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OVEREXPRESSION OF Bcl-xl PROTECTS SEPTAL NEURONS FROM PROLONGED HYPOGLYCEMIA AND FROM ACUTE ISCHEMIA-LIKE STRESS

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Abstract—Overexpression of Bcl-xl, a member of the Bcl-2 protein family, is reported to protect from a variety of stresses involving delayed cell death. We tested the ability of Bcl-xl overexpression to protect primary cultures of embryonic rat septal neurons subjected to one of four different stresses: 6 h of combined oxygen–glucose deprivation, which produces rapid cell death, or a 24 h exposure to hypoglycemia, hyperglycemia, or 1 mM 3-nitropropionic acid (an inhibitor of mitochondrial respiration), which results in a more slowly-developing death. Prior to the stress neurons were transiently transfected to overexpress either green fluorescent protein only or green fluorescent protein along with wild-type Bcl-xl. Immediately after oxygen–glucose deprivation, many neurons expressing green fluorescent protein only showed process blebbing and disintegration, with only 49% of the initial cells remaining intact with processes. Neurons expressing both green fluorescent protein and Bcl-xl showed less damage (68% intact post-stress, $P<0.05$). This result indicates that Bcl-xl's saving effects are not due solely to blocking delayed (apoptotic) death, because death following oxygen–glucose deprivation was rapid and was not accompanied by increased activation of caspase-3. Bcl-xl expression also significantly protected against the hypoglycemic stress (23% intact 24 h post-stress with green fluorescent protein only, compared with 70% with Bcl-xl and green fluorescent protein), but did not protect from hyperglycemia or 3-nitropropionic acid. Thus Bcl-xl does not protect against all forms of delayed death. Bcl-xl's protective effects may include blocking early damaging events, perhaps by increasing mitochondrial function in the face of low levels of energy substrates. Bcl-xl's protective effects may require an intact electron transport chain.

Key words: Bcl-xl, hypoglycemia, hyperglycemia, ischemia, transfection, neuron.

Neurons are sensitive to reduced oxygen/glucose levels because they have high metabolic rates and little energy reserve. Mitochondria generate most of the energy consumed by neurons. Disturbances of normal mitochondrial function can reduce neuronal survival both because of the resulting ATP depletion and via release of apoptotic factors from mitochondria. Mitochondrial release of apoptotic factors is influenced by the Bcl-2 family of proteins, which includes proteins favoring apoptosis (Bax, Bak, BAD, BIM, Bid) as well as proteins that are usually anti-apoptotic (Bcl-2, Bcl-xl) (reviewed by Danial and Korsmeyer, 2004). The Bcl-x gene is a member of the Bcl-2 family and gives rise to two proteins, Bcl-xl and Bcl-xs, via alternative mRNA splicing (Boise et al., 1993). Bcl-xl protein, like Bcl-2, inhibits delayed cell death (or apoptosis), whereas Bcl-xs can promote apoptosis (Boise et al., 1993).

While Bcl-xl is expressed at low levels during early embryonic development, its expression level increases during development and remains high in the adult nervous system (Gonzalez-Garcia et al., 1994; Merry et al., 1994). Stresses for which Bcl-xl has been shown to have anti-apoptotic effects include fas-mediated apoptosis (Zhang et al., 1996), ischemia (Cao et al., 2002; Huang et al., 2003), oxidative stress (Cherbonnel-Lasserre and Dosanjh, 1997) neurotrophin deprivation (Vander Heiden et al., 1999), excitotoxicity (Matsuoka et al., 2002), phosphatase inhibitor-induced stress (Nonner et al., 2004) and glucocorticoid-induced stress (Chao et al., 1995). However, Bcl-xl has only mild or no protective effects following other stresses including H_2O_2-induced cell death in SH-SY5Y neuroblastoma cells (Luetjens et al., 2001), lactacystin-induced cell death in U937 monocytic leukemia cells (Abdelhaleem, 2002), and ceramide-induced death in D283 medulloblastoma cells (Poppe et al., 2002).

While the mechanism(s) of Bcl-xl's protective action is as yet unknown, it is hypothesized that Bcl-xl binds to BAX (a pro-apoptotic protein) and prevents BAX from translocating from the cytosol to the mitochondria. BAX is thought to form a channel in the outer mitochondrial membrane and thereby release pro-apoptotic molecules including cytochrome c and caspase-3 (Henshall et al., 2002). Bcl-xl is also known to complex with the pro-apoptotic BAD. Upon phosphorylation on Ser^{133}, BAD detaches from Bcl-xl and complexes with 14-3-3 proteins (Zha et al., 1996, 1997) and the released Bcl-xl exerts its protective effects. Bcl-xl also has other effects that can protect cells, including upregulation of anti-oxidant molecules glutathione and superoxide dismutase (Steinman, 1995), and regulation of the mitochondrial voltage-dependent anion channel (VDAC) (Degterev et al., 2001). In non-neuronal cells such as FL5.12 hematopoietic cells, Bcl-xl protects from growth factor deprivation by increasing cellular ATP levels or by sustaining an optimal level of mitochondrial ATP (Vander Heiden et al., 1999). Thus, in addition to Bcl-xl's protective effects via the classical anti-apoptotic pathways, it may

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Abbreviations: GFP, green fluorescent protein; OGD, oxygen–glucose deprivation; PI, propidium iodide; VDAC, voltage-dependent anion channel; 3-NP, 3-nitropropionic acid.

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also protect cells more directly by regulating mitochondrial function.

In the present study we tested the protective effects of Bcl-xl during several stresses including both acute and prolonged energy stress and stress involving inhibition of the mitochondrial electron transport chain. We show that overexpression of Bcl-xl can protect neurons from acute oxygen–glucose deprivation (OGD) and also from delayed death induced by prolonged hypoglycemia. However, Bcl-xl did not protect from damage induced by hyperglycemia or 3-nitropropionic acid (3-NP), which inhibits succinate dehydrogenase (complex II of the electron transport chain, Beal et al., 1993).

EXPERIMENTAL PROCEDURES

Neuronal cell culture

Neuron-rich cultures were prepared as described in Nonner et al. (1996). Briefly, septal regions were dissected from embryonic day 15 (E15) Sprague–Dawley rats, dissociated by gentle trituration and plated in Neurobasal medium supplemented with an acid-stable 55 kDa fraction of horse serum (1 mg protein/ml, Kaufman and Barrett, 1983) which contains selenoprotein-P (Yan and Barrett, 1998). Cultures were plated in poly-L-lysine-coated Nunc Terasaki microwell plates. Cells were maintained at 37 °C in 95% O_2/5% CO_2. Procedures followed guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the local animal care committee (IACUC) of the University of Miami, Miami, FL. For preparation of neuronal cultures, pain and suffering of animals were minimized by use of anesthetics as approved by IACUC. The minimum number of animals required to achieve statistical significance was used for the study.

Transient transfection

Plasmids for green fluorescent protein (GFP) (pIRES-2EGFP, Clontech, Palo Alto, CA, USA) and Bcl-xl (pGL3, gift from Dr. Larry Boise, University of Miami, FL, USA; Boise et al., 1995) were amplified using XL1-blue cells (Stratagene, La Jolla, CA, USA). Plasmid DNA was extracted using a QIAgen Plasmid kit (Qiagen, Valencia, CA, USA) and DNA was quantitated using CyQuant (Molecular Probes, Eugene, OR, USA). Neurons (10–12 days *in vitro*) were co-transfected with plasmids containing cDNA for GFP along with Bcl-xl using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, with plasmid DNA concentration not exceeding 0.05 mg/1000 cells. Following transfection cultures were washed with medium 24 h later and then fed with a mixture (1:1) of normal medium and neuronal conditioned medium. Stress experiments were conducted 48 h following transfection. The transfection efficiency was approximately 5% and all transfection experiments were repeated with multiple platings.

Imaging of transfected neurons

Transfected neurons containing GFP were imaged using a Leica DMIRBE fluorescence microscope equipped with a monochromator, Hamamatsu ER CCD camera and Prior electrical stage, using low levels of excitation light. An incubator chamber on the microscope maintained CO_2 and temperature during imaging. The use of microwell cultures together with a computer-controlled microscope stage enabled us to follow the same transfected cells over 72 h. Programs for automated collection of fluorescence images utilized macros written in V++ (Digital MicroOptics/Roper Inc., Brown Bay, Auckland, NZ) and Image Pro Visual Basic (Media Cybernetics/Roper).

GFP-transfected neurons were imaged before, immediately after stress termination, and 24–48 h after stress termination. In the absence of any stress, transfected cells maintained normal morphology with a well-defined cell body and intact neuronal processes. Survival of transfected neurons was determined by

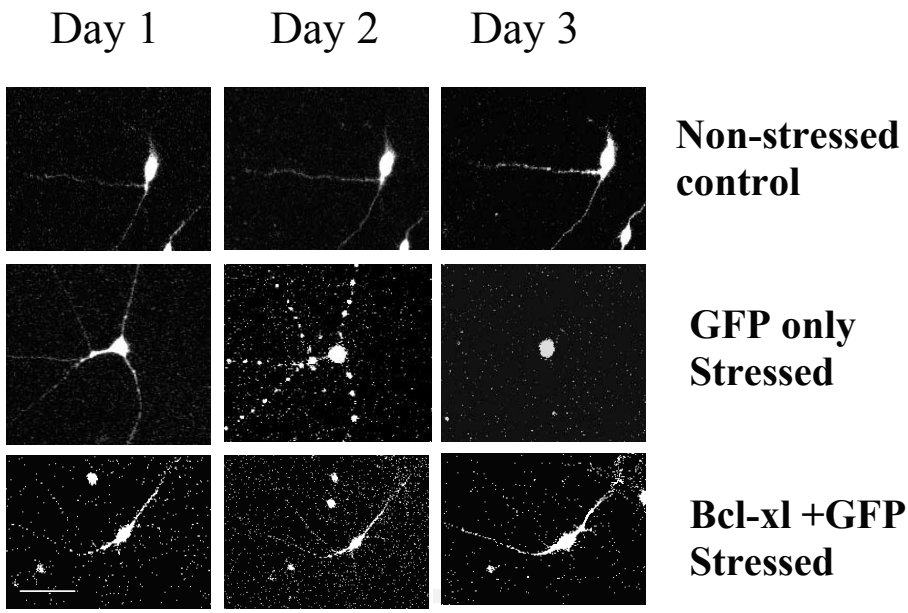

Fig. 1. Overexpression of Bcl-xl can prevent loss of neurites following a hypoglycemic stress. Panels show sequential fluorescence micrographs of representative septal neurons transfected with GFP alone (upper two rows) or with GFP plus Bcl-xl (lower row). Images were collected before the stress in control medium on day 1 followed by a 24 h hypoglycemic stress. The same neurons were re-imaged immediately after the stress (day 2), then returned to normal conditioned medium and imaged again on day 3. Non-stressed controls were washed as often as stressed cultures, but maintained in normal medium throughout. Summed results from this experiment are plotted in Fig. 2. Scale bar=50 μm.