Cyanide-Induced Apoptosis of Dopaminergic Cells Is Promoted by BNIP3 and Bax Modulation of Endoplasmic Reticulum-Mitochondrial Ca^{2+} Levels^[S]

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ABSTRACT

Cyanide is a potent neurotoxicant that can produce dopaminergic neuronal death in the substantia nigra and is associated with a Parkinson-like syndrome. In this study involvement of Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3), a BH3-only Bcl-2 protein, in cyanide-induced death of dopaminergic cells was determined in mice and Mes 23.5 cells. Treatment of mice with cyanide up-regulated BNIP3 and Bax expression in tyrosine hydroxylase (TH)-positive cells of the substantia nigra, and progressive loss of TH-positive neurons was observed over a 9-day period. In Mes 23.5 dopaminergic cells, cyanide stimulated translocation of BNIP3 to both endoplasmic reticulum (ER) and mitochondria. In ER, BNIP3 stimulated release of Ca^{2+} into the

cytosol, followed by accumulation of mitochondrial Ca^{2+} , resulting in reduction of mitochondrial membrane potential ($\Delta\psi_m$) and eventually cell death. Cyanide also activated Bax to colocalize with BNIP3 in ER and mitochondria. Forced overexpression of BNIP3 activated Bax, whereas gene silencing reduced Bax activity. Knockdown of Bax expression by small interfering RNA blocked the BNIP3-mediated changes in ER and mitochondrial Ca^{2+} to block cyanide-induced mitochondrial dysfunction and cell death. These findings show that BNIP3 mediates cyanide-induced dopaminergic cell death through a Bax downstream signal that mobilizes ER Ca^{2+} stores, followed by mitochondrial Ca^{2+} overload.

Cyanide is a rapid-acting toxicant for which the primary target organ is the central nervous system. Acute, sublethal intoxication with cyanide is associated with a secondary Parkinsonism characterized by selective dopaminergic neuronal loss in basal ganglia (Rosenberg et al., 1989; Sarikaya et al., 2006). Parkinsonism and cognitive impairment have also been associated with long-term occupational exposure to cyanide (Di Filippo et al., 2008). Although cyanide induces death of dopaminergic cells in both in vivo and in vitro models, the mechanism is not clear.

BNIP3 is a BH3-only Bcl-2 protein that plays a critical role in regulating death in a variety of cell types (Althaus et al., 2006; Tracy and Macleod 2007; Chinnadurai et al., 2009). Our previous studies showed that BNIP3 was up-regulated by cyanide which then contributed to dissipation of mitochondrial membrane potential ($\Delta\psi_m$) and ultimately to cell death (Prabhakaran et al., 2007; Zhang et al., 2007). A recent study performed in

myocardial cells suggests that Bak and Bax are downstream effectors of BNIP3-mediated mitochondrial dysfunction (Kubli et al., 2007). Cells lacking Bax and Bak were more resistant to apoptotic stimuli, even in the presence of substantial BNIP3 up-regulation. BH3-only proteins can activate Bax by direct interaction or indirectly by sequestration of antiapoptotic Bcl-2 proteins such as Bcl-2 and Bcl-X_L (Letai et al., 2002; Kuwana et al., 2005; Li et al., 2007). Thus, Bax dependence of BNIP3-mediated cell death was examined in the current study.

Bax is a proapoptotic Bcl-2 family protein that enhances ER and mitochondrial Ca^{2+} cross-talk. Nutt et al. (2002a) reported that when Bax was overexpressed in prostate cancer cells, it localized to the ER membrane to induce Ca^{2+} mobilization, followed by increased mitochondrial Ca^{2+} accumulation. The subsequent mitochondrial Ca^{2+} overload triggered mitochondrial cytochrome *c* release and subsequent activation of downstream apoptotic events. Exogenous apoptotic stimuli, including staurosporine and doxorubicin, also induced ER-mitochondrial Ca^{2+} redistribution. The redistribution was abolished in Bax-deficient cells and was restored by forced expression of Bax, thus indicating Bax is critical in regulating Ca^{2+} mobilization.

We have shown in cyanide-treated cortical cells that Bax

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ABBREVIATIONS: BNIP3, Bcl-2/adenovirus E1B 19-kDa interacting protein 3; BNIP3ΔTM, transmembrane domain deleted form of BNIP3; ER, endoplasmic reticulum; HM, heavy membrane; LM, light membrane; KCN, potassium cyanide; R123, rhodamine 123; siRNA, small interfering RNA; SN, substantia nigra; TG, thapsigargin; TH, tyrosine hydroxylase; K-R, Krebs-Ringer.

undergoes up-regulation, followed by translocation from cytosol to mitochondria (Shou et al., 2003). The cyanide-induced translocation of Bax was downstream of p38 MAPK activation. In this study, it is shown that Bax and BNIP3 interact to increase release of the ER Ca^{2+} pool, and then death of dopaminergic cells is executed following mitochondrial Ca^{2+} accumulation.

Materials and Methods

Potassium Cyanide Treatment of Mice. Male non-Swiss albino mice (20–25 g) were purchased from Harlan (Indianapolis, IN). Animal maintenance and experimental protocols were in accordance with National Institutes of Health Guidelines and approved by the Purdue University Institutional Committee on Animal Care. For cyanide intoxication, mice received a sublethal dose of KCN (6 mg/kg twice daily) in saline by intraperitoneal injection as described by Mills et al. (1999). Control animals received an equivalent volume of sterile saline solution. Animals were treated with KCN for 0, 1, 2, 3, 4, 5, and 9 days and then sacrificed 16 h after the last injection by cervical dislocation. The ventral midbrains were dissected, frozen immediately on dry ice, and stored in -80°C for Western blotting and real-time polymerase chain reaction analysis. For immunocytochemical analysis, animals were anesthetized and then immediately perfused transcardially with ice-cold phosphate-buffered saline (PBS) (0.1 M, pH 7.4) for 1 min, followed by 4% paraformaldehyde in PBS for 20 min. Brains were removed and postfixed in the same solution for at least 12 h, then were imbedded in paraffin blocks for further analysis.

Immunocytochemistry. Paraffin blocks of mouse brains were thinly sectioned (5 μm) and mounted onto Superfrost glass slides (Thermo Fisher Scientific, Waltham, MA). Slides were first processed by hydration and then rinsed in PBS before 1 h of incubation with blocking solution, consisting of 20% goat serum dissolved in PBS, pH 7.4. Slides were then treated with 0.2% Triton X-100 for permeabilization, followed by incubation with primary antibodies, including mouse anti-BNIP3, mouse anti-Bax, and/or rabbit anti-TH antibodies for 2 h at 37°C . After three washes in PBS, the sections were incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody and/or Alexa Fluor 568 conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA) at room temperature for 1 h. After a wash in PBS, slides were rinsed in dH_2O and mounted on cover slips. Sections were examined and photographed by use of an inverted fluorescence microscope (Nikon TE-2000) with objectives and band-pass filter set for and Alexa Fluor 488, and Alexa Fluor 568. Nonspecific staining was assessed by omitting the primary antibody. In these controls, only light autofluorescence and no cross-reactive immunostaining were observed.

Mes 23.5 cells were grown on poly(L-lysine) precoated cover slips were labeled with 100 nM MitoTracker Red (Invitrogen) for 30 min at 37°C . After washing with PBS, cells were fixed in paraformaldehyde and permeabilized with 0.2% Triton X-100 for 30 min at room temperature. Cells were then exposed to anti-BNIP3 primary antibody followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody as described above.

Double-labeling immunofluorescence was used to characterize cellular distribution of BNIP3-immunoreactive proteins in cells. Rabbit polyclonal anticalnexin antibody was added with the BNIP3 antibody. After 3 h of incubation, Alexa Fluor 568-conjugated goat anti-rabbit secondary antibody was applied with the Alexa Fluor 488-conjugated goat anti-mouse secondary antibody. After 1 h of incubation at room temperature in the dark, cover slips were then mounted onto glass slides and examined by use of laser-scanning confocal microscopy as described previously (Zhang et al., 2009).

Cell Culture. The Mes 23.5 cell line, obtained from Dr. C. Rochet, Purdue University, was derived from somatic cell fusion of rat embryonic mesencephalon cells and the murine neuroblastoma-glioma cell line N18TG2 (Le et al., 1995). Cells were seeded on poly(L-lysine)-precoated plates and maintained in Dulbecco's modified Eagle's me-

dium supplemented with 5% fetal bovine serum, 2% newborn calf serum, 15 mM HEPES containing insulin (5 $\mu\text{g}/\text{ml}$), transferrin (5 $\mu\text{g}/\text{ml}$), pyruvic acid (48.6 $\mu\text{g}/\text{ml}$), putrescine (4 $\mu\text{g}/\text{ml}$), sodium selenite (5 ng/ml), and progesterone (6.3 ng/ml) at 37°C in an atmosphere of 5% CO_2 and 95% air.

Cell Death Assay. Apoptosis was detected by nuclear staining with Hoechst 33258 as described previously (Zhang et al., 2007). Cells were washed twice with PBS, fixed in 4% paraformaldehyde in PBS, then treated with 2 μM Hoechst 33258 dye, and examined by fluorescence microscopy. Cells with condensed and fragmented DNA were considered apoptotic. Cell death was expressed as the percentage of apoptotic cells relative to total number of cells.

Western Blot Analysis. After treatments, whole-cell lysates were prepared by use of a lysis 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 MgCl_2 , 1 mM dithiothreitol, 0.1% Triton X-100, and protease inhibitors. Western blotting was carried out by use of the ECF Western blot kit (GE Healthcare, Waukesha, WI) as described by the manufacturer. The primary antibodies were: mouse anti-BNIP3 antibody, mouse anti- β -actin antibody (Sigma-Aldrich, St. Louis, MO), mouse anti-cytochrome *c* oxidase (COX IV), rabbit anticalnexin antibodies (Abcam Inc., Cambridge, MA), rabbit anti-Bax antibody (Millipore, Billerica, MA), and mouse anti-Bax antibody (Santa Cruz Biotechnology, San Cruz, CA).

Subcellular Fractionation. Isolation of mitochondria and ER subcellular fractions were performed as described by Wang and Chan (2006) with modifications. Cells were collected and suspended in hypotonic buffer, containing 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA protease inhibitor cocktail and homogenized by passing through a syringe needle (26-gauge). Homogenates were first centrifuged at 800g for 10 min at 4°C , and the supernatant was centrifuged at 10,000g for 20 min at 4°C . The pellet corresponded to the mitochondrial fraction (heavy membrane, HM), and the supernatant was centrifuged at 100,000g for 1.5 h at 4°C . The final pellet corresponded to the fraction enriched with the endoplasmic reticulum (light membrane, LM). The quality of the fractionation experiments was confirmed by assessing the presence of COX IV for mitochondria and calnexin for ER.

Plasmid Constructs and Transient Transfection. The BNIP3 wild-type and the transmembrane domain-deleted form of BNIP3 (BNIP3 ΔTM) plasmids was constructed as described previously (Zhang et al., 2007). Transient transfections in Mes 23.5 cells were performed by use of Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. The transfection efficiency was approximately 90% when assessed by use of eYFP (data not shown).

Gene Knockdown. Small interfering RNA (siRNA) corresponding to BNIP3 was designed and synthesized by Ambion (Austin, TX), and selectivity for knocking down BNIP3 expression in Mes 23.5 has been shown (Zhang et al., 2007). The gene-specific sequences were: sense, 5'-GCU ACU CUC AGC AUG AGA Att-3' and antisense, 5'-UUC UCA UGC UGA GAG UAG Ctg-3'. Predesigned siRNA for Bax (Cell Signaling Technology, Danvers, MA) was used to knock down gene expression. A second Bax siRNA used to verify knockdown of Bax was produced in Mes 23.5 cells (see Supplemental Figs. S1–S3). The silencer negative-control siRNA, which does not target rat, mouse, or human genes, was used as negative control (Ambion). Transient transfection of siRNA was performed with Lipofectamine 2000 (Invitrogen).

Measurement of Cytosolic-Free Ca^{2+} . Cells were loaded with 2.5 μM fluorescence Ca^{2+} indicator dye fluo-4AM (Invitrogen) in Krebs-Ringer (K-R) buffer consisting of 125 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 5 mM NaHCO_3 , and 25 mM HEPES, pH 7.4, for 30 min at 37°C . Then, cells were washed with K-R buffer and incubated for 30 min at room temperature to allow for de-esterification of the fluo-4 AM dye. Fluorescence was recorded with a FluoMax stirring cell spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ) at 37°C and fluo-4 was excited with a 485-nm laser, and emission was detected at 517 nm. After obtaining the basal signal (F_0), fluorescence intensities were acquired at 3-s

intervals for 5 to 10 min with continuous stirring of the cell suspension. The relative fluorescence intensity (F/F_0) was used as an indicator of $[Ca^{2+}]_i$, where F represents the fluorescence intensity at a given time, and F_0 represents initial fluorescence. In some experiments, fluorescence intensities were converted to $[Ca^{2+}]_i$ by use of the calcium calibration buffer kit 2 (Invitrogen) and the formula: $[Ca^{2+}]_i = K_d[(F - F_{min})/(F_{max} - F)]$, where $K_d = 410$ nM. F_{min} was the minimal fluorescent intensity after removal of free Ca^{2+} deletion by ionomycin (10 μ M) and EGTA (2 mM). F_{max} was obtained from cells incubated with excess Ca^{2+} ($CaCl_2$, 10 mM).

For indirect measurement of the ER calcium pool, fluo-4 AM-loaded cells were bathed in Ca^{2+} free K-R buffer. After establishing a baseline $[Ca^{2+}]_i$, ER Ca^{2+} was mobilized by addition of thapsigargin (TG, 2 μ M). The measured fluo-4 fluorescence ratio between basal and peak fluorescence after TG stimulation was an index of mobilizable $[Ca^{2+}]_{ER}$.

To measure mitochondrial-to-cytosolic Ca^{2+} levels, cells were loaded with the low-affinity Ca^{2+} dye rhod-2 AM (4 μ M) and fluo-4 AM (2.5 μ M) for 30 min at 37°C. Specific mitochondria staining of rhod-2 AM was confirmed in cell culture plates by colocalization with the mitochondria-specific probe MitoTracker green FM and confirmed by producing >95% loss of rhod-2 fluorescence within 5 min after addition of 1 μ M carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to disrupt $\Delta\Psi_m$. Mitochondrial Ca^{2+} and cytosolic Ca^{2+} fluorescence were then monitored at excitation wavelength, 485 and 550 nm, and emission, 517 and 580 nm.

Mitochondrial Membrane Potential. $\Delta\Psi_m$ was monitored with rhodamine 123 (R123) as described previously (Prabhakaran et al., 2005). Uptake of R123 into mitochondria directly reflects the membrane potential; thus, the increase in R123 fluorescence reflects a lowering of $\Delta\Psi_m$. After treatment, cells were loaded with 10 μ M R123 and incubated at 37°C for 30 min in the dark. After washing twice with PBS, changes in R123 fluorescence were monitored with a fluorescence plate reader at 485 nm excitation and 525 nm emission.

Statistical Analyses. Data were expressed as mean $\pm$ S.E.M. One-way analysis of variance followed by Tukey-Kramer procedure for multiple comparisons were used to determine statistical differ-

ences between treatments. Differences were considered significant at $P < 0.05$.

Results

BNIP3 Up-Regulation in Mouse Midbrain TH-Positive Cells. Cyanide toxicity can manifest as a Parkinson-like syndrome as a result of dopaminergic cell death in basal ganglia (Uitti et al., 1985). In the present study, cell death was observed in midbrains of mice repeatedly treated with cyanide as described by Mills et al. (1999) with use of the same duration and dose of KCN treatment. Figure 1A shows distribution of TH-immunoreactive cells in the SN at the level of the interpeduncular nucleus. Normal SN exhibited dense TH-positive cells in pars compacta and fewer numbers of TH-positive cells in pars reticulata, indicating normal architecture of dopaminergic neurons. Loss of TH-positive neurons was first apparent after 3 days of cyanide treatment and progressed over the 9-day treatment period (Fig. 1, B–D).

To determine involvement of BNIP3 in cyanide-mediated death of dopaminergic neurons in mouse brain, the temporal expression of BNIP3 was examined. BNIP3 mRNA increased in the SN within 1 day of treatment, peaked at 4 days, and remained elevated over the 9-day observation period (Fig. 2A). BNIP3 protein was elevated in SN within 3 days and progressively increased over the 9 days (Fig. 2B). Coronal sections from cyanide-treated animals were then examined for cellular distribution of the protein. An increase of BNIP3 immunoreactivity was observed in TH-positive neurons within 3 days of initiating cyanide treatment and persisted throughout the study, whereas mice receiving vehicle control exhibited no detectable BNIP3 (Fig. 3A). An increase of Bax expression was also observed in TH-positive cells after KCN treatment (Fig. 3B).

Cyanide Induces BNIP3-Dependent Cell Death. In Mes 23.5 cells, cyanide induced a BNIP3-dependent apopto-

Fig. 1. Cyanide-induced loss of TH-positive neurons in the SN. Representative photomicrographs of coronal sections through ventral midbrain of animals with 0 (A), 3 (B), and 9 (C) days of cyanide treatment (magnification, 40 $\times$). Bright immunofluorescence corresponds to TH-positive cells. (D) The number of TH-positive cells in ventral-midbrain sections after 3 and 9 days of cyanide treatment (6 mg/kg twice daily). Data represent mean of determinations from two mice.

Fig. 2. Cyanide-induced BNIP3 up-regulation in the ventral-midbrain. Mice were treated with saline or KCN (6 mg/kg twice daily). At indicated days of treatment, the ventral midbrain was collected, and BNIP3 mRNA and protein expression were determined by real-time polymerase chain reaction (A) or Western blot analysis (B). Data represent mean $\pm$ S.E.M. of determinations from three mice. *, Significantly different from saline control group, $P < 0.05$.

sis (Fig. 4A). Overexpression of wild-type BNIP3 by transient transfection increased cell death above control, and cyanide potentiated the BNIP3-mediated cytotoxicity. Overexpression of BNIP3 Δ TM, which functions as a dominant negative mutant, blocked the cyanide-induced cell death, thus showing that cyanide-induced apoptosis was BNIP3-dependent. To determine the intracellular distribution pattern of up-regulated BNIP3 after cyanide, expression was examined by subcellular fractionation-immunoblot analysis. BNIP3 was observed in both ER and mitochondrial fractions within 6 h of initiating cyanide treatment and protein expression progressively increased over the next 24 h (Fig. 4B). Parallel results were observed by double immunofluorescence staining of the cells. In the absence of cyanide, constitutive BNIP3 was expressed at a low level, as indicated by weak green fluorescence (Fig. 4, C and D). In contrast, when BNIP3 was up-regulated by cyanide, it partially colocalized with MitoTracker Red in mitochondria, as indicated by yellow fluorescence. Cyanide-treated cells also exhibited a colocalization of BNIP3 with calnexin, an ER marker. It was concluded that cyanide-induced localization of BNIP3 in both mitochondria and ER.

BNIP3-Mediated ER Ca^{2+} Depletion and Mitochondrial Ca^{2+} . The effect of cyanide on ER Ca^{2+} was evaluated

Fig. 3. Effect of cyanide on BNIP3 and Bax immunoreactivity in TH-positive cells of the SN. Mice were treated with KCN (6 mg/kg twice daily) for 3 days. A, photomicrographs of the SN in which cells were double-immunofluorescence-stained for TH (red) and BNIP3 (green). Overlay of dual staining (orange-yellow) indicates the colocalization of TH and BNIP3. B, photomicrographs of SN in which cells were stained for TH (red) and Bax (green). Arrows indicate colocalization of TH and Bax. Scale bar = 25 μm .

by monitoring TG-induced Ca^{2+} release in cells in which BNIP3 expression was knocked down. The siRNA knockdown was used as an alternate approach to reducing BNIP3 function as opposed to use of the dominant negative mutant BNIP3 Δ TM described above. Figure 5 shows that the siRNA effectively reduced cellular BNIP3 expression. Cells were treated with cyanide and loaded with fluo-4 AM, and then ER Ca^{2+} release was initiated by TG, an ER Ca^{2+} ATPase inhibitor that induces release of Ca^{2+} . Representative fluorometric Ca^{2+} traces obtained from cells treated with control siRNA or BNIP3 siRNA 12 h after cyanide are shown in Fig. 6A. The TG-releasable pool of ER Ca^{2+} was significantly reduced as early as 12 h after cyanide (Fig. 6B). To confirm that reduction in the TG-inducible $[\text{Ca}^{2+}]_i$ spike was the result of ER Ca^{2+} depletion, cells were exposed to exogenous Ca^{2+} . Cyanide-treated cells showed a higher capacitative Ca^{2+} entry compared with untreated control cells, indicating a lower $[\text{Ca}^{2+}]_{\text{ER}}$ and thus a higher store-operated Ca^{2+} entry.

Pseudosynaptic contacts between ER and mitochondria facilitate rapid Ca^{2+} uptake by mitochondria after release of Ca^{2+} from the ER pool (Rizzuto et al., 1993). Therefore, the effect of cyanide on mitochondrial Ca^{2+} was determined by loading cells with rhod-2 AM and fluo-4 AM to simultaneously measure mitochondrial and cytosolic free Ca^{2+} . The ratio of rhod-2 to fluo-4 fluorescence served as an index of the mitochondrial Ca^{2+} level. Intracellular cytosolic free Ca^{2+} levels were not changed at 6 h, 12 h, and 24 h after cyanide compared with control cells (data not shown). On the other hand, treatment

Fig. 4. Cyanide-induced apoptosis and subcellular localization of BNIP3 expression. **A**, Mes 23.5 cells were transfected with empty vector (EV), BNIP3 wild-type, or BNIP3 Δ TM for 24 h, followed by KCN (400 μ M) for 24 h; then, apoptosis was determined. Data represent mean $\pm$ S.E.M. of four separate determinations. *, Significantly different from control (EV without KCN); #, significantly different from EV with KCN, $P < 0.05$. **B**, cells were treated with KCN for varying times, followed by subcellular fractionation. BNIP3 expression was then determined by Western blotting in mitochondrial (HM) or ER (LM) fractions. Fraction purity was confirmed by assessing the presence of COX IV (mitochondrial biomarker) and calnexin (ER biomarker). **C**, **D**, cells were treated with KCN for 24 h and then BNIP3 expression determined by immunostaining with anti-BNIP3 antibodies (green). Mitochondria were labeled with MitoTracker red and ER with anticalnexin antibodies (red). Confocal microscopy shows colocalization of BNIP3 and mitochondria (**C**) or ER (**D**). Scale bar, 10 μ m.

with cyanide significantly increased mitochondrial Ca^{2+} within 12 h (Fig. 6C). The rise in mitochondrial Ca^{2+} was reduced by BNIP3 knockdown and by Ru-360, a mitochondrial Ca^{2+} uniporter inhibitor (Fig. 6, C and D). It was concluded that BNIP3 is critical for mitochondrial Ca^{2+} uptake in cyanide-treated cells.

Bax Is a Downstream of BNIP3 in Depleting ER Ca^{2+} . Because Bax has been reported to be activated after BNIP3

Fig. 5. Cyanide-induced BNIP3 expression was knocked down by siRNA. Western blot analysis of whole cell homogenates was conducted 24 h after transfection with siRNA. Densitometric data were normalized with the loading control β -actin and represents the mean $\pm$ S.E.M. of three separate determinations. Mock = cells undergoing transfection with Lipofectamine alone. *, Significantly different from the mock transfection alone. #, Significantly different from the KCN + control siRNA group.

overexpression (Kubli et al., 2007), the relationship of Bax expression to BNIP3 was determined in cyanide-treated cells. At 24 h after transfection with control or BNIP3 siRNA, cells were subjected to cellular fractionation and Bax expression determined by immunoblot analysis. The Bax siRNA was specific for Bax knockdown, as determined by use of two different siRNA that produced parallel effects on expression (see Supplemental Fig. S1). Bax activation was determined by a rabbit anti-Bax antibody (Millipore) that specifically recognizes the active conformation of Bax (Desagher et al., 1999). In the control siRNA group, activated Bax was detected in both the ER and mitochondrial fractions within 12 h after cyanide (Fig. 7A). In contrast, BNIP3 knockdown significantly attenuated Bax activation. To further confirm that BNIP3 acts upstream of Bax, cells were transiently transfected with wild-type BNIP3, and, 24 h later, Bax immunoreactivity was determined. BNIP3 overexpression significantly increased Bax activity in both ER and mitochondria (Fig. 7B). Furthermore, the effect of Bax knockdown was examined in cells transfected with Bax siRNA. Bax expression was reduced in control and cyanide-treated cells by siRNA, whereas BNIP3 expression was not altered (Fig. 8A). It is noteworthy that knockdown of Bax blocked cyanide-induced release of ER Ca^{2+} and, in turn, blocked the mitochondrial Ca^{2+} uptake (Fig. 8B). Note that a parallel study was conducted with a second Bax siRNA and yielded similar Ca^{2+} responses, thus demonstrating the target specificity of the siRNA (Fig. S2). It is concluded that BNIP3 is critical for mitochondrial Ca^{2+} uptake in cyanide-treated cells and Bax is downstream of BNIP3.

Bax Contributes to Mitochondrial Dysfunction and Cell Death. Mitochondrial Ca^{2+} overload can produce permeabilization of the outer mitochondrial membrane and subsequent activation of the apoptotic cascade (Halestrap 2006). To determine whether $\Delta\Psi_m$ dissipation accompanies cyanide-induced mitochondrial Ca^{2+} overload, cells were treated with KCN in the presence or absence of Ru-360 (mitochon-

Fig. 6. BNIP3-mediated depletion of ER Ca²⁺ and mitochondrial Ca²⁺ overload in cyanide-treated Mes 23.5 cells. Cells were transfected with control or BNIP3 siRNA for 24 h, followed by KCN (400 μ M). **A**, representative traces showing transfected cells at 12 h after KCN. Cells were loaded with fluo-4 AM, and ER Ca²⁺ release was measured. Addition of TG is indicated by the arrow. After reaching baseline (control), CaCl₂ (10 mM) was added to the extracellular medium, and cytosolic Ca²⁺ was quantified. **B**, levels of TG-releasable ER Ca²⁺ after KCN treatment. **C**, the ratio of mitochondrial to cytosolic Ca²⁺ (Mito: Cyto Ca²⁺) was estimated by measuring rhod-2/fluo-4 fluorescence. **D**, the ratio of mitochondrial to cytosolic Ca²⁺ following KCN in the presence or absence of Ru-360. Data represent mean $\pm$ S.E.M. of three to six separate determinations. *, Significantly different from control group; #, significantly different from KCN + RNAi control group; &, significantly different from KCN group; $P < 0.05$.

drial Ca²⁺ uniporter inhibitor), and the level of $\Delta\Psi_m$ was examined by the R123 method. The cyanide-induced decrease of $\Delta\Psi_m$ was blocked by Ru-360, showing that Ca²⁺ overload contributes to the mitochondrial dysfunction (Fig. 9A). The action of Bax on mitochondrial function was confirmed by transfecting cells with Bax siRNA (Fig. 9B). Bax knockdown blocked cyanide-induced $\Delta\Psi_m$ dissipation, confirming involvement of Bax in the effect on $\Delta\Psi_m$.

The relationship of mitochondrial Ca²⁺ and Bax to cyanide-induced cell death was also examined. Cyanide-induced apoptosis within 24 h and Ru-360 significantly attenuated cyanide-induced cytotoxicity (Fig. 10A), consistent with our previous report (Zhang et al., 2007). Furthermore, knockdown of Bax by siRNA reduced the extent of cell death (Fig. 10B), indicating Bax expression is necessary for cyanide-induced apoptosis.

Discussion

In the present study, cyanide-induced mitochondrial dysfunction and death of dopaminergic cells was mediated by up-regulation and localization of BNIP3 to mitochondria and ER. After exposure to a cell death stimulus, BNIP3 undergoes insertion into the outer mitochondrial membrane, leading to mitochondrial membrane permeability and activation of the cell death cascade (Vande Velde et al., 2000). This study provides an alternative explanation for BNIP3-mediated cell death by showing that BNIP3 and Bax activation modulate Ca²⁺ mobilization in the ER. Subsequent mitochondrial Ca²⁺ overload then promotes mitochondrial dysfunction and execution of apoptosis.

Cyanide is a classic mitochondrial toxicant associated with a

secondary Parkinsonism that occurs after severe, acute intoxication (Uitti et al., 1985; Sarikaya et al., 2006). In sublethal toxicity, a postintoxication sequelae can manifest as akinesia and rigidity. Selective degeneration of dopaminergic basal ganglia pathways have been reported (Rosenberg et al., 1989; Rosenow et al., 1995). The primary action of cyanide is inhibition of cytochrome c oxidase in complex IV of the oxidative phosphorylation chain, thereby blocking intracellular oxygen utilization (histotoxic hypoxia) and reducing cellular ATP generation (Leavesley et al., 2008). The central nervous system has a limited anaerobic metabolic capacity and high energy dependence, making it one of the most susceptible organs to cyanide toxicity. In the mouse, sublethal doses of cyanide produced neuronal death in the SN (Mills et al., 1999). This study shows that TH-positive cells of the SN are the most sensitive to cyanide, with substantial loss of cells observed within 3 days of initiation of cyanide exposure.

Differential expression and subcellular localization of BNIP3 play significant roles in regulating cell responses to various stimuli. In hypoxia-mediated cell death, BNIP3 translocates to mitochondria to reduce $\Delta\Psi_m$ and initiate mitochondrial-mediated cell death (Kubli et al., 2007). In cultured mesencephalic cells BNIP3 can also localize to the ER, thereby stimulating mobilization of ER Ca²⁺ to initiate cell death (Zhang et al., 2009). Burton et al. (2006) observed in glial and glioblastoma tumor cells that BNIP3 is expressed in the nucleus, and hypoxia stimulates BNIP3 translocation to cytosol and mitochondria to participate in execution of cell death. Similarly in normoxic hepatocytes, constitutive BNIP3 is localized to the nucleus and after hypoxia, BNIP3 levels increased in cyto-

Fig. 7. Relationship of BNIP3 expression to Bax activation. A, cells were transfected with control or BNIP3 siRNA and 24 h later were treated with KCN (400 μ M) for 12 h. Bax expression in mitochondria (HM) and ER (LM) was detected by Western blotting. Densitometric data were normalized with the loading control COX IV (mitochondria) or calnexin (ER) and represent the mean $\pm$ S.E.M. of three separate determinations. #, Significantly different from the KCN + control siRNA group in HM; &, significantly different from the KCN + control siRNA group in LM. B, cells were transfected with wild-type BNIP3 cDNA, and 24 h later, Bax and BNIP3 expression was determined by Western blotting in HM and LM fractions. EV = empty vector.

plasm (Metukuri et al., 2009). Sequestering BNIP3 in the nucleus blocks BNIP3's association with mitochondria and other organelles, thereby suppressing execution of cell death.

In the present study, forced overexpression of wild-type BNIP3 enhanced the level of cell death produced by KCN and increased mitochondrial and ER BNIP3 levels. Using a transmembrane domain deleted mutant (BNIP3 Δ TM), which functions as a dominant negative mutant, KCN-induced cell death was blocked, thus showing BNIP3 is involved in execution of the cell death. Our previous study, using BNIP3 mutants containing ER and mitochondrial targeting signals, showed that both ER and mitochondrial localization are critical in executing BNIP3-mediated cell death (Zhang et al., 2009). Mutants targeted to ER stimulate Ca^{2+} mobilization, resulting in an overload of mitochondrial Ca^{2+} and cell death. On the other hand, selective targeting of BNIP3 to

Fig. 8. Effect of Bax knockdown on ER and mitochondrial Ca^{2+} levels. A, cells were transfected with either control or Bax siRNA, and 24 h later they were treated with KCN for 12 h. Bax and BNIP3 expression was then determined in whole-cell lysates, and the level of TG-releasable ER Ca^{2+} was measured in intact cells. B, cells were transfected with control or BNIP3 siRNA, followed by KCN treatment for 12 h and then the ratio of mitochondrial to cytosolic Ca^{2+} (Mito:Cyto Ca^{2+}) determined. Data represent mean $\pm$ S.E.M. of three to six separate determinations. *, Significantly different from control group; #, significantly different from KCN + control siRNA group; $P < 0.05$.

mitochondria produces a Ca^{2+} -independent cell death. It would be interesting to study the role of other subcellular sites in BNIP3-mediated cell death by using BNIP3 mutants lacking ER and/or mitochondrial localization signals.

Cyanide-induced histotoxic hypoxia induces BNIP3 up-regulation by the hypoxia-inducible factor-1 dependent pathway (Zhang et al., 2007). It was observed that cyanide up-regulated BNIP3 in TH-positive cells in mouse brain and in the Mes 23.5 cell line, thus providing strong evidence for involvement of BNIP3 in the dopaminergic cell death. Subcellular fractionation showed that both BNIP3 and Bax expression increased in ER and mitochondria after cyanide treatment. It is possible that the colocalized proteins interact to initiate cell death by modulating ER Ca^{2+} mobilization. Overexpression of BNIP3 stimulated Bax localization in both ER and mitochondria. As expected, BNIP3 knockdown reduced the level of Bax at the two sites. The Bax activation was critical for BNIP3-mediated cell apoptosis since Bax knockdown blocked cyanide-induced mitochondrial dysfunction and cell death.

It was shown that Bax is a downstream effector of BNIP3 in mitochondria-mediated apoptosis, but the relationship of increased BNIP3 expression to Bax activation is not clear. Regulation of Bax activity by BNIP3 seems not to be at the transcription level, because Bax mRNA and protein levels

Fig. 9. Blockade of mitochondrial Ca^{2+} uniporter or Bax silencing attenuated KCN-induced loss of $\Delta\Psi_m$. A, cells were treated with KCN (400 μM) for 24 h in the presence or absence of 10 μM Ru-360. The relative $\Delta\Psi_m$ was then monitored by the R123 method. Data represent mean $\pm$ S.E.M. of six separate determinations. *, Significantly different from control (no treatment) group; #, significantly different from KCN treatment group; $P < 0.05$. B, cells were transfected with control or Bax siRNA, followed by KCN for 24 h; then, the relative $\Delta\Psi_m$ was determined. Data represent mean $\pm$ S.E.M. of six separate determinations. *, Significantly different from RNAi control group; #, significantly different from KCN + control RNAi group; $P < 0.05$.

remain unchanged after cyanide treatment (Zhang et al., 2007). Under normal conditions Bax is a cytosolic protein that translocates to the ER/mitochondria in response to apoptotic stimuli (Lahiry et al., 2008; Liao et al., 2008). On the other hand, overexpressed BNIP3 can localize to both ER and mitochondria (Ray et al., 2000; Zhang et al., 2009). The mechanism underlying the BNIP3-dependent activation and translocation of Bax is not clear. Bax activation may occur after heterodimerization with BNIP3, either at the ER or mitochondria membrane or in the cytosol before membrane insertion. Studies with cell-free systems and recombinant proteins have shown that BH3-peptides derived from Bim and Bid can directly bind and subsequently activate Bax to induce cytochrome c release from mitochondria (Cartron et al., 2004; Kuwana et al., 2005). Alternatively, BNIP3 binding of Bax in the cytosol may induce a conformational change in Bax to expose the organelle targeting signal that is normally embedded in the protein structure. This would enhance Bax translocation from cytosol to ER/mitochondria. On the other hand, BH3-only proteins may insert into outer membranes of

Fig. 10. Blockade of mitochondrial Ca^{2+} uniporter or Bax silencing attenuated KCN-induced apoptosis. A, cells were treated with KCN (400 μM) for 24 h in the absence or presence of Ru-360 (10 μM), and apoptotic cell death was determined. Data represent mean $\pm$ S.E.M. of four separate determinations. *, Significantly different from control (no treatment) group; #, significantly different from KCN treatment group; $P < 0.05$. B, cells were transfected with control or Bax siRNA, followed by exposure to KCN for 24 h; then, apoptosis was determined. Data represent mean $\pm$ S.E.M. of four separate determinations. *, Significantly different from control RNAi group; #, significantly different from KCN + RNAi control group; $P < 0.05$.

organelles and induce a conformational change in the membrane to facilitate insertion of activated Bax. Finally, most BH3-only proteins, including BNIP3, have a strong binding affinity for Bcl-2 or Bcl-X_L (Willis and Adams 2005), suggesting BNIP3 may sequester Bcl-2 or Bcl-X_L from Bax to indirectly activate Bax.

Forced overexpression of BNIP3 mutants targeted to the ER can produce ER Ca^{2+} depletion (Zhang et al., 2009). In this study, up-regulation of BNIP3 in response to cyanide exerted the same effect on ER Ca^{2+} . Transfection of cells with BNIP3 siRNA effectively knocked down BNIP3 expression and blocked Ca^{2+} release from ER, thus confirming that BNIP3 disrupts ER Ca^{2+} homeostasis. Bax was required for this action of BNIP3 because cells deficient in Bax (Bax knockdown) were resistant to BNIP3-mediated Ca^{2+} mobilization. Knockdown of Bax did not change BNIP3 expression, confirming Bax acts downstream of BNIP3.

A number of studies have shown that translocation of Bax to the ER can modulate ER Ca^{2+} homeostasis to induce release of Ca^{2+} into the cytosol (Jones et al., 2007; Stout et al., 2007). In turn, increased mitochondrial uptake of Ca^{2+} can lead to dissipation of the membrane potential and then execution of cell death. For instance, overexpression of Bax caused re-distribution of Ca^{2+} from ER to mitochondria, which then produced mitochondrial membrane transition and release of cytochrome c (Nutt et al., 2002b). This was

followed by activation of downstream apoptotic signaling. It seems that Bax activation is necessary for the transfer of Ca^{2+} from ER to mitochondria and then execution of cell death. In Bax/Bak-deficient cells, the mitochondrial Ca^{2+} uptake and subsequent mitochondrial dysfunction is significantly reduced after stimuli that induce release of Ca^{2+} from ER (Demaurex and Distelhorst, 2003). In addition, microinjection of Bax into astrocytes evokes Ca^{2+} wave propagation in cells by stimulating Ca^{2+} to relocate from ER to mitochondria (Carvalho et al., 2004). Recently Jones et al., (2007) reported that T cells lacking both Bax and Bak display defects in inositol 1,4,5-trisphosphate-dependent Ca^{2+} mobilization.

Present results demonstrate that Bax activation is essential for BNIP3-mediated mitochondrial dysfunction and apoptosis in response to cyanide. We have previously shown that cyanide-induced apoptosis in Mes 23.5 cells is caspase-independent (Zhang et al., 2007). This is consistent with the observation that Bax can stimulate release of apoptosis-induced factor from mitochondria to initiate mitochondrial-mediated death in the absence of caspase activation (Kawakami et al., 2008). Bax may also induce caspase-independent cell death through mitochondrial fission (Arnoult et al., 2005). In this case, Bax undergoes oligomerization on the mitochondrial membrane and then induces a shift in the DRP1 (a fission protein) cycling dynamics, thereby promoting fission of mitochondrial membrane, ultimately leading to cell death.

In conclusion, the current study provides evidence that mobilization of Ca^{2+} in the ER and subsequent mitochondrial Ca^{2+} overload occurs during cyanide-induced cell death. BNIP3 initiates a Bax-dependent release of Ca^{2+} from the ER to activate mitochondria-mediated apoptosis.

References

- Althaus J, Bernaudin M, Petit E, Toutain J, Touzani O, and Rami A (2006) Expression of the gene encoding the pro-apoptotic BNIP3 protein and stimulation of hypoxia-inducible factor-1alpha (HIF-1alpha) protein following focal cerebral ischemia in rats. *Neurochem Int* **48**:687–695.
- Arnoult D, Rismanchi N, Grodet A, Roberts RG, Seeburg DP, Estaquier J, Sheng M, and Blackstone C (2005) Bax/Bak-dependent release of DDP/TIMM8a promotes Drp1-mediated mitochondrial fission and mitoptosis during programmed cell death. *Curr Biol* **15**:2112–2118.
- Burton TR, Henson ES, Baijal P, Eisenstat DD, and Gibson SB (2006) The pro-cell death Bcl-2 family member, BNIP3, is localized to the nucleus of human glial cells: implications for glioblastoma multiforme tumor cell survival under hypoxia. *Int J Cancer* **118**:1660–1669.
- Cartron PF, Gallenne T, Bougras G, Gautier F, Manero F, Vusio P, Meflah K, Vallette FM, and Juin P (2004) The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. *Mol Cell* **16**:807–818.
- Carvalho AC, Sharpe J, Rosenstock TR, Teles AF, Youle RJ, and Smaili SS (2004) Bax affects intracellular Ca^{2+} stores and induces Ca^{2+} wave propagation. *Cell Death Differ* **11**:1265–1276.
- Chinnadurai G, Vijayalingam S, and Gibson SB (2009) BNIP3 subfamily BH3-only proteins: mitochondrial stress sensors in normal and pathological functions. *Oncogene* **27**:S114–S127.
- Demaurex N and Distelhorst C (2003) Cell biology. Apoptosis—the calcium connection. *Science* **300**:65–67.
- Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B, and Martinou JC (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol* **144**:891–901.
- Di Filippo M, Tambasco N, Muzi G, Balucani C, Saggese E, Parnetti L, Calabresi P, and Rossi A (2008) Parkinsonism and cognitive impairment following chronic exposure to potassium cyanide. *Mov Disord* **23**:468–470.
- Halestrap AP (2006) Calcium, mitochondria and reperfusion injury: a pore way to die. *Biochem Soc Trans* **34**:232–237.
- Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals*, 7th ed. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington DC.
- Jones RG, Bui T, White C, Madesh M, Krawczyk CM, Lindsten T, Hawkins BJ, Kubek S, Frauwirth KA, Wang YL, et al. (2007) The proapoptotic factors Bax and Bak regulate T cell proliferation through control of endoplasmic reticulum Ca^{2+} homeostasis. *Immunity* **27**:268–280.
- Kawakami M, Inagawa R, Hosokawa T, Saito T, and Kurasaki M (2008) Mechanism of apoptosis induced by copper in PC12 cells. *Food Chem Toxicol* **46**:2157–2164.
- Kubli DA, Ycaza JE, and Gustafsson AB (2007) Bnip3 mediates mitochondrial dysfunction and cell death through Bax and Bak. *Biochem J* **405**:407–415.
- Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, and Newmeyer DD (2005) BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* **17**:525–535.
- Lahiry L, Saha B, Chakraborty J, Bhattacharyya S, Chattopadhyay S, Banerjee S, Choudhuri T, Mandal D, Bhattacharyya A, Sa G, et al. (2008) Contribution of p53-mediated Bax transactivation in the flavin-induced mammary epithelial carcinoma cell apoptosis. *Apoptosis* **13**:771–781.
- Le WD, Colom LV, Xie WJ, Smith RG, Alexianu M, and Appel SH (1995) Cell death induced by beta-amyloid 1–40 in MES 23.5 hybrid clone: the role of nitric oxide and NMDA-gated channel activation leading to apoptosis. *Brain Res* **686**:49–60.
- Leavesley HB, Li L, Prabhakaran K, Borowitz JL, and Isom GE (2008) Interaction of cyanide and nitric oxide with cytochrome c oxidase: implications for acute cyanide toxicity. *Toxicol Sci* **101**:101–111.
- Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, and Korsmeyer SJ (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2**:183–192.
- Li R, Boehm AL, Miranda MB, Shangary S, Grandis JR, and Johnson DE (2007) Targeting antiapoptotic Bcl-2 family members with cell-permeable BH3 peptides induces apoptosis signaling and death in head and neck squamous cell carcinoma cells. *Neoplasia* **9**:801–811.
- Liao PC, Tan SK, Lieu CH, and Jung HK (2008) Involvement of endoplasmic reticulum in paclitaxel-induced apoptosis. *J Cell Biochem* **104**:1509–1523.
- Metukuri MR, Beer-Stolz D, Namas RA, Dhupar R, Torres A, Loughran PA, Jefferson BS, Tsung A, Billiar TR, Vodovotz Y, et al. (2009) Expression and subcellular localization of BNIP3 in hypoxic hepatocytes and liver stress. *Am J Physiol Gastrointest Liver Physiol* **296**:G499–G509.
- Mills EM, Gunasekar PG, Li L, Borowitz JL, and Isom GE (1999) Differential susceptibility of brain areas to cyanide involves different modes of cell death. *Toxicol Appl Pharmacol* **156**:6–16.
- Nutt LK, Chandra J, Pataer A, Fang B, Roth JA, Swisher SG, O'Neil RG, and McConkey DJ (2002a) Bax-mediated Ca^{2+} mobilization promotes cytochrome c release during apoptosis. *J Biol Chem* **277**:20301–20308.
- Nutt LK, Pataer A, Pahler J, Fang B, Roth J, McConkey DJ, and Swisher SG (2002b) Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca^{2+} stores. *J Biol Chem* **277**:9219–9225.
- Prabhakaran K, Li L, Mills EM, Borowitz JL, and Isom GE (2005) Up-regulation of uncoupling protein 2 by cyanide is linked with cytotoxicity in mesencephalic cells. *J Pharmacol Exp Ther* **314**:1338–1345.
- Prabhakaran K, Li L, Zhang L, Borowitz JL, and Isom GE (2007) Upregulation of BNIP3 and translocation to mitochondria mediates cyanide-induced apoptosis in cortical cells. *Neuroscience* **150**:159–167.
- Ray R, Chen G, Vande Velde C, Cizeau J, Park JH, Reed JC, Gietz RD, and Greenberg AH (2000) BNIP3 heterodimerizes with Bcl-2/Bcl-X(L) and induces cell death independent of a Bcl-2 homology 3 (BH3) domain at both mitochondrial and nonmitochondrial sites. *J Biol Chem* **275**:1439–1448.
- Rizzuto R, Brini M, Murgia M, and Pozzan T (1993) Microdomains with high Ca^{2+} close to IP_3 -sensitive channels that are sensed by neighboring mitochondria. *Science* **262**:744–747.
- Rosenberg NL, Myers JA, and Martin WR (1989) Cyanide-induced Parkinsonism: clinical, MRI, and 6-fluorodopa PET studies. *Neurology* **39**:142–144.
- Rosenow F, Herholz K, Lanfermann H, Weuthen G, Ebner R, Kessler J, Ghaemi M, and Heiss WD (1995) Neurological sequelae of cyanide intoxication - the pattern of clinical, magnetic resonance imaging, and positron emission tomography findings. *Ann Neurol* **38**:825–828.
- Sarikaya I, Apaydin H, Topal U, and Karaoglan O (2006) Cyanide-induced Parkinsonism and F-18 FDG PET/CT findings. *Clin Nucl Med* **31**:363–364.
- Shou Y, Li L, Prabhakaran K, Borowitz JL, and Isom GE (2003) p38 Mitogen-activated protein kinase regulates Bax translocation in cyanide-induced apoptosis. *Toxicol Sci* **75**:99–107.
- Stout BA, Melendez K, Seagrave J, Holtzman MJ, Wilson B, Xiang J, and Tesfayigzi Y (2007) STAT1 activation causes translocation of Bax to the endoplasmic reticulum during the resolution of airway mucous cell hyperplasia by IFN-gamma. *J Immunol* **178**:8107–8116.
- Tracy K and Macleod KF (2007) Regulation of mitochondrial integrity, autophagy and cell survival by BNIP3. *Autophagy* **3**:616–619.
- Uitti RJ, Rajput AH, Ashenhurst EM, and Rozdilsky B (1985) Cyanide-induced parkinsonism: a clinicopathologic report. *Neurology* **35**:921–925.
- Vande Velde C, Cizeau J, Dubik D, Alimonti J, Brown T, Israels S, Hakem R, and Greenberg AH (2000) BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol Cell Biol* **20**:5454–5468.
- Wang W and Chan JY (2006) Nr1f1 is targeted to the endoplasmic reticulum membrane by an N-terminal transmembrane domain. Inhibition of nuclear translocation and transacting function. *J Biol Chem* **281**:19676–19687.
- Willis SN and Adams JM (2005) Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol* **17**:617–625.
- Zhang L, Li L, Liu H, Borowitz JL, and Isom GE (2009) BNIP3 mediates cell death by different pathways following localization to endoplasmic reticulum and mitochondrion. *FASEB J* **23**:3405–3414.
- Zhang L, Li L, Liu H, Prabhakaran K, Zhang X, Borowitz JL, and Isom GE (2007) HIF-1alpha activation by a redox-sensitive pathway mediates cyanide-induced BNIP3 upregulation and mitochondrial-dependent cell death. *Free Radic Biol Med* **43**:117–127.

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