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Acidosis Regulates the Stability, Hydrophobicity, and Activity of the BH3-Only Protein Bnip3

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ABSTRACT

Bnip3 is a prodeath member of the so-called BH3-only subfamily of Bcl-2 proteins. A major function of this class of proteins is to regulate the permeability state of the outer mitochondrial membrane by forming homo- and hetero-oligomers inside the membrane. We reported previously that Bnip3 accumulates in cardiac myocytes during exposure to hypoxia, but coincident acidosis is required to activate the death program. Acidosis increased the rate of intracellular accumulation of Bnip3 and promoted a tighter association with mitochondria. Here we report that acidic pH mediates increased half-lives of Bnip3 dimers and monomers (>3×) as well as that of a faster-migrating fragment (>10×) and confers protection against degradation by protease. Hydrophobic partitioning experiments revealed that Bnip3 monomers and oligomers from hypoxia-acidic cell fractions associated significantly with the detergent layer, whereas protein from hypoxia-neutral myocytes did not. Acidosis promoted homodimerization of Bcl-xL but did not increase its association with detergent. Neutralization of the extracellular medium of cardiac myocyte cultures under hypoxia-acidosis resulted in rapid degradation of accumulated Bnip3 (half life, <2 h), coincident with cessation of the death program. Bnip3 monomers appear to be the active species because substitution of alanine for histidine at position 173 within the transmembrane (TM) domain prevented homodimerization but did not inhibit the death function. These results demonstrate a pH-sensitive shift in the stability and apparent hydrophobicity of Bnip3 monomers that correlates closely with membrane binding and function. *Antioxid. Redox Signal.* 8, 1625–1634.

INTRODUCTION

THE BCL-2 FAMILY PROTEINS REGULATE programmed death interacting with intracellular organelles, in particular mitochondria, endoplasmic reticulum (ER), and possibly nuclear membranes, thereby determining the exchange of signaling molecules between these organelles and the cytoplasm. The family is divided into three groups based on structure and function [reviewed in (22, 31, 38]. The first group contains four Bcl-2 homology (BH) domains, has antiapoptotic/prosurvival properties, and includes Bcl-2, Bcl-xL, and Mcl-1. The second has two or three BH domains, promotes apoptosis and includes Bax, Bak, and Bad. The third, known as the BH3-only group, also has proapoptotic functions and includes Bik, Bid, Bim, Nix, NOXA, HGTD-P, RTP801, and Bnip3. The BH3-only proteins may modulate the function of the first two groups by physical interactions or may function independently to promote apoptosis (7, 16, 21). Multiple lines of evidence connect the function of Bcl-2 family proteins with their subcellular localization and association with membranes (8, 31). Most of the Bcl-2 proteins including the BH3-only group have C-terminal transmembrane (TM) domains that facilitate membrane anchoring and integration (22, 31). Antiapoptotic Bcl-2 and Mcl-1 are integral membrane proteins permanently inserted into the outer mitochondrial, ER, or nuclear envelope membranes. Most of the other proteins are cytoplasmic or loosely membrane associated in healthy cells, undergoing membrane translocation in re-

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sponse to death signals (22). Membrane translocation may involve conformational change (Bax, Bcl-w) (5, 18, 19, 25, 45), proteolytic cleavage (Bid) (2, 24), or phosphorylation (Bad, Bim, Bmf) (1, 9, 14).

Most studies have found that the BH3 region is essential for the proapoptotic functions of the BH3-only proteins (7, 16). This region is required for binding to other Bcl-2 family members (29). The BH3-only proteins Bid and Bim permeabilize mitochondria directly by hetero-oligomerizing with proapoptotic Bax and Bak. Bad and Bik, conversely, permeabilize by antagonizing antiapoptotic proteins, Bcl-xL and Bcl-2 (21, 23, 26). Homo-oligomers of the cleaved form of Bid (tBid) form transmembrane pores that permeabilize mitochondria independently of other Bcl-2 proteins or the mitochondrial permeability transition pore (MPTP) (13). These studies implicate BH3-only proteins in three types of interaction: (a) complexes with other Bcl-2 proteins and the MPTP, (b) hetero-oligomeric pore-forming complexes, and (c) homo-oligomeric pore-forming complexes. The independent pore-forming properties of Bcl-2 family oligomers are supported by structural studies and permeability studies of lipid vesicles (21, 46).

Bnip3 contains a region of limited homology to the consensus BH3 peptide sequence, but this region is not required for the death-promoting function (28, 37). Bnip3 also contains a TM region, and this is required for membrane association, dimerization, and the death function. Induction of Bnip3 by hypoxia generates a loosely membrane-associated protein that becomes tightly bound during acidosis (20). We show here that acidosis mediates a pronounced increase in the stability of both the Bnip3 monomer and the SDS-resistant dimer. This is associated with increased resistance to proteases, enhanced degree of hydrophobicity, and activation of the death function. Mutations that eliminated homodimerization but maintained membrane association also retained the death function. The results are consistent with a pH-dependent insertion of Bnip3 into the membrane that activates monomer-dependent death functions. These changes are reminiscent of those that occur when Bax translocates to mitochondria in response to death-promoting stimuli (13, 18).

MATERIALS AND METHODS

Reagents

Antibodies to Bcl-xL and actin were from Santa Cruz Biotechnology and anti-Bnip3 was from R. K. Bruick (3). Cycloheximide, protease K and Triton X-114 were from Sigma Chemical Co. (St. Louis, MO).

Cell culture

Methods for primary culture of neonatal rat cardiac myocytes have been previously described (39, 40). In brief, enriched cultures of myocytes were obtained from 1- to 2-day-old neonatal rats by stepwise trypsin dissociation. They were plated at a density of 4×10^6/60-mm dish. Incubations were in defined serum-free DMEM/M-199 (4:1) medium supplemented with transferrin, vitamin B_{12}, and insulin. The final cultures contained >97% cardiac myocytes contracting at >200 beats/min. HEK 293 cells were grown in Minimal Essential Media (Cellgro) supplemented with 10% FBS.

Hypoxia and acidosis

Details of our methods for exposing cells to hypoxia in an environmental chamber at neutral or acidic pH have been described previously (20, 41). To maintain a neutral extracellular pH during hypoxia, the culture medium was either adjusted to pH 7.4 with NaOH dissolved in DMEM or the culture medium was replaced with fresh hypoxic medium every 12 h. For hypoxia–acidosis, the medium was adjusted to the required pH with a mixture of lactic and phosphoric acids dissolved in culture medium as described previously (41) or by allowing acid to build up in the medium and maintaining glucose at >5 m*M* by addition of 100× glucose stock (27). Oxygen was continuously monitored with an oxygen electrode inside the chamber, pH was measured using an Orian micro-pH electrode, contractility was monitored by edge detection as described previously (39), and glucose was measured with standard glucometer strips (40). The chamber oxygen concentration was maintained at <10 mm Hg. Cultures were lysed under hypoxia using ice-cold deoxygenated buffers for analyses.

Western blots

Our procedures for Western blots have been described in detail elsewhere (20, 41). Equal amounts of protein were fractionated on 12% SDS-polyacrylamide gels and electroblotted to nitrocellulose (BioRad). Blots were stained with Ponceau Red to monitor the transfer of proteins. Membranes were blocked as described previously and incubated with specific antibodies for 2–4 h. After washing, the blots were reacted with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (ECL; Pierce).

Northern blots

Northern blot procedures were exactly as described previously (25) with full-length rat BNIP3 and β-actin probes.

DNA Fragmentation and nuclear condensation

Genomic DNA fragmentation (DNA ladders) was analyzed exactly as described previously (10, 20, 41). Samples (7 μg) were subjected to electrophoresis in 2% agarose gels and imaged by ethidium bromide staining and digital photography. Cells were examined for morphologic evidence of apoptosis or necrosis after staining with the fluorescent DNA-binding dyes Hoechst 33342 and propidium iodide (PI) (10, 20). Live cells were incubated with 1-mg/ml Hoechst 33342 and 1-mg/ml PI for 20 min, washed twice with PBS, and analyzed by microscopy. Nuclear diameters were quantified using Image J software to estimate nuclear condensation.

Subcellular fractionation

Cell fractionation and alkali treatments were performed as described (37). In brief, after treatments, cells were washed with PBS and incubated in buffer containing 100 m*M* manni-

tol, 10 m*M* Tris, 5 m*M* $MgCl_2$, 1 m*M* EGTA, 1 m*M* DTT and lysed by 50 strokes of a Dounce homogenizer with tight-fitting pestle. Samples were split in two, and the pH of one half was adjusted to 11.0 with 1 *M* Na_2CO_3 and incubated on ice for 20 min. Both samples were fractionated by differential centrifugation. Intact cells and nuclei were separated by centrifugation at 120 *g* for 5 min; supernatants were centrifuged at 10,000 *g* for 10 min to collect the heavy (mitochondrial) membrane pellet. Cytoplasmic fractions were obtained by centrifuging supernatants at 100,000 *g* for 30 min.

Triton-X114 phase partitioning

To examine the hydrophobicity of Bnip3, Triton X-114 phase partitioning was used as described previously (19). In brief, cytosolic extracts prepared in isotonic buffers at either pH 7.4 or 6.6 were treated with 2% (vol/vol) Triton X-114, and the hydrophobic detergent phase was partitioned by incubation at 37°C followed by high-speed centrifugation. Bnip3 was analyzed in the fractions by Western blot.

Site-directed mutagenesis

Site-directed mutagenesis was performed in wild-type pBnip3 (24) using the Quick-Change kit (Strategene, La Jolla, CA) described previously (15, 44). The His_{173}-Ala mutation was confirmed by sequencing.

Transfections

HEK 293 cells and cardiac myocytes were transfected as described previously (10). The 50% confluent plates were co-transfected with 7 μg of the indicated Bnip3 plasmid and 1 μg of luciferase expression plasmid using Lipofectamine 2000 reagent (Invitrogen). At 6 h after transfection, the medium was changed, and at indicated times, the cells were harvested for luciferase assays, as described previously (25).

Statistics

Western blots were scanned and analyzed by NIH Image. Error bars represent SEM; significance was calculated using analysis of variance (ANOVA) software.

RESULTS

Acidosis promotes Bnip3 accumulation and increased stability

Figure 1A shows a typical time course of Bnip3 accumulation after exposure of cardiac myocytes to hypoxia. Protein can be seen after 2 h and peaks between 24 and 36 h at neutral pH. Under conditions of progressive acidosis mediated by prolonged hypoxia without media change or by manipulating the medium pH as described in Methods, more Bnip3 accumulated and an additional band that migrated slightly faster than the monomer was evident (Fig. 1A; 36-h time point, pH 6.6). The abundance of the Bnip3 monomer increased by 5.7- ± 1.8-fold ($n = 6$; $p < 0.01$) under acidic conditions and the rapidly migrating form by >15-fold ($n = 6$; $p < 0.001$). Messenger RNA levels of Bnip3 were not affected by acidosis; therefore to investigate the nature and kinetics of the regulation, protein synthesis was inhibited with cycloheximide, and protein levels were measured at progressive time points after cycloheximide addition (Fig. 1B). These results are quantified in Fig. 1C. Under hypoxia and pH neutral conditions, the Bnip3 dimer had an apparent half-life of 3.9 ± 1 h, and the monomer, 3.5 ± 1 h ($n = 3$). Under hypoxia–acidosis these half-lives were increased several fold with no loss of protein at 4 h ($n = 3$), and only a slight reduction at 12 h (not shown). Longer time points were not investigated because of cycloheximide toxicity.

Acidosis protects against protease digestion

Increased stability in response to low pH could result from protection against proteases, perhaps due to altered conformational or intracellular localization. To determine whether pH affected proteolysis, extracts were prepared from cardiac myocytes subjected to pH neutral hypoxia or hypoxia–acidosis as described in Fig. 1. The extracts were subjected to increasing concentrations of proteinase K, and the products analyzed by Western blots. These results are shown in Fig. 2A and B. Bnip3 monomer from acidotic myocytes was significantly more resistant to proteinase K digestion than were the pH neutral samples. The Bnip3 dimer was less sensitive to digestion than was the monomer, and the sensitivity was not affected by pH (Fig. 2C). Like the Bnip3 dimer, actin digestion was not affected by pH. These results support a low pH–mediated change in the conformation or physical location of the Bnip3 monomer that makes it less accessible to proteolysis.

Increased TX144 partitioning of low-pH Bnip3

Hydrophobic proteins partition preferentially into detergent layers after mixing and separation of aqueous and detergent phases. To determine whether the partitioning of Bnip3 was affected by pH, cardiac myocytes from hypoxia–pH neutral (HX) or hypoxia–acidotic (HA) conditions were lysed and the extracted proteins subjected to TX-144 partitioning as described in Methods.

Figure 3A confirms our previous report that the majority of Bnip3 accumulated under hypoxia–pH-neutral conditions is associated with mitochondria, but a fraction of this is washed into the cytoplasmic fraction by alkali treatment. In contrast, alkali treatment had no effect on the intracellular membrane localization of Bnip3 under hypoxia–acidic conditions. The consistent recovery of alkali-solubilized Bnip3 only from hypoxia–pH-neutral extracts supports the presence of a loosely bound fraction that is eliminated by acidosis. Detergent partitioning was implemented to determine whether this correlated with increased hydrophobicity of Bnip3 at acidic pH that may indicate increased membrane penetration. After the partitioning procedure, Bnip3 species corresponding to monomer, dimer, and higher molecular-weight complexes were detected in different fractions (Fig. 3B). Bnip3 from hypoxia–pH-neutral conditions was predominantly present as a dimer of ~60 kDa in the aqueous fraction with <20% associated with detergent. In contrast, a significant fraction of Bnip3 monomer and dimer from hypoxia–acid conditions was recovered in the detergent layer. Higher molecular-weight species were about evenly distributed between aqueous and

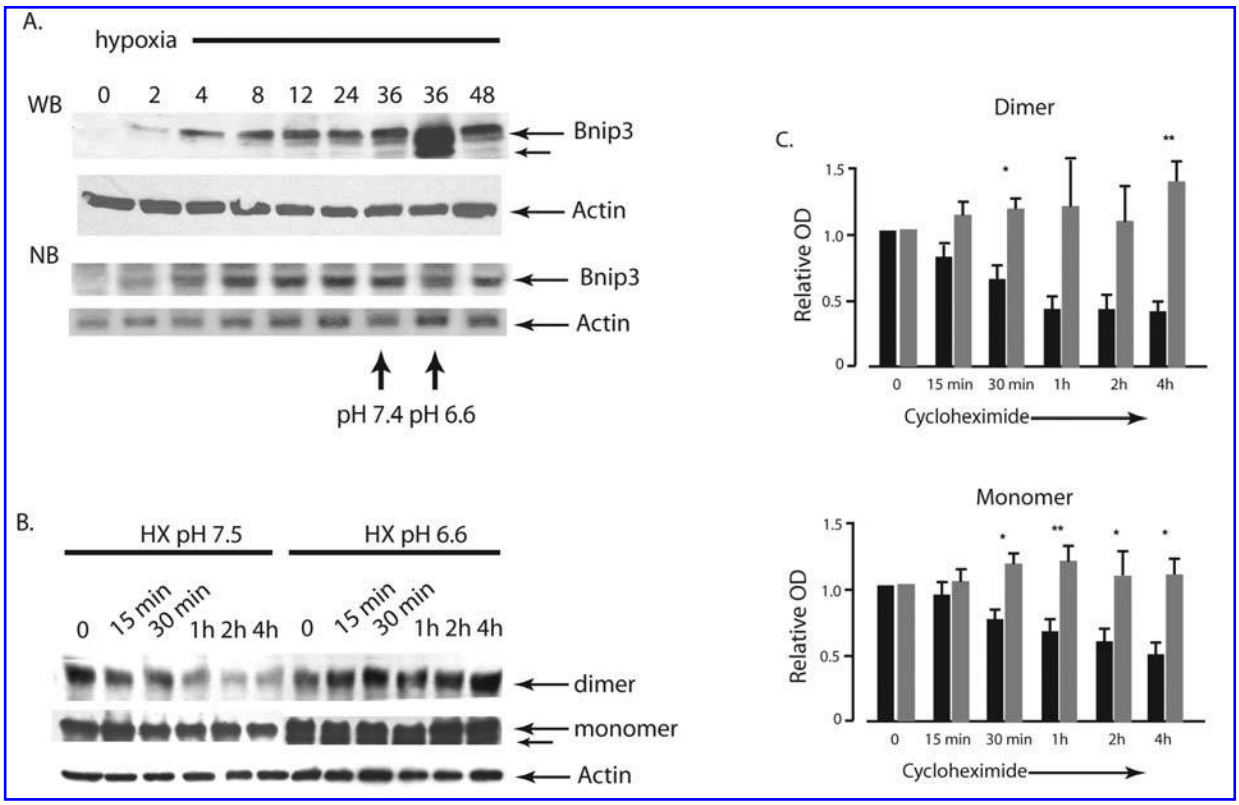

FIG. 1. Enhanced stability of Bnip3 by low pH. (**A**) Cardiac myocytes were subjected to hypoxia or hypoxia–acidosis, as described in Methods, and harvested at the indicated times. Samples containing equal amounts of protein were analyzed by Western blots (WBs) probed with anti-Bnip3. As a loading control, the blot was stripped and reprobed with anti-actin. The pH of the media at the time of harvesting is indicated for the 36-h hypoxia–acid sample; the pH of hypoxia-only cultures was 7.4. The lower two panels show Northern blots (NBs) of RNA from the same time points probed with Bnip3 and actin cDNAs. (**B**) Cardiac myocytes were subjected to hypoxia with or without medium change for 36 h, treated with 5 μg/ml cycloheximide for the indicated periods, and harvested for Western blot analysis with anti-Bnip3 antibody. Lower gel shows the same blot probed with anti-actin. The pH of the media at the time of treatment is indicated. (**C**) Densitometry of the Bnip3 dimer (top), and monomer (middle), relative to the untreated sample in hypoxia–neutral (black bars) and hypoxia–acidotic (gray bars) conditions. [$*p < 0.05$; $**p < 0.01$ for hypoxia (HX) compared with hypoxia–acid (HA) at the indicated cycloheximide incubation period; $n = 3$].

detergent fractions. Quantification of Bnip3 hybridized from the detergent fraction from three different experiments confirmed that acidosis significantly shifted Bnip3 from aqueous to detergent layers (Fig. 3C). In contrast to Bnip3, Bcl-xL partitioned independently of pH into the aqueous (85%) and detergent (15%) phases. Acidosis promoted a shift of Bcl-xL monomer into dimer, both associated with the aqueous phase. This is consistent with a previous report that acidic pH promotes dimerization of Bcl-xL (43). These results support a low pH-dependent increase in the hydrophobicity of Bnip3 that is consistent with the enhanced membrane association and stability that is mediated by acidosis.

Kinetics of Bnip3 regulation by pH

To examine rates of Bnip3 turnover in response to a change in extracellular pH, cardiac myocytes were exposed to hypoxia or hypoxia–acid for 36 h, and Bnip3 levels were measured at progressive time points after the medium was acidified or neutralized, respectively (Fig. 4). Acidification of 36-h hypoxic cultures caused an immediate induction of Bnip3 that reached 6 ± 1.2-fold above the pH-neutral level after 4 h. Neutralization of 36-h acidic cultures mediated a decrease of Bnip3 with similar but inverse kinetics. These results are quantified in Fig. 4B. Note that some lanes in Fig. 4A are overexposed to allow visualization of all time points; quantification was implemented on lower exposures within the linear range. To determine whether the acid-activated death program was interrupted by neutralization, DNA fragmentation and nuclear condensation assays were implemented (Fig. 4C and D). DNA ladders become progressively more intense with time during hypoxia–acidosis (Fig. 4C, lanes 4 and 9). Neutralization of the pH at 36 h inhibited the progression of DNA fragmentation (compare lanes 4, 5, and 9). Conversely, acidification of pH rapidly promoted DNA fragmentation (lane 6). These results confirm the pH-dependence of this death pathway and the dynamic relation between pH, Bnip3 levels, and progression of cell death. Results from DNA ladders were supported by analyses of Hoechst-stained nuclei (Fig. 4D). Nuclear diameters were reduced by 64% after 72-h hypoxia–acidosis ($p < 0.01$ comparing treated with air controls) similar to staurosporine treatments (Fig. 4E). The diam-

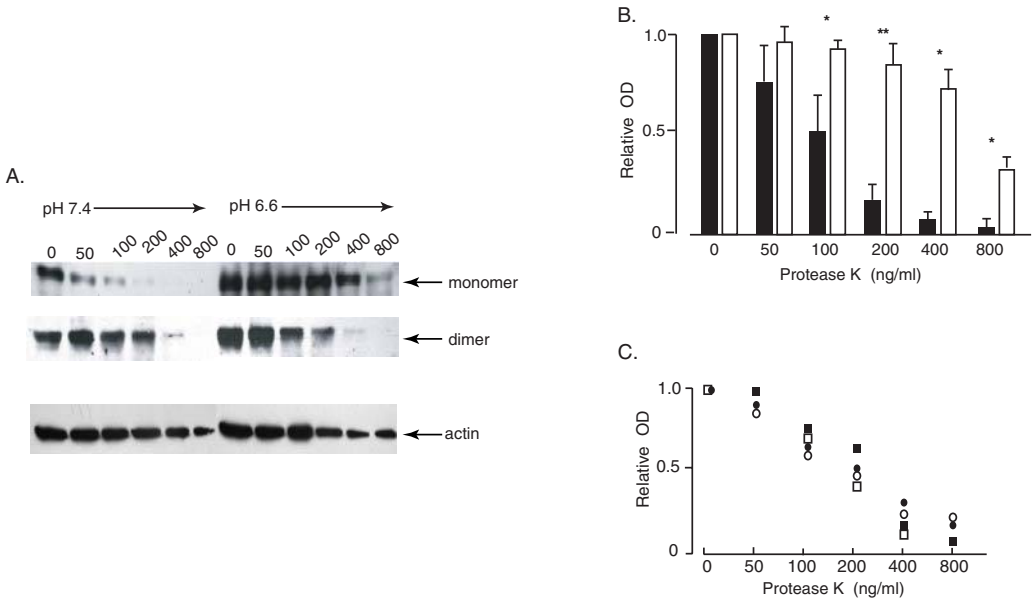

FIG. 2. Bnip3 susceptibility to proteinase K digestion. **(A)** Cardiac myocytes were subjected to hypoxia with or without medium change for 36 h. Cell lysates were treated with the indicated amounts of proteinase K (ng/ml) for 5 min; reactions were stopped with 10 m*M* PMSF, and samples were analyzed by Western blotting with anti-Bnip3 antibody. Lower panel in 1A shows the same blot probed with anti-actin. The pH of the media at the time of treatment is indicated**. (B)** Densitometry of the Bnip3 monomer; *solid* and *open* bars refer to hypoxia–neutral and hypoxia–acidic, respectively**. (C)** Densitometry of Bnip3 dimer (*squares*) and actin (*circles*), *solid* symbols are pH 7.4, *open* symbols, pH 6.6. (*$p < 0.05$; **$p < 0.01$ for HX compared with HA at the indicated PK concentration; $n = 4$).

eters of nuclei from 36 h of hypoxia–acid and 36 h hypoxia–acid with 36-h hypoxia–neutral pH were reduced by 19 ± 3.3% and 21 ± 4.1%, respectively (not significantly different from each other).

Bnip3 dimers are not required for the death function

Our results indicate that acidosis mediates increased stability and possibly hydrophobicity of Bnip3 monomers and oligomers. Bax and tBid homodimers have been assigned direct death-promoting functions through their ability to permeabilize the mitochondrial outer membrane, independent of other Bcl-2 proteins (11, 13, 30, 35). To determine whether dimerization is required for the death function of Bnip3, we replaced the histidine residue at position 273 within the TM domain (His_{273}) with alanine and tested for oligomerization and the death function. We reported previously that transfected wild-type Bnip3 did not induce significant cell death of cardiac myocytes unless the transfected cells were subjected to hypoxia and/or acidosis (25). This is not the case for HEK 293 cells; therefore these cells were used in luciferase reporter cotransfection assays to determine the death-promoting function of the His_{173}-Ala mutation. As shown in Fig. 5, wild-type Bnip3 but neither Bnip3ΔTM nor His_{273}-Ala generated the slower migrating band with a size consistent with a homodimer. The rapidly migrating Bnip3 species were also present in all three transfections (Fig. 5A, short arrows), indicating that their formation was not pH-dependent in HEK 293 cells, and not necessarily linked to the death function because Bnip3ΔTM does not kill. Cotransfection of Bnip3 expression constructs into HEK 293 with a luciferase reporter (8:1 ratio of Bnip3 plasmid to reporter plasmid) showed persistent expression of the reporter in cells transfected with the reporter only, or cells cotransfected with Bnip3ΔTM but significant loss of expression from contransfections with wild-type Bnip3 or the His_{273}-Ala mutant construct. Independent transfections using GFP instead of luciferase confirmed the potent killing of cells by wild-type and His_{273}-Ala mutant but not ΔTM (latter data not shown; see reference 24).

DISCUSSION

Most of the Bcl-2 proteins contain C-terminal transmembrane (TM) domains consisting of a short chain (15–22) of hydrophobic amino acids that facilitate membrane targeting and/or insertion (reviewed in reference 30). The TM domains of Bcl-2, Mcl-1, and Bak are probably exposed and direct the proteins for insertion into membranes immediately after or during translation, where they reside permanently as integral membrane proteins in the ER and mitochondria (2, 31). The TM of Bax is folded back into a hydrophobic pocket so that the protein is soluble and can reside in the cytosolic compartment. In this case, membrane translocation is induced by apoptotic stimuli and involves a conformational change that unfolds the TM (4, 5). Bcl-xL and Bcl-w have intermediate properties and can exist as soluble or loosely membrane-bound proteins or integrated into the membrane (18, 42). Our

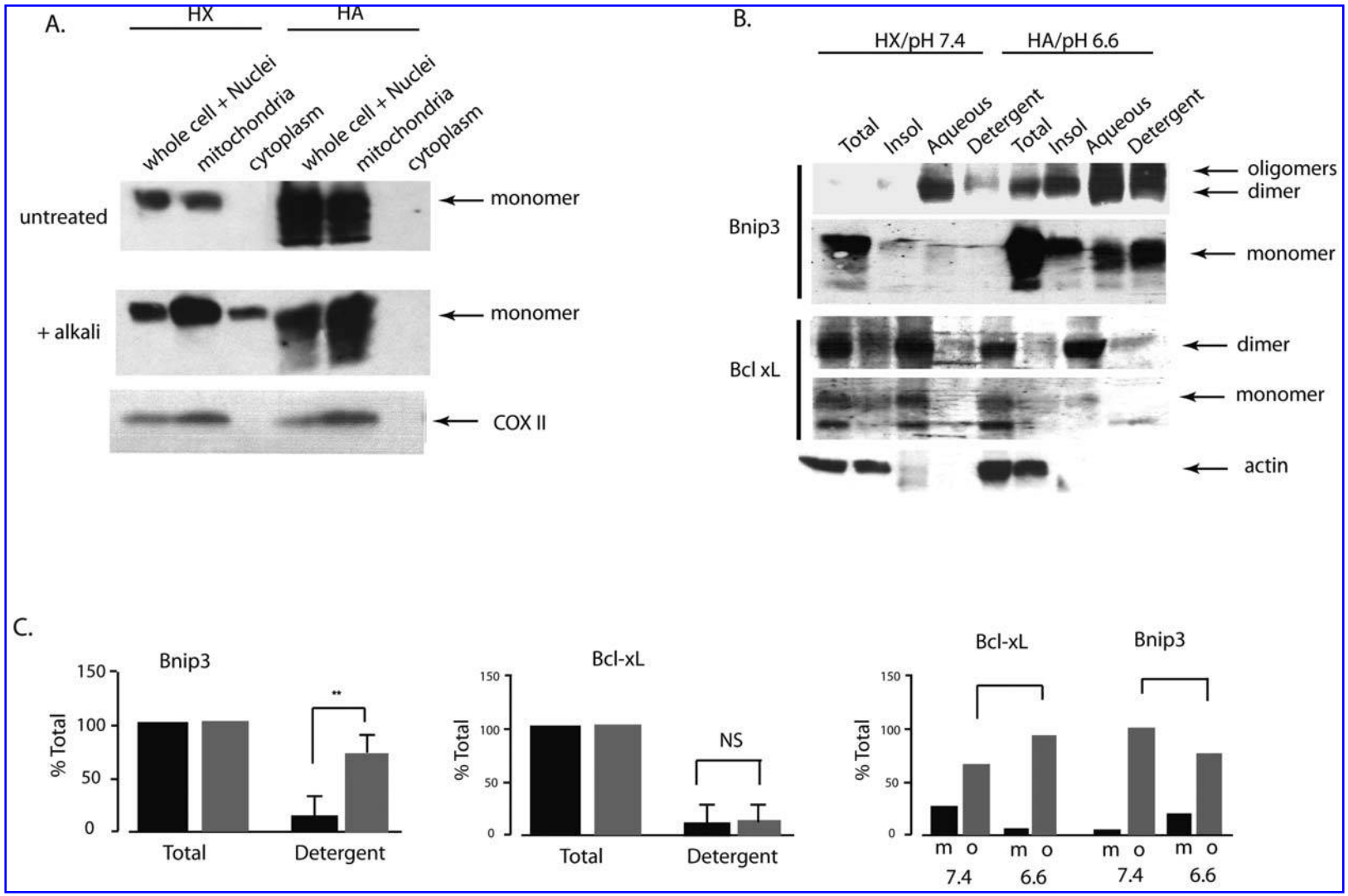

FIG. 3. Bnip3 Triton X-114 phase partitioning. **(A)** Cardiac myocytes were exposed to hypoxia with (HX) or without (HA) medium change for 36 h, as described in Methods, and subsequently harvested in hypotonic lysis buffer. Cell lysates were treated with alkali (+ alkali) or not (untreated) and fractions separated by differential centrifugation, as described previously (25). Fractions were analyzed by Western blots probed with anti-Bnip3. To determine fractional purity, blots were reprobed with anti-cytochrome oxidase (COX). **(B)** Cardiac myocytes were subjected to hypoxia with (pH 7.4) or without (pH 6.6) medium change for 36 h, and 200 μg of total protein was subjected to Triton X-114 phase partitioning as described in Methods. Insoluble, aqueous, and detergent fractions and 200 μg of total cell lysate were analyzed by Western blots with anti-Bnip3 or Bcl-xL antibodies. Lower gel shows the same blot probed with anti-actin. **(C)** Densitometry of total Bnip3 (left) and total Bcl-xL (right) partitioning to the detergent fraction in hypoxia–neutral (*black bars*) and hypoxia–acidotic (*gray bars*) conditions. (**$p < 0.01$ for HX compared with HA in the detergent fraction; $n = 3$). Right shows quantization of Bcl-xl and Bnip3 monomers (m) and oligomers (o) at neutral and acidic pH. All data represent three separate experiments.

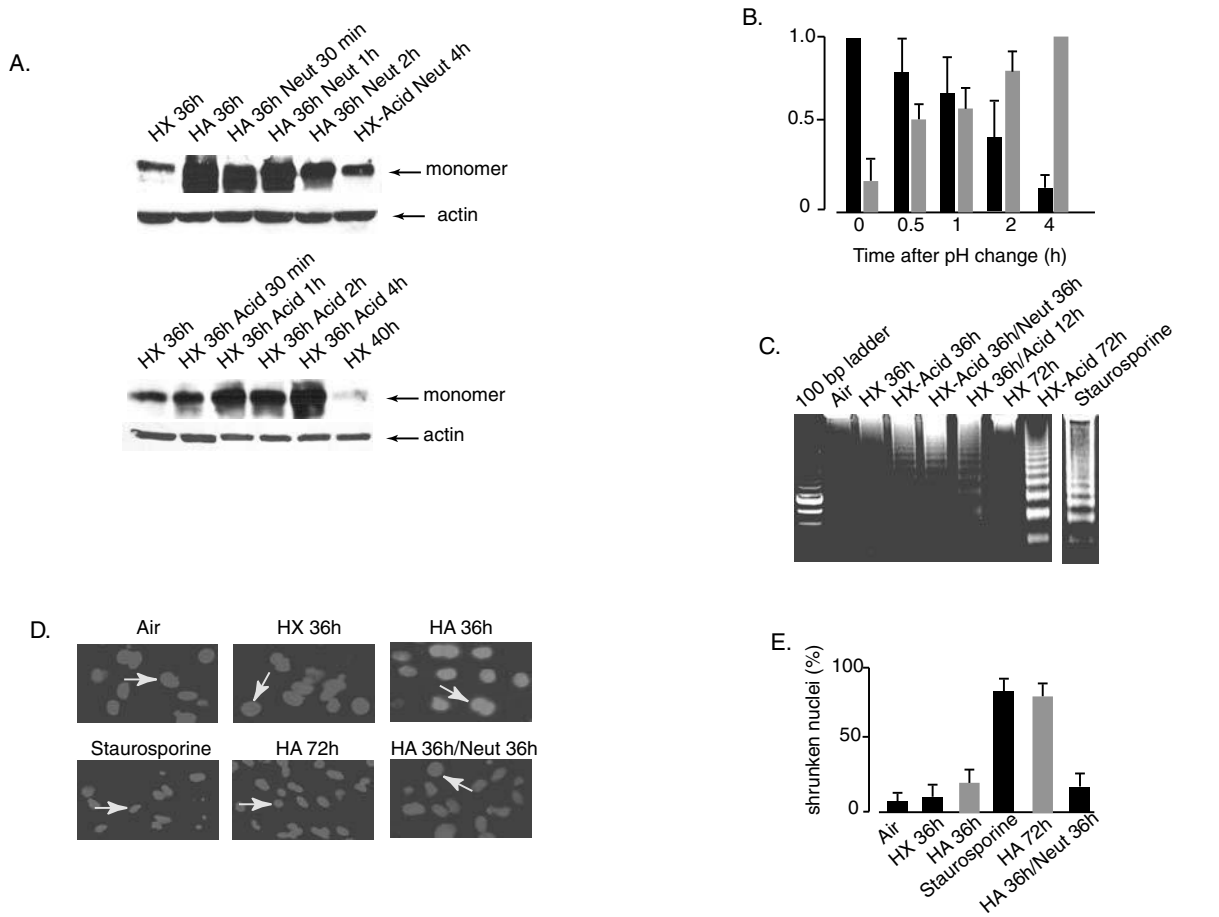

FIG. 4. Kinetics of Bnip3 stability regulation by pH. (A) Cardiac myocytes were subjected to hypoxia with (HX) or without (HA) medium change for 36 h. After 36 h, HA plates were neutralized with fresh hypoxic media (upper panel), and HX plates were acidified with conditioned media (lower panel), as described in (29), and incubated for 0.5–4 h. At the indicated times, cells were harvested and proteins analyzed by Western blotting with anti-Bnip3 antibody. Lower blot in each panel shows the same blot probed with anti-actin. **(B)** Densitometric quantification of Bnip3 in neutralized hypoxia–acidotic samples (*black bars*) and acidified hypoxia–neutral samples (*gray bars*). Results are representative of four separate experiments. **(C)** Cardiac myocytes were subjected to hypoxia and neutralization/acidification as in **B** and incubated for the indicated times. Cell lysates were harvested for genomic DNA fragmentation and analyzed by ethidium bromide staining. **(D, E)** Cardiac myocytes were subjected to hypoxia and neutralization/acidification, as in **B** and **C,** and stained with Hoechst 33342 after the indicated times; *arrows* indicate typical large nuclei under the indicated conditions compared with highly shrunken nuclei in cultures treated with staurosporine or HA for 72 h. **(E)** represents quantification from at least 100 nuclei per sample; shrunken nuclei are <50% diameter of normal nuclei measured from aerobic cultures. All results are representative of at least three separate experiments.

results indicate that endogenous Bnip3 also has intermediate properties, with a structure that is loosely membrane associated and inactive at neutral pH but tightly membrane associated and possibly integrated at low pH. This conclusion is supported by the following observations:

1. When proteins were extracted from hypoxic myocytes at neutral pH, Bnip3 was membrane-associated, but ≥30% of this was solubilized by alkali treatment. In contrast, the same alkali treatment did not release Bnip3 from the membrane fraction of hypoxia–acidotic extracts. It is not clear why only ~30% of the Bnip3 protein was released from the pH-neutral membranes; however, the significance of the exclusive partitioning to the membrane fraction in the hypoxia–acidic condition is accentuated by the greater than fivefold higher abundance of total Bnip3 in this condition.
2. In hydrophobic partitioning experiments, significantly more Bnip3 from hypoxia–acidotic extracts partitioned into the detergent fraction compared with hypoxia–neutral pH extracts. In contrast, the partitioning of neither Bcl-xL nor actin was affected by pH.
3. Bnip3 monomers in extracts from hypoxia–acidotic cells were significantly more resistant to proteinase K digestion compared with monomers from equivalent neutral pH extracts. The dimer was more resistant than the monomer at neutral pH, and pH did not influence the sensitivity of either dimer or actin to proteinase K.

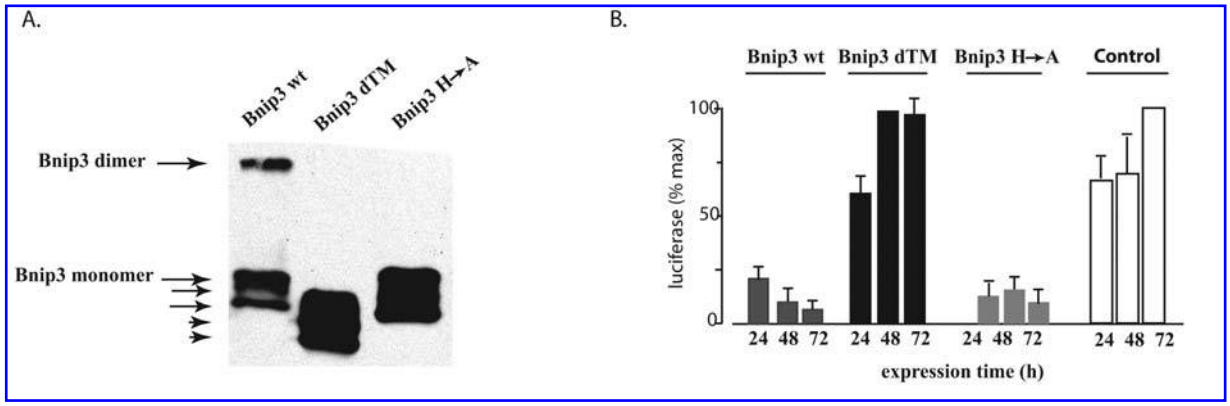

FIG. 5. Mutation of His_{173} inhibits homodimerization but not death. HEK-293 cells were transfected with the respective Bnip3 plasmids (7 μg) and pSVGL2 (Promega) (1 μg), as described in Methods. From 24 to 72 h after transfections, cells were harvested, and proteins analyzed by Western blot **(A)** or luciferase assay **(B)** using equal amounts of protein. Expression of luciferase in the presence of Bnip3ΔTM or control (without cotransfected Bnip3 DNA) was significantly higher at all time points than the cotransfections with wild-type or the H173-A mutation ($p < 0.01$; $n = 4$). No significant difference was found between wild-type and H173-A mutations.

4. The highly significant increase in the half-life of Bnip3 under hypoxia–acidotic conditions determined by the cycloheximide inhibition time course indicates a pH-dependent change in state of the protein and is consistent with a conformational change and/or membrane integration.

Our results also confirm the close correlation between Bnip3 stabilization by low pH and activation of the death program. The rate of acid production by cardiac myocytes during hypoxic culture is >10-fold higher than in equivalent aerobic culture. After ~24 h of hypoxic culture, the buffer capacity begins to fail, and the pH decreases steeply. Bnip3 accumulation, tighter membrane association, and initiation of the death program all closely parallel the decline of pH.

We demonstrated previously that this death program could be inhibited by antisense Bnip3 (20). When hypoxic cardiac myocytes were acidified, Bnip3 levels increased by 8.5-fold within 4 h, and the death program was rapidly activated. Conversely, when hypoxia–acidotic cultures were neutralized, Bnip3 levels declined to the preacidotic levels within 4 h and halted the death program. Therefore, sustained levels of "activated" Bnip3 are required to complete the death program.

In other BH3-only proteins, oligomerization through the BH3 domain is required for the death function; our results indicate that this is not the case for Bnip3. A previous study demonstrated that substitution of alanine for His173 in the isolated Bnip3 TM prevented its oligomerization but not membrane insertion (33). We confirmed the former result for the intact Bnip3 protein and further demonstrated that the His_{273}-Ala Bnip3 mutant was competent to induce cell death. Therefore, oligomerization of Bnip3 either with itself or with other Bcl-2 proteins may not be required for the death pathway.

Consistent with this independence from protective (Bcl-2, Bcl-XL) proteins, previous studies have also shown that tBid induced pore formation within mitochondrial membranes independent of other Bcl-2 proteins (13).

The activation of proapoptotic functions by membrane targeting has been described for Bax and the BH3-only proteins Bad and Bid (reviewed in 16, 22, 30). Bax is loosely membrane associated in healthy cells, and insertion into the mitochondrial membranes involves conformational changes that are initiated by diverse death signals (4, 5, 17). During Bax activation, the TM domain is released from its hydrophobic pocket, exposing other BH-domains that promote membrane insertion and oligomerization. Previous studies have also revealed a pH-regulated component in the conformational transition and membrane insertion of Bax in response to TNF-α, staurosporine, ceramide, and interleukin-7 withdrawal (6, 19, 34). The conformational change can be elicited by moderately increased or decreased pH, and multiple studies including biophysical analyses of purified protein support a critical role for pH. Changes in pH were shown to regulate the conformation, hydrophobicity, and membrane insertion of Bax (19). Our studies indicate that this also may be true for Bnip3; acidic pH increased the membrane association and hydrophobicity but decreased the sensitivity to protease digestion, possibly indicating a compact conformation and/or direct membrane sequestration. We have not been able to analyze the conformational changes of Bnip3 directly because, like Bcl-2, intact Bnip3 has poor solubility *in vitro* [data not shown, see (27)].

The property of Bnip3 to exist in an inactive, latent configuration explains why cells can tolerate high levels of Bnip3 and remain viable. Hypoxic cardiac myocytes maintained at neutral pH accumulate Bnip3 but remain viable and functional (contractile). High levels of Bnip3 have been reported in skeletal muscle (37) as well as in the hypoxic zone of solid tumors (32), and the cells are viable in both cases. We have found that Bnip3 levels fluctuate in the late embryonic heart, and Bnip3 is expressed strongly in adult heart without apparent adverse effects (Spiga M-G, Yeasky T, and Webster KA, unpublished data). The requirement for activation of death-promoting proteins by a secondary death signal is not uncommon: Bad must be dephosphorylated to be released from 14–3-3 proteins, Bid is activated by cleavage, and Bax requires conformational change as well calpain cleavage to produce full activity (36).

In conclusion, we provide evidence that Bnip3 can exist in a latent form that is bound to membranes in an inactive state at neutral pH. In response to a pH decrease of less than 1 unit, Bnip3 becomes more hydrophobic and assumes a physical structure or state that is resistant to proteolysis. Hypoxia and acidosis routinely accompany ischemia, and a pH decrease within the range that activates Bnip3 is associated with myocardial ischemia as well as with apoptotic initiation in response to multiple other stimuli (6, 12). We propose that membrane integration of Bnip3 monomers is the initial step of the death program initiated by Bnip3. The requirement for an activation step for Bnip3 can explain why high levels of Bnip3 can accumulate in viable tissues. It is not clear whether the maintenance of Bnip3 in adult tissues (skeletal and cardiac muscle) has a physiologic function, but it seems possible that strategies to control acidosis during myocardial ischemia may help preserve tissue and reduce infarction by neutralizing the Bnip3 pathway.

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