perifocal tissue corresponded with the ischemic times typically required to initiate infarct formation in these same regions. This association is consistent with the mitochondrial glutathione loss being one factor contributing to the tissue damage, perhaps by providing conditions promoting induction of the permeability transition or other deleterious mitochondrial changes.

The glutathione depletion in ischemia was temporarily blocked or reversed by a single intracerebral injection of glutathione monoethylester but this treatment did not modify infarct volume (Anderson et al., 2004b). However, more prolonged treatment with this glutathione ester via intracerbroventricular infusion initiated at the time of temporary arterial occlusion reduced infarct volume by more than 60% (Anderson et al., 2004a). Further studies are needed to demonstrate that mitochondrial glutathione is indeed contributing to this protective response and to identify the critical period(s) during which the glutathione ester is protective. Nonetheless, the results obtained so far are consistent with mitochondrial glutathione loss being an important factor in the vulnerability of cells in ischemic and postischemic brain.

IMPLICATIONS FOR NEUROPROTECTION

The findings from investigations of the brain in vivo and of cell populations derived from this tissue are consistent with a key role for mitochondrial glutathione in promoting cell viability under some pathological conditions. Additional studies are needed to better understand the normal controls influencing the content of glutathione in brain mitochondria and to further evaluate the possible contribution of mitochondrial glutathione changes in stroke and other brain disorders. Findings to date indicate that treatments to replenish or increase this endogenous antioxidant pool might limit cell death in some brain disorders and could possibly be useful as a prophylactic treatment in situations (such as major surgery) that are associated with an increased risk of brain damage.

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