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Effect of Overexpression of Protective Genes on Mitochondrial Function of Stressed Astrocytes

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Antiapoptotic members of the Bcl-2 family have been shown to reduce ischemic brain injury in vivo and in vitro. Understanding early changes in respiration are important in understanding the cells response to stress and the mechanisms of protection afforded by overexpression of protective genes. This mini-review summarizes current knowledge regarding early responses of astrocytes to ischemia-like stress and the effects of overexpression of protective Bcl-2 family genes on astrocyte mitochondrial function. Overexpression of Bcl-x_L improves mitochondrial respiratory function, normalizes mitochondrial membrane potential, and reduces production of free radicals early after the imposition of a stress in primary cultured murine astrocytes.

KEY WORDS: Astrocytes; Bcl-2; Bcl-x_L; cytochrome *c*; mitochondria; mitochondrial membrane potential; reactive oxygen species.

Mitochondria are central to both normal cell function and the regulation of cell death. Within the brain astrocytes are crucial for neuronal metabolic, antioxidant, and trophic support, as well as normal synaptic function. In the setting of stress, such as during cerebral ischemia, astrocyte dysfunction may compromise the ability of neurons to survive. Despite their central importance, the response of astrocyte mitochondria to stress has not been extensively studied. Limited data already suggest clear differences in the response of neuronal and astrocytic mitochondria to oxygen–glucose deprivation. Measurement of mitochondrial enzymatic activity by Almeida *et al.* (2002) demonstrated oxygen glucose deprivation (OGD) induced mitochondrial dysfunction in neurons at durations that left astrocyte activity intact. Loss of astrocyte mitochondrial membrane potential with OGD was not associated with irreversible injury, but required about 1 h to recover once oxygen and glucose were restored (Reichert *et al*., 2001).

Prominent mitochondrial alterations during stress that can contribute to cell death include reduced ATP production, increased production of reactive oxygen species (ROS), and release of death regulatory and signaling molecules from the intermembrane space. In response to stress mitochondrial respiratory function and membrane potential can also change, and these changes depend in part on cell type. Bcl-2 family proteins are the best-studied regulators of cell death, and mitochondria are a major site of action for these proteins (Gross *et al*., 1999). The Bcl-2 family is divided into antiapoptotic members including Bcl-2 and Bcl-x_L and proapoptotic members such as BAX and BID (Adams and Cory, 1998; Tsujimoto, 1998; Tsujimoto and Shimizu, 2000a,b). Although much data supports the role of Bcl-2 family proteins in the regulation of some of these mitochondrial alterations, this remains an area of active investigation.

To better understand the ability of Bcl-x_L to protect astrocytes we examined mitochondrial function early after the imposition of oxidative stress. We previously reported that overexpression of Bcl-x_L increases astrocyte survival after both hydrogen peroxide exposure and glucose deprivation (GD) injury (Xu *et al*., 1999a). Here we describe our recent findings on the effects of Bcl-x_L on accumulation of reactive oxygen species (ROS), cell respiration, and

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Key to abbreviations: GD, glucose deprivation; OGD, oxygen glucose deprivation; ROS, reactive oxygen species; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TMRE, tetramethylrhodamine ethylester.

Table I. Effect of Bcl-x_L Overexpression on Astrocyte Mitochondrial Membrane Potential With Glucose Deprivation

	Control, −G	Lac-Z, −G	Bcl-x_L, −G	Control, +G
0 h GD	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.01
3 h GD	1.25 ± 0.03*	1.26 ± 0.04*	0.98 ± 0.03#	1.00 ± 0.01#
5 h GD	0.84 ± 0.01*	0.80 ± 0.02*	0.96 ± 0.02#	0.41 ± 0.01[a]

Note. TMRE mitochondrial fluorescence with glucose deprivation (−G) in uninfected, Lac-Z, and Bcl-x_L overexpressing astrocytes was normalized to the basal fluorescene for each cell at the start of the experiment. Values are means ± SD of at least 50 astrocytes per conditon. # indicates $P < 0.05$ compared with the Lac-Z control at the same time, * indicated $P < 0.05$ compared with 0 h same condition by ANOVA followed by Scheffe's test.

[a] CCCP (5 μM) was used to induce a collapse of mitochondrial membrace potential in control cultures not deprived of glucose (+G).

mitochondrial membrane potential in astrocytes exposed to hydrogen peroxide or GD. Overexpression of Bcl-x_L or a control gene was achieved in primary astrocyte cultures from mouse cortex using retroviral vectors (Xu *et al.*, 1999a). This resulted in cultures in which essentially all the cells express the gene of interest.

Tetramethylrhodamine ethylester (TMRE), a potentiometric fluorescent dye that incorporates into mitochondria was used at 100 nM to determine the time course of changes in mitochondrial membrane potential (Ouyang *et al.*, 2002). Distinct changes in astrocyte mitochondrial membrane potential were observed in response to H_2O_2 exposure as compared to GD. H_2O_2 induced a decrease in mitochondrial membrane potential (Ouyang *et al.*, 2002), while GD caused an initial increase (at about 3 h, Table I, Fig. 1) followed by a decrease (between 4 and 5 h) (Ouyang *et al.*, 2002). Although both injuries involve oxidative stress, peroxide exposure may more rapidly damage oxidation sensitive mitochondrial proteins. Both the transient increase in the mitochondrial membrane potential after 2–3 h of glucose deprivation, and subsequent decrease in mitochondrial membrane potential were prevented by Bcl-x_L overexpression (Fig. 1) (Ouyang *et al.*, 2002).

Oxygen consumption was measured in cortical astrocytes after increasing durations of GD (Ouyang and Giffard, 2003) using a modification of the method of Fiskum and colleagues (Fiskum *et al.*, 2000; Moreadith and Fiskum, 1984). Astrocytes were permeabilized by adding 0.01% digitonin and state III respiration was initiated by adding 0.4 mM ADP. Oligomycin (2 μg/mL) was used to initiate state IV respiration and the uncoupled rate was determined by addition of carbonylcyanide *m*-chlorophenylhydrazone (CCCP, 0.1 μM). State III respiration decreased significantly as early as 3 h after removal of glucose (Table II). At this time point state IV

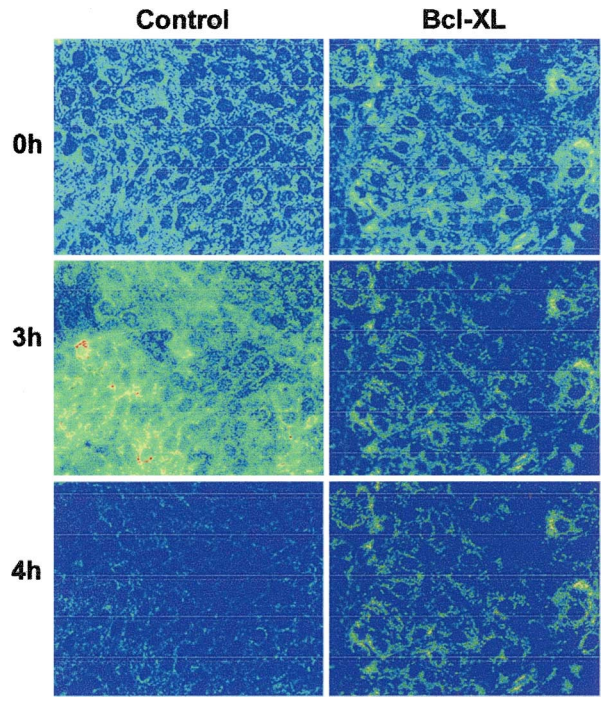

Fig. 1. TMRE was used to image mitochondrial membrane potential in astrocytes subjected to GD. Pseudocolor images at 0 h (upper panel), 3 h (middle panel), and 5 h (lower panel) of control uninfected (left column) and Bcl-x_L overexpressing (right column) astrocytes are shown.

respiration and uncoupled respiration did not change. After 5 h of GD state IV respiration increased significantly and state III respiration declined further. In contrast, uncoupled respiration did not change much compared with 0 h GD. Although overexpression of Bcl-x_L did not change basal respiratory rates (Ouyang and Giffard, 2003), when astrocytes were stressed, Bcl-x_L overexpression prevented

Table II. Mitochondrial Respiration of Astrocytes During Glucose Deprivation

	State III	State IV	Uncoupled
Lac-Z			
O h GD	25.2 ± 3.1	5.1 ± 0.4	35.9 ± 3.6
3 h GD	18.6 ± 2.3*	4.9 ± 0.3	34.8 ± 4.2
5 h GD	16.4 ± 1.2*	8.1 ± 0.5*	31.1 ± 3.3
Bcl-X_L			
0 h GD	27.8 ± 2.5	5.5 ± 0.3	38.9 ± 2.5
3 h GD	29.9 ± 1.6#	5.7 ± 0.3#	40.4 ± 2.6
5 h GD	28.3 ± 2.3#	6.9 ± 0.4*#	35.7 ± 2.1

Note. Values are means ± SD nmol O_2/min/mg protein. # indicates $P < 0.05$ compared with the Lac-Z control at the same time, * indicates $P < 0.05$ compared with 0 h same condition by ANOVA followed by Scheffe's test.

the decrease in state III respiration and moderated the increase in state IV respiration (Table II).

Using the ROS-sensitive fluorescent dye hydroethidine, we recently demonstrated that with GD or H_2O_2, cultured astrocytes showed immediate and rapid increases in ROS accumulation which were markedly reduced by overexpressing Bcl-x_L (Ouyang *et al*., 2002). We previously found that peroxide exposure was associated with an increase in intracellular calcium as measured with Fura-2 (Ouyang *et al*., 2002). However, the improved survival with Bcl-x_L overexpression at 400 μM peroxide did not correlate with a reduction in the increase in intracellular calcium observed (Ouyang *et al*., 2002).

Fluorescence immunocytochemistry was performed to detect cytochrome *c* release (Ouyang and Giffard, 2003). At 3 h GD cytochrome *c* is localized in mitochondria while at 5 h about 1/4 of control astrocytes showed an evenly distributed immunostaining pattern demonstrating release of cytochrome *c* from the mitochondria to the cytosol. Mitochondrial morphology changed from elongated to punctate in those cells. We observed that Bcl-x_L overexpression prevented loss of cytochrome *c* from mitochondria and the change in mitochondrial morphology (Ouyang and Giffard, 2003). We assessed cell death at these times by staining with Hoechst dye 33258 and propidium iodide. While essentially no cells stained with propidium iodide at the beginning of the experiment, at 3 h of GD $4.2 \pm 0.1\%$ and after 5 h GD $15.4 \pm 0.4\%$ of the control cells stained with propidium iodide. This suggests that the control-injured cells releasing cytochrome *c* are dead or dying. GD induced astrocyte death is more rapid in cells being imaged than in sister cultures maintained in the dark.

We conclude that in addition to the well-established ability of the antiapoptotic Bcl-2 family members to block release of apoptotic factors from mitochondria, Bcl-x_L also improves mitochondrial respiratory function, normalizes membrane potential, and reduces production of free radicals by astrocytes subjected to oxidative stress. These improvements in astrocyte mitochondrial function may in part explain the ability of Bcl-x_L overexpressing astrocytes to protect wild type neurons co-cultured with them against GD and OGD (Xu *et al*., 1999b).

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