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UPREGULATION OF BNIP3 AND TRANSLOCATION TO MITOCHONDRIA MEDIATES CYANIDE-INDUCED APOPTOSIS IN CORTICAL CELLS

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Abstract

BNIP3, a BH3 domain only Bcl-2 protein, has been identified as a mitochondrial mediator of hypoxia-induced cell death. Since cyanide produces histotoxic anoxia (chemical hypoxia), the present study was undertaken in primary cortical cells to determine involvement of the BNIP3 signaling pathway in cyanide-induced death. Over a 20 h exposure KCN increased BNIP3 expression, followed by a concentration-related apoptotic death. To determine if BNIP3 plays a role in the cell death, expression was either overexpressed with BNIP3 cDNA (BNIP3⁺) or knocked down with small interfering RNA (RNAi). In BNIP3⁺ cells, cyanide-induced apoptotic death was markedly enhanced and preceded by reduction of mitochondrial membrane potential ($\Delta\psi_m$), release of cytochrome *c* from mitochondria and elevated caspase 3 and 7 activity. Pretreatment with the pan caspase inhibitor zVAD-fmk suppressed BNIP3⁺-mediated cell death, thus confirming a caspase-dependent apoptosis. On the other hand, BNIP3 knock down by RNAi or antagonism of BNIP3 by a transmembrane-deleted dominant-negative mutant (BNIP3 Δ TM) markedly reduced cell death. Immunohistochemical imaging showed that cyanide stimulated translocation of BNIP3 from cytosol to mitochondria and displacement studies with BNIP3 Δ TM showed that integration of BNIP3 into the mitochondrial outer membrane was necessary for the cell death. In BNIP3⁺ cells, cyclosporin-A, an inhibitor of mitochondrial pore transition, blocked the cyanide-induced reduction of $\Delta\psi_m$ and decreased the apoptotic death. These results demonstrate in cortical cells that cyanide induces a rapid upregulation of BNIP3 expression, followed by translocation to the mitochondrial outer membrane to reduce $\Delta\psi_m$. This was followed by mitochondrial release of cytochrome *c* to execute a caspase-dependent cell death.

Keywords

cyanide; BNIP3; apoptosis; neurotoxicity

INTRODUCTION

BNIP3 (Bcl-2/adenovirus E1B 19-kDa-interacting protein 3) is a member of the Bcl-2 mitochondrial protein family that has a single Bcl-2 homology 3 (BH3) domain and is a pro-

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death factor (Lee and Paik, 2006). Under control conditions BNIP3 is expressed at low levels in a variety of tissues, including brain where it is primarily localized to the cytosol (Vande Velde *et al.*, 2000; Lee and Paik, 2006). BNIP3 appears to be a key regulator of hypoxia-ischemic cell death and activation can initiate either apoptotic or non-apoptotic modes of cell death, depending on cell type and stimulus (Crow, 2002; Regula *et al.*, 2002). In hypoxia, BNIP3 undergoes a rapid up-regulation, followed by translocation from cytosol to mitochondria for initiation of the cell death cascade. BNIP3 is inserted in the outer mitochondrial membrane via its C-terminus, with the N-terminus remaining in the cytosol (Ray *et al.*, 2000). Following membrane insertion, BNIP3 mediates mitochondrial dysfunction to induce cell death (Chen *et al.*, 1997; Vande Velde *et al.*, 2000; Yan *et al.*, 2006).

Following hypoxia, BNIP3 may mediate either necrotic or apoptotic deaths of cardiac myocytes and brain cells (Graham *et al.*, 2004). The overexpression of BNIP3 induces apoptotic cell death (Chen *et al.*, 1997; Chen *et al.*, 1999; Vande Velde *et al.*, 2000). Overexpression opens the mitochondrial permeability transition (MPT) pore which disrupts the $\Delta\psi_m$ and activates the cell death cascade (Vande Velde *et al.*, 2000; Kim *et al.*, 2002). It has been postulated that BNIP3 may exert an effect through either functional or physical interaction with multiple death factors at mitochondrial and extra-mitochondrial sites. BNIP3 can induce a caspase-dependent apoptosis by heterodimerizing with Bcl-2/Bcl-XL through binding to the BH3 domain, thus neutralizing the anti-apoptotic activity (Chen *et al.*, 1997; Ray *et al.*, 2000). On the other hand, BNIP3-mediated cell death can be caspase-independent producing an atypical cell death exhibiting necrotic morphological features (Vande Velde *et al.*, 2000). The mode of death activated by BNIP3 is dependent on level of expression, functional interaction with Bcl-2 and/or non-Bcl-2 proteins and the level of mitochondrial dysfunction (dissipation of $\Delta\psi_m$ and MPT pore opening) (Itoh *et al.*, 2003; Zhang *et al.*, 2003).

Cyanide is a rapid-acting neurotoxicant that inhibits cytochrome oxidase (complex IV) to block mitochondrial respiration, leading to lowering of $\Delta\Psi_m$, reduced ATP levels and enhanced intracellular reactive oxygen species (ROS) generation (Jones *et al.*, 2003). Cyanide can produce two distinct modes of death in the nervous system, depending on cell type and level of oxidative insult (Mills *et al.*, 1999; Prabhakaran *et al.*, 2002). In primary rat cortical cells, cyanide induces predominately apoptotic death, whereas mesencephalic cells undergo necrotic death. Cyanide-induced apoptosis of cortical cells is caspase-dependent and involves loss of $\Delta\psi_m$ and MPT pore opening (Shou *et al.*, 2000; Prabhakaran *et al.*, 2002). Pharmacological inhibition of either the caspases or the MPT pore blocked cyanide-induced cortical cell death, thus establishing a link between mitochondrial dysfunction and apoptosis. Subsequent studies have shown that the Bcl-2 protein family is involved in regulation of mitochondrial cytochrome *c* release and activation of the caspase cascade in cyanide-induced cortical cell death (Shou *et al.*, 2002).

Since we have previously shown in mice that cyanide produces apoptotic death of brain cortical cells (Mills *et al.*, 1999), the present study was undertaken to examine the role of BNIP3 in cyanide-induced death of primary rat cortical cells.

EXPERIMENTAL PROCEDURES

Cell culture

Primary cortical cells were prepared from embryonic day 16 Sprague-Dawley rats as previously described (Prabhakaran *et al.*, 2002). In brief, cerebral cortices were dissected and cells dissociated in 0.025% trypsin at 37°C for 15 min. Digestion was stopped by adding trypsin inhibitor and DNase I. Cells were passed through a Pasteur pipette several times and plated at a density of 5×10^5 cells/cm² onto 6-well culture plates or 75 mm² culture flasks pre-coated with 10 µg/ml poly-L-lysine. Cells were grown in high glucose Delbecco's modified Eagle's

medium supplemented with 10% fetal bovine serum, 10% horse serum, 2 mM glutamine and 1 ml penicillin/streptomycin (5000 U/ml) at 37°C in an atmosphere of 5% CO₂ and 95% air. Cytosine arabinofuranoside (10 µM) was added at 6 days *in vitro* for 24 h to limit growth of glial cells. Medium was changed twice a week until experiments were carried out at 10–12 days *in vitro*. At the time of experimentation, the percent astrocytes in the culture was approximately 30% as estimated by immunostaining of the astrocyte specific marker glial fibrillary acidic protein (GFAP) and 70% neurons as determined by immunostaining of the neuron-specific marker microtubule associated protein-2 (MAP2).

Transient transfection of primary cortical cells

The BNIP3 cDNA was cloned into the expression vector pCDNA3.1 as previously described [10]. Transient transfections of BNIP3 plasmid or BNIP3ΔTM containing the transmembrane-deleted BNIP3 cDNA or siRNA were performed with Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA) as described by Krichevsky and Kosik (2002). Lipofectamine diluted in Opti-MEM (Invitrogen, Carlsbad, CA, USA) was applied to the plasmids or dsRNA and incubated for 45 min. To each microtiter plate well containing cells, 1 µg of plasmid or 0.2 µg of 21-bp dsRNA (siRNA) containing Lipofectamine 2000™ was applied in a final volume of 1 ml. The medium was changed to regular cortical cell culture medium (previously described in the cell culture section) 5 h after transfection, and cells were treated with KCN 24 h after transfection. The overall transfection efficiency, as assessed by GFP positive cells, was estimated to be 10–12%.

siRNA preparation and transient transfection

siRNA corresponding to the BNIP3 was synthesized by Ambion, Inc. (Austin, TX, USA) with 5' phosphate, 3' hydroxyl, and two base overhangs on each strand. The gene-specific sequences used for BNIP3 interference were: sense 5'-GCUGCCUGCUACCUCUAtt-3' and antisense 5'-UGAGAGGUAGCAGGGCAGCtt-3' annealing for duplex siRNA formation was performed as described by the manufacturer. Lipofectamine 2000™ was used to transfect siRNA or negative control RNAi containing a 21-base mRNA sequence starting with the AA dinucleotide into cortical cells (Krichevsky and Kosik, 2002).

Cytotoxicity analysis

Cortical cells were incubated with 100–400 µM KCN for 24 h and cell death was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Kitazawa *et al.*, 2001). After treatment with KCN, cells were incubated in serum-free medium containing 0.25 mg/ml MTT for 3 h at 37°C. Formation of formazan from tetrazolium was measured at 570 nm with a reference wavelength at 630 nm using a microplate reader.

TUNEL assay for apoptosis

Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end-labeling (TUNEL) was performed on paraformaldehyde (4% in phosphate buffered saline)-fixed cells using the Apoptag™ *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD, USA). Briefly, cells were pre-incubated in equilibration buffer containing 0.1 M potassium cacodylate (pH 7.2), 2 mM CaCl₂, and 0.2 mM dithiothreitol for 10 min at room temperature and then incubated in TUNEL reaction mixture (containing 200 mM potassium cacodylate (pH 7.2), 4 mM MgCl₂, 2 mM 2-mercaptoethanol, 30 µM biotin-16-dUTP, and 300 U/ml TdT) in a humidified chamber at 37°C for 1 h. After incubating in stop/wash buffer for 10 min, the elongated digoxigenin-labeled DNA fragments were visualized using anti-digoxigenin peroxidase antibody solution followed by staining with DAB/H₂O₂ (0.2 mg/ml diaminobenzidine tetrachloride and 0.005% H₂O₂ in PBS, pH 7.4). Cells were then counterstained with hematoxylin. The selectivity of the assay is based on the presence of 3-OH DNA fragment ends in apoptotic cells. Based on morphology,

astrocytic cells were excluded from counting. The percent of non-astrocytic cells that were TUNEL positive were determined from four randomly selected fields for the estimation of apoptosis. To verify apoptosis cells were stained with Hoechst 33258 and examined with a fluorescence microscope. Apoptotic cells were identified on the basis of changes in nuclear morphology, such as chromatin condensation and fragmentation.

Subcellular fractionation

After different treatments, cortical cells were harvested in phosphate-buffered saline (PBS) and centrifuged at 500 g (Shou *et al.*, 2003). Cell pellets were resuspended in isotonic mitochondrial buffer (MB: 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM HEPES, pH 7.5), supplemented with a protease inhibitor cocktail (Calbiochem, CA, USA) and homogenized with a polytron homogenizer. Samples were centrifuged at 500 g for 5 min at 4°C and the resulting supernatant was centrifuged at 10,000 g for 30 min at 4°C to obtain the heavy membrane pellet (HM) enriched for mitochondria. The 10,000 g supernatant was used as crude cytosol and the HM pellet resuspended in 50 µl MB buffer.

To assess whether BNIP3 integrates into mitochondrial membranes, the mitochondrial fractions were subjected to alkali extraction using freshly prepared 0.01 M Na₂CO₃ (pH 11.5) to dissociate and solubilize proteins that are not integrated into the membrane (Goping *et al.*, 1998).

Whole cell extraction

Control and cyanide-treated cells were prepared by washing the cells three times with PBS and harvested by centrifugation at 500×g for 5 min. Cell pellets were lysed in a buffer containing 220 mM mannitol, 68 mM sucrose, 20 mM HEPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 1 mM EDTA, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1% Triton-X100 and protease inhibitors on ice for 15 min. After centrifugation, supernatants were taken as whole cell protein extraction.

Western blot analysis

Western analysis was performed according to the protocol supplied with the ECF Western blot kit (Amersham, Piscataway, NJ, USA). The protein content in the extractions was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). The samples containing 30–50 µg protein were boiled in Laemmli buffer for 5 min and subjected to electrophoresis in 12% SDS-polyacrylamide gel, followed by transfer to a nitrocellulose membrane. After blocking with PBS containing 5% nonfat dry milk and 0.1% Tween 20, the membrane was exposed to primary antibody for 3 h at room temperature on a shaker. Reactions were detected with a fluorescein-linked anti-mouse IgG (second antibody) conjugated to horseradish peroxidase using enhanced chemiluminescence. Primary antibodies used were mouse monoclonal anti-BNIP3 antibody, mouse monoclonal anti-β-actin antibody (Sigma Chemical Co, St. Louis, MO, USA), mouse monoclonal anti-cytochrome c antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and bovine monoclonal anti-cytochrome oxidase subunit IV antibody (Invitrogen, Carlsbad, CA, USA).

Immunocytochemistry

Cortical neurons were grown on poly-L-lysine coated coverslips and after treatment, cells were labeled with 500 nM Mitotracker (Invitrogen, Carlsbad, CA, USA) for 30 min at 37°C. After washing three times with PBS, cells were fixed for 15 min with 4% paraformaldehyde and then permeabilized with 0.2% Triton X-100 for 30 min at room temperature. Cultures were then washed with PBS and exposed to blocking solution (5% goat serum in PBS). After PBS washing, cells were incubated with monoclonal anti-BNIP3 antibody (1:200 diluted in PBS) at room temperature for 3 h. Cells were washed twice in PBS and incubated with Alexa Fluor

488-conjugated goat anti-mouse secondary antibody (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. Coverslips were then mounted onto glass slides and images were acquired and analyzed using a BioRad MRC 1024ES laser scanning confocal microscope (Hercules, CA, USA).

Caspase activity

The generation of 7-amino-4-methylcoumarin (AMC) from the caspase tetrapeptide substrate DEVD-AMC (Bachem Bioscience, King of Prussia, PA, USA) was used as an index of caspase 3 and 7 activity (See and Loeffler, 2001). Briefly, cells grown in 6 well culture dishes were washed with PBS and lysed with 100 μ l of lysis buffer [0.05% Igepal NP-40 (Sigma Chemical Co., St. Louis, MO, USA), 5 mM EDTA, 150 mM NaCl, 50 mM Tris, pH 7.5] on ice. 50 μ l of lysate were added to a reaction mixture containing 100 μ l of 2 X reaction buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 2.5 mM dithiothreitol), 40 μ l of H₂O, and 10 μ l of DEVD-AMC (final concentration 50 μ M) and incubated for 1 h at 37°C. The reaction was stopped by a 10 X dilution with ice-cold lysis buffer. Fluorescence of free AMC was measured (excitation and emission wavelengths: 360 and 465 nm, respectively) using a plate reader.

Mitochondrial membrane potential

The relative $\Delta\psi_m$ was monitored with rhodamine 123 (R123) as previously described (Prabhakaran *et al.*, 2002). After treatment, cells were loaded with 10 μ M R123 and incubated at 37°C for 30 min in the dark. Uptake of R123 into mitochondria is a direct reflection of its permeability; an increase of R123 fluorescence reflects a lowering of $\Delta\psi_m$. Loaded cells were washed twice with Krebs-Ringer buffer, and changes in R123 fluorescence were monitored using a fluorescence plate reader at 498 nm excitation and 525 nm emission.

Statistics

Data were expressed as mean $\pm$ SEM and statistical significance was assessed by one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple range test. Differences were considered significant at $P < 0.05$.

RESULTS

Cyanide-induced apoptosis correlates with increased BNIP3 expression

A concentration-dependent decrease in cell viability was detected in cortical cells exposed to 100–400 μ M KCN (Figure 1A). As determined by TUNEL assay, the mode of death was predominantly apoptotic, consistent with previous studies on cyanide-induced death in primary cortical cells (Figure 1B) (Li *et al.*, 2002; Prabhakaran *et al.*, 2002). Apoptosis was confirmed by cell staining with Hoechst 33258 to visualize nuclear changes (data not shown). It should be noted that in these studies mixed cultures or cell composed of neurons and astrocytes were used and the contribution of astrocytes to the cytotoxic response was minimal. We have observed that astrocytic cells are resistant to the cytotoxic action of cyanide and at the concentrations used, minimal astrocytic cell damage or death was detectable. On the other hand, pure neuronal cultures (grown in serum free neurobasal-B27 medium yielding >95% neurons) were very sensitive to cyanide (data not shown).

To establish whether cyanide could alter BNIP3 expression, cells were exposed to KCN (100–400 μ M) for 20 h and expression analyzed by Western blotting. Cyanide induced a concentration- and time- dependent increase of BNIP3 expression (Figure 2), paralleling the cell death response. BNIP3 was expressed at a low level under control conditions, whereas cyanide rapidly increased expression with enhanced levels noted within 3 h and continued to increase over the 24 h observation period.

Altered BNIP3 expression modulates cyanide-induced apoptosis

To link BNIP3 to apoptosis, cells were transiently transfected with BNIP3 cDNA (BNIP3⁺) for forced overexpression or siRNA (RNAi) to knockdown BNIP3 (Vande Velde *et al.*, 2000; Kubasiak *et al.*, 2002), and then 24 h later treated with cyanide (400 μ M). In BNIP3⁺ cells cyanide produced a marked up-regulation of BNIP3 expression that was accompanied by an increased apoptotic death (Figure 3A,C). RNAi blocked the KCN-induced up-regulation of BNIP3 and reduced cell death to control levels. Interestingly constitutive expression of BNIP3 did not change after transfection with RNAi, suggesting a low turnover rate and/or a very low basal expression of BNIP3 in the wild type cells. To confirm that RNAi selectively knocked down BNIP3, transfection with a negative control RNAi did not alter either BNIP3 expression or the level of cell death induced by cyanide (Figure 3B,C). It was concluded that the RNAi sequences were specific for BNIP3.

BNIP3 upregulation produces cytochrome c release and caspase activation

Release of the mitochondrial protein, cytochrome *c*, into the cytosol regulates apoptotic death by promoting caspase activation, followed by execution of apoptosis (Li *et al.*, 1997). To determine whether BNIP3 overexpression stimulates cytochrome *c* release from mitochondria, expression of BNIP3 was increased by transfection (BNIP3⁺) and subcellular distribution of cytochrome *c* examined. Cyanide released cytochrome *c* into the cytosol in both BNIP3⁺ and wildtype cells (Figure 4A). To determine if the cell death was caspase-dependent, expression of BNIP3 was altered by transfection (BNIP3⁺ or RNAi) and caspase activity determined following cyanide. In BNIP3⁺ cells, cyanide increased caspase 3 and 7 activity and zVAD-fmk significantly reduced cell death (Figure 4C), thus linking caspase activity and apoptosis in BNIP3⁺ cells. Also, RNAi knock down of BNIP3 decreased cyanide-induced caspase activation in wildtype cells, in parallel with the reduction of apoptosis (Figure 4B,C). zVAD-fmk blocked both the increase of caspase 3 and 7 activity and apoptotic death, establishing that the cyanide-induced apoptosis was caspase-dependent.

BNIP3-mediated mitochondrial dysfunction and cell death

To determine the effect of BNIP3 expression on mitochondrial function, cells were transfected with BNIP3 or RNAi prior to cyanide and then the relative $\Delta\psi_m$ was monitored using rhodamine-123. Cyanide significantly reduced $\Delta\psi_m$ as reflected by increased R-123 fluorescence and the reduction was enhanced in BNIP3⁺ cells (Figure 5A). On the other hand the KCN-induced reduction of $\Delta\psi_m$ was blocked by RNAi knockdown of BNIP3. Pretreatment with CsA (MPT pore inhibitor) blocked the cyanide-induced reduction in $\Delta\psi_m$ and apoptotic death in BNIP3⁺ cells (Figure 5A,B). These observations establish an association between cyanide-induced apoptosis and BNIP3-mediated mitochondrial dysfunction.

Cyanide stimulates translocation and integration of BNIP3 into mitochondria

The initial step in BNIP3-mediated cell death is translocation of the protein to mitochondria, followed by insertion into the outer membrane (Chen *et al.*, 1997). To determine if cyanide stimulated BNIP3 translocation, confocal imaging of BNIP3 was conducted by use of a fluorescence labeled anti-BNIP3 antibody and mitochondria were imaged with Mitotracker Red. In KCN treated cells, BNIP3 was localized to mitochondria, whereas in wildtype control cells the protein remained localized in the cytosolic compartment (Figure 6A). Integration of BNIP3 into the outer mitochondrial membrane was determined by Western blot analysis of isolated mitochondria from control and cyanide treated cells. Isolated mitochondria were subjected to alkali extraction to dissociate and solubilize loosely associated membrane proteins (Goping *et al.*, 1998). Western blots of alkali extracted mitochondria showed that BNIP3 was readily stripped from mitochondrial membranes of control wildtype cells, whereas BNIP3 remained at high levels in the mitochondrial fraction of cyanide treated cells, showing that the protein

had integrated into mitochondrial membranes after exposure to cyanide (figure 6B). Transfection with a carboxyl terminal transmembrane deletion mutant of BNIP3 (BNIP3 Δ TM) blocked insertion of BNIP3.

To demonstrate that integration of BNIP3 into mitochondrial membrane is necessary for cell death, cytotoxicity was assessed in BNIP3 Δ TM⁺ cells. Cyanide-induced cell death was significantly reduced in BNIP3 Δ TM⁺ cells as compared to wild type cells treated with KCN (Figure 7A). The magnitude of the reduction of cell death was comparable to that produced by RNAi (Figure 3C). BNIP3 Δ TM⁺ also blocked the KCN-induced caspase activation and dissipation of $\Delta\psi_m$ (Figure 7B,C). Western blot analysis showed that the mutant blocked mitochondrial release of cytochrome c (data not shown). As compared to control cells, BNIP3 Δ TM⁺ did not produce mitochondrial dysfunction and cell death, thus it was apparent that the mutant was not toxic under control conditions. It was concluded that in primary cortical cells, BNIP3 Δ TM reduces cyanide cytotoxicity by a dominant negative antagonism of BNIP3 translocation and insertion into mitochondrial outer membrane.

DISCUSSION

BNIP3 is a unique pro-death factor that is a member of the BH3-only subfamily of Bcl-2 proteins that regulates cell death. BNIP3 lacks the BH1 and BH2 domains characteristic of the Bcl-2 family, but has a common BH3 interaction domain (Chen *et al.*, 1997; Webster *et al.*, 2005). BNIP3 expression is regulated transcriptionally and when induced, protein levels rise rapidly, followed by translocation to mitochondria and insertion of the C-terminal domain into the outer mitochondrial membrane (Kubasiak *et al.*, 2002). The mode of cell death initiated by BNIP3 is stimulus and cell type specific. In a number of cell models a caspase-dependent apoptosis has been reported, whereas a caspase-independent necrosis-like death has been observed following hypoxia (Vande Velde *et al.*, 2000; Kubasiak *et al.*, 2002).

In the present study, it was shown in primary cortical cells that cyanide-induced a caspase dependent, apoptotic death linked to induction of BNIP3 expression. The apoptotic cell death was mediated by mitochondrial release of cytochrome *c* which then activated the caspase apoptotic death cascade. Knockdown studies with BNIP3 RNAi demonstrated that up-regulation of BNIP3 was directly linked with apoptosis. Also, forced overexpression of BNIP3 sensitized the cells to cyanide. After up-regulation, BNIP3 translocated from the cytosol and integrated into the mitochondrial outer membrane to induce MPT pore opening. Temporally this was followed by dissipation of the mitochondrial membrane proton electrochemical gradient and release of cytochrome *c* to initiate caspase activation, the execution step of the cell death.

The mechanism by which BNIP3 initiates mitochondria-mediated cell death is not clear and may be different from the typical cell death cascade mediated by the Bcl-2 family. Following insertion into the outer membrane, BNIP3 can heterodimerize with Bcl-2 and Bcl-X(L) to neutralize their anti-apoptotic properties to initiate cell death (Goping *et al.*, 1998). This mode of death is caspase-dependent and mediated by secretion of cytochrome *c* from mitochondria. Additionally, BNIP3 can produce rapid mitochondrial dysfunction by directly interacting with VDAC/porin and ANT to stimulate opening of the MPT pore, thereby dissipating $\Delta\psi_m$ (Shimizu *et al.*, 2000; Zhang *et al.*, 2003). The rapid loss of $\Delta\psi_m$ can lead to ATP depletion, calcium ion imbalance and eventually loss of plasma membrane integrity to produce a necrosis-like morphology. Similarly, cyanide induced a rapid loss of mitochondrial function as reflected by the loss of $\Delta\psi_m$. This is accompanied by a catastrophic decrease of cellular ATP, elevation of cytosolic calcium and then execution of cell death (Sun *et al.*, 1997; Prabhakaran *et al.*, 2005)

In the nervous system, BNIP3 expression is normally not detectable or expressed at low levels. However the protein may play a role in brain cell death under select conditions. Following global brain ischemia, expression is rapidly induced in CA1 neurons and undergoes nuclear localization, suggesting a novel role in regulating cell death (Schmidt-Kastner *et al.*, 2004). A recent study indicates that BNIP3 may be a mediator of brain developmental apoptosis (Sandau *et al.*, 2006). *In situ* hybridization analysis of BNIP3 mRNA showed that a transient increase in mRNA expression correlated with developmental cell death in neonatal rat cortex and hippocampus. Involvement of BNIP3 in cyanides action on the nervous system is of interest since exposure can produce a unique region-specific neurodegeneration (Mills *et al.*, 1999). Cyanide produces a pattern of cell death in the brain in which the mesencephalic areas undergo a necrotic death, whereas in the cortex apoptosis has been observed (Prabhakaran *et al.*, 2002). Based on the present study, it is concluded that BNIP3 induction and translocation to mitochondria plays a role in the cortical apoptosis, whereas a different cell signaling system or execution cascade may be activated in mesencephalic necrosis.

The mode of cortical cell death observed with cyanide is similar to that produced by hypoxia, however the initiation signal may be different (Tanaka *et al.*, 1994; Halterman and Federoff, 1999). Cell hypoxia increases BNIP3 expression by activating hypoxia inducible factor 1 alpha (HIF-1 α), thus stimulating BNIP3 gene transcription by binding to the hypoxia-response element (HRE) present in the BNIP3 promoter. In hypoxia, low cellular oxygen directly stimulates an increased HIF-1 α activity and its DNA binding to increase BNIP3 transcription (Guo *et al.*, 2001). On the other hand, cyanide induces histotoxic anoxia in which cellular concentration of oxygen is normal, only oxygen utilization in oxidative phosphorylation is inhibited (Way, 1984), thus the initiation pathway for BNIP3 induction in cyanide-induced apoptosis may be independent of the normal HIF-1 α pathway. Other studies have shown that BNIP3 induction can be activated by non-hypoxia pathways by activating HIF-1 α signaling by non-oxygen mediated pathways involving kinase signaling pathways perhaps stimulated by oxidative stress (Yook *et al.*, 2004; Kanzawa *et al.*, 2005; An *et al.*, 2006). The intense oxidative stress produced by cyanide as a result of complex IV inhibition may be the BNIP3 activation stimulus (Jones *et al.*, 2003; Chen *et al.*, 2003).

In order for BNIP3 to initiate mitochondrial dysfunction, the protein must be integrated into the outer mitochondrial membrane (Chen *et al.*, 1999; Ray *et al.*, 2002). It was observed that under basal conditions (controls) BNIP3 was loosely associated with mitochondria, but following addition of cyanide the protein could not be stripped from the mitochondrial surface by alkali treatment. This “tight” binding or integration into the membrane apparently may have led to an intra-membrane interaction necessary for initiation of mitochondrial dysfunction. This is supported by the observation that expression of BNIP3 Δ TM, a mutant lacking the transmembrane insertion domain, blocked BNIP3 translocation and integration into the mitochondrial outer membrane to block apoptosis. Previous studies have shown that BNIP3 Δ TM functions as a dominant negative mutant that blocks translocation to mitochondria (Kubasiak *et al.*, 2002; Regula *et al.*, 2002). These observations provide evidence that in cortical cells, cyanide induces a mitochondria-mediated apoptosis by up-regulating BNIP3 and stimulating its translocation.

In summary, cyanide produces an apoptotic cell death in primary cortical cells by a BNIP3 inducible pathway. This potent neurotoxin rapidly stimulates up-regulation of BNIP3 expression which in turn translocates to the mitochondrial outer membrane, followed by mitochondrial dysfunction, release of cytochrome c and then execution of a caspase dependent apoptosis. It is likely that a unique initiation signal is stimulated by cyanide since cellular oxygen levels are not altered by the histotoxic anoxia.

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Abbreviations

AMC	7-amino-4-methylcoumarin
BNIP3	Bcl-2/adenovirus E1B 19-kDa-interacting protein 3
BNIP3ΔTM	BNIP3 protein with deletion of transmembrane domain
BH3	Bcl-2 homology domain 3
CsA	cyclosporin A
$\Delta \psi_m$	mitochondrial membrane potential
DEVD-AMC	caspase tetrapeptide substrate
HIF-1α	hypoxia-inducible factor 1 alpha
HM	heavy membrane pellet
MTP	mitochondrial transition pore
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
R123	rhodamine 123
siRNA (RNAi)	small interfering RNA
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
zVAD-fmk	N-benzyloxycarbonyl-Ala-Asp-fluoromethyl ketone

Figure 1. Cyanide-induced cell death in cortical cells. Cells were exposed to cyanide (100–400 μ M) for 24 h and cell death quantitated. **A)** Cell viability determined by MTT assay. **B)** Apoptotic death determined by TUNEL assay. Data represents mean $\pm$ SE of three or more experiments. *Significantly difference from control ($P < 0.05$).

Figure 2.

Cyanide-induced expression of BNIP3 in cortical cells. **A)** Western blot analysis of cells treated with KCN (100–400 μ M) for 20 h. **B)** Western blot of cells treated with KCN (400 μ M) for 3–24 h. Densitometric analysis was by Scion Image software. *Significantly difference from control ($P < 0.05$).

Figure 3.

Effect of transfection on BNIP3 expression and apoptosis. **A**) Cells were transfected with BNIP3 expression plasmid or BNIP3 siRNA (knockdown) and 24 h later treated with KCN (400 μ M) for 12 h. Cell lysates were subjected to Western blot analysis with an anti-BNIP3 antibody. **B**) Cells were transfected with BNIP3 siRNA or BNIP3 siRNA⁻ (negative control) prior to Western analysis. Densitometric analysis was by Scion Image software. **C**) Percent apoptotic (TUNEL positive) cells were determined after 24 h of KCN exposure in transfected cells. Data represents mean $\pm$ SE of three or more experiments. *Significantly difference from control; #significantly different from KCN; +significant different from KCN alone and BNIP3⁺ + KCN group, ($P < 0.05$).

Figure 4.

Expression of BNIP3 promotes cytochrome *c* release, caspase activation and apoptosis. Cells were transfected with BNIP3 expression plasmid or BNIP3 siRNA for 24 h and then treated with KCN (400 μ M). **A**) Six h after KCN, mitochondrial and cytosolic extracts were subjected to Western blotting with an anti-cytochrome *c* antibody. **B**) Transfected cells were pre-treated with z-VAD-fmk for 1 h before addition of KCN (400 μ M) for 6 h, followed by determination of caspase activity. **C**) Percent apoptotic (TUNEL positive) cells following 24 h of KCN exposure. Data represent mean $\pm$ SE of three or more experiments. *Significantly different from control; #significantly different from KCN; +significantly different from BNIP3⁺ + KCN group, ($P < 0.05$).

Figure 5.

Effect of BNIP3 on $\Delta\Psi_m$ and apoptosis. **A)** Cells were transfected with a BNIP3⁺ plasmid or BNIP3 siRNA for 24 h and then treated with CsA for 1 h before addition of KCN (400 μ M). Two h later the $\Delta\Psi_m$ was monitored by the R123 method. **B)** Percent apoptotic (TUNEL positive) cells was determined 24 h after treatments. Data represent mean $\pm$ SE of six or more experiments. *Significantly different from control; # significantly different from KCN; +significantly different from BNIP3⁺ + KCN group, (P<0.05).

Figure 6.

Cyanide-induced mitochondrial translocation and membrane integration of BNIP3. **A)** Immunofluorescence imaging of the subcellular distribution of BNIP3. After transfection and KCN treatment for 12 h, cells were exposed to a fluorescent labeled anti-BNIP3 antibody (green) and with Mitotracker red which localizes in mitochondria. Confocal microscopy illustrates localization of mitochondria (a–c) and BNIP3 (d–f). Co-localization of BNIP3 and mitochondria is shown as yellow in merged images (g–i). **B)** Alkali extracts of mitochondria from control and treated cells were subjected to Western blotting with an anti-BNIP3 antibody.

Figure 7.

Expression of BNIP3 TM reduces KCN-induced cytotoxicity. Cells were transfected with BNIP3 TM for 24 h before cyanide treatment. **A)** Percent apoptotic (TUNEL positive) cells. **B)** $\Delta\Psi_m$ was monitored by the R123 fluorescence method. **C)** Caspase activity was determined in cellular lysates following the specific treatments. Data represent mean $\pm$ SEM of six or more experiments. *Significantly different from control; #significantly different from KCN, ($P < 0.05$).