Bnip3 functions as a mitochondrial sensor of oxidative stress during myocardial ischemia and reperfusion

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Bnip3 functions as a mitochondrial sensor of oxidative stress during myocardial ischemia and reperfusion

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Kubli DA, Quinsay MN, Huang C, Lee Y, Gustafsson ÅB. Bnip3 functions as a mitochondrial sensor of oxidative stress during myocardial ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* 295: H2025–H2031, 2008. First published September 12, 2008; doi:10.1152/ajpheart.00552.2008.—Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3 (Bnip3) is a member of the Bcl-2 homology domain 3-only subfamily of proapoptotic Bcl-2 proteins and is associated with cell death in the myocardium. In this study, we investigated the potential mechanism(s) by which Bnip3 activity is regulated. We found that Bnip3 forms a DTT-sensitive homodimer that increased after myocardial ischemia-reperfusion (I/R). The presence of the antioxidant *N*-acetylcysteine reduced I/R-induced homodimerization of Bnip3. Overexpression of Bnip3 in cells revealed that most of exogenous Bnip3 exists as a DTT-sensitive homodimer that correlated with increased cell death. In contrast, endogenous Bnip3 existed mainly as a monomer under normal conditions in the heart. Screening of the Bnip3 protein sequence revealed a single conserved cysteine residue at position 64. Mutation of this cysteine to alanine (Bnip3C64A) or deletion of the NH_2-terminus (amino acids 1-64) resulted in reduced cell death activity of Bnip3. Moreover, mutation of a histidine residue in the COOH-terminal transmembrane domain to alanine (Bnip3H173A) almost completely inhibited the cell death activity of Bnip3. Bnip3C64A had a reduced ability to interact with Bnip3, whereas Bnip3H173A was completely unable to interact with Bnip3, suggesting that homodimerization is important for Bnip3 function. A consequence of I/R is the production of reactive oxygen species and oxidation of proteins, which promotes the formation of disulfide bonds between proteins. Thus, these experiments suggest that Bnip3 functions as a redox sensor where increased oxidative stress induces homodimerization and activation of Bnip3 via cooperation of the NH_2-terminal cysteine residue and the COOH-terminal transmembrane domain.

Bcl-2 homology domain 3 proteins; apoptosis; reactive oxygen species; cysteine residues

MYOCARDIAL ISCHEMIA-REPERFUSION (I/R) injury is associated with an extensive loss of myocardial cells due to both necrosis and apoptosis. Bcl-2 family members are important regulators of cell death in myocardial cells (16). This family is divided into antiapoptotic members, such as Bcl-2 and Bcl-x_L, and proapoptotic members, which include Bax and Bak as well as Bcl-2 homology domain 3 (BH3)-only proteins (i.e., Bad, Noxa, and Puma). BH3-only proteins function as sensors of stress in the cell and transduce these signals to the downstream effectors Bax and/or Bak. At least 10 different BH3-only proteins have been identified, and they differ in their mode of activation. Their proapoptotic activity is regulated by transcription and/or posttranslational modification (16). Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3 (Bnip3) is a BH3-only protein that is localized primarily to the mitochondria (7, 18, 35). Overexpression of Bnip3 leads to the activation of Bax/Bak, opening of the mitochondrial permeability transition pore, and cell death (21, 22, 28, 31). Bnip3 is substantially upregulated in the heart after acute ischemia, in chronic heart failure after myocardial infarction in vivo (12, 28), and in patients with ischemic cardiomyopathy and end-stage heart failure (13). In addition, Bnip3 contributes to cell death during remodeling of the heart after myocardial infarction (9).

Bnip3 is constitutively expressed in the adult heart, suggesting that Bnip3 exists in an "inactive" state under nonstressed conditions (18). Bnip3 is a significant contributor to I/R injury by inducing mitochondrial dysfunction (13, 18); however, exactly how I/R causes the activation of Bnip3 is not known. In this study, we investigated the potential mechanism(s) by which Bnip3 is activated in cardiac cells in response to I/R. We provide evidence that Bnip3 functions as a mitochondrial sensor of oxidative stress where an increase in reactive oxygen species (ROS) induces the homodimerization and activation of Bnip3 via a conserved cysteine residue in the NH_2 terminus.

MATERIALS AND METHODS

All animal experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with the approval of the Institutional Animal Care and Use Committee of San Diego State University. Male Sprague-Dawley rats (225–250 g) acquired from Harlan were used in this study.

Langendorff perfusion. Rat hearts were perfused in the Langendorff mode using a protocol adapted from Tsuchida et al. (30). Hearts were perfused with Krebs-Ringer buffer at a constant pressure of 60 mmHg. Hearts were stabilized for 20 min and then subjected to 30 min of no-flow ischemia and 15 min of reperfusion, during which time significant amounts of ROS are produced (14). Control hearts were continuously perfused for 65 min. *N*-acetylcysteine (NAC; 5 mM) was added to the perfusion buffer and was present throughout the experiment. Creatine kinase activity in the coronary effluent was measured to verify the efficacy of I/R using a diagnostic kit (Stanbio Laboratory, Boerne, TX).

Isolation of cardiac myocytes. Adult cardiac myocytes were prepared as previously described (11). Briefly, hearts were quickly excised, cannulated via the aorta, and perfused with heart medium (J-MEM supplemented with 10 mM HEPES, 30 mM taurine, 2 mM carnitine, and 2 mM creatine) for 5 min. After digestion of the hearts by perfusion with 0.1% collagenase II (Worthington Biochemicals, Lakewood, NJ) plus 0.1% BSA and 25 μM $CaCl_2$ in heart medium, the ventricles were minced, and myocytes were dispersed by gentle

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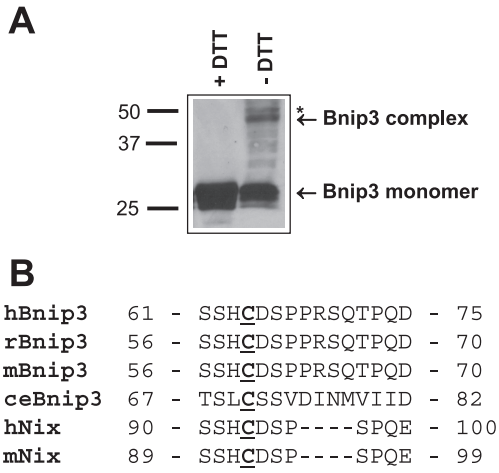

Fig. 1. Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3 (Bnip3) forms an ~48-kDa DTT-sensitive protein complex in heart lysates. *A*: Western blot (WB) analysis of rat heart lysates under reduced (+DTT) and nonreduced (−DTT) conditions showing that Bnip3 formed a DTT-sensitive protein complex (*nonspecific band). *B*: analysis of Bnip3 and homolog Bnip3L/Nix protein sequences revealed a single conserved cysteine residue in the NH_2-terminal domain. hBnip3, human Bnip3; rBnip3, rat Bnip3; mBnip3, mouse Bnip3; ceBnip3, *Caenorhabditis elegans* Bnip3; hNix, human Nix; mNix, mouse Nix.

pipetting. Cells were filtered through a nylon mesh and washed twice at 50 *g* for 5 min. Calcium was reintroduced to isolated cells to select for calcium-tolerant cells, which were plated on laminin-coated dishes for experiments. After 2 h of plating, myocytes were subjected to simulated ischemia by incubation in ischemic buffer (125 mM NaCl, 8 mM KCl, 1.2 mM KH_2PO_4, 1.25 mM $MgSO_4$, 1.2 mM $CaCl_2$, 6.25 mM $NaHCO_3$, 20 mM 2-deoxyglucose, 5 mM Na-lactate, and 20 mM HEPES; pH 6.6) in hypoxic pouches (GasPak EZ, BD Biosciences) for 1 h at 37°C. Reperfusion was initiated by a change to Krebs-Henseleit buffer (110 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.25 mM $MgSO_4$, 1.2 mM $CaCl_2$, 25 mM $NaHCO_3$, 15 mM glucose, and 20 mM HEPES; pH 7.4) for 1 h. Since ROS are produced during both ischemia and reperfusion (32), NAC (5 mM) was added to both the ischemic and Krebs-Henseleit buffers. Alternatively, after 2 h of plating, cells were treated with hydrogen peroxide, which rapidly enters the cell, where it is converted to more reactive species of oxygen metabolites such as the hydroxyl radical (5, 34). Cells were incubated with 100 μM hydrogen peroxide in Krebs-Henseleit buffer, a concentration that induces apoptosis in cardiac myocytes (24). Cells were treated for 30 min, which is the time point where mitochondrial membrane potential to starts decrease and release of cytochrome *c* is observable with 100 μM hydrogen peroxide (1). Cell lysates were prepared in RIPA buffer [50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS] and cleared by centrifugation at 20,000 *g* for 20 min.

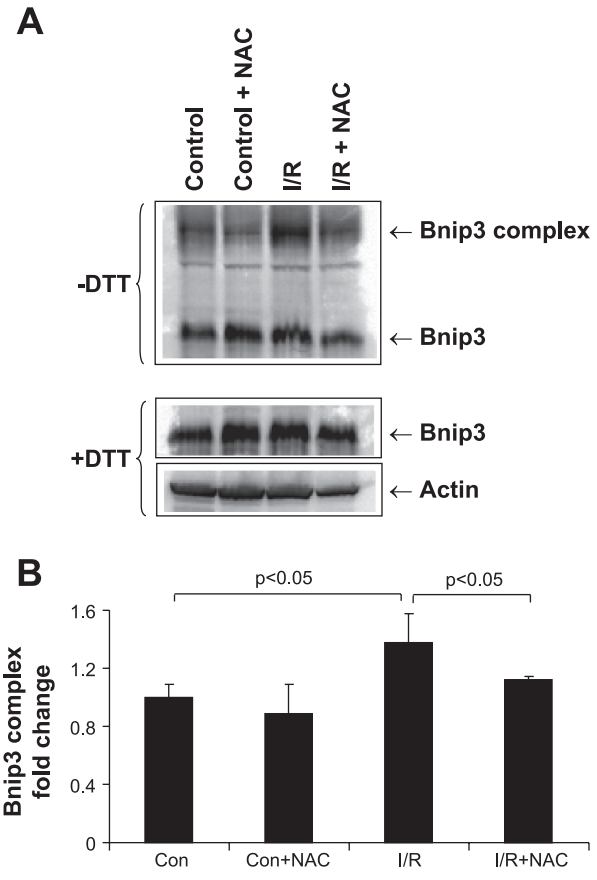

Fig. 2. Ischemia-reperfusion (I/R) causes an increase in the Bnip3 DTT-sensitive complex. *A*: Langendorff-perfused rat hearts were subjected to continuous perfusion or 30 min of ischemia and 15 min of reperfusion with or without 5 mM *N*-acetylcysteine (NAC) in the perfusion buffer. Bnip3 expression was analyzed by WB analysis under reduced and nonreduced conditions. *B*: quantitation of the Bnip3 complex in heart lysates. After densitometric analysis, the dimer was normalized to actin, and the ratio compared with control perfused hearts ($n = 3$). Con, control.

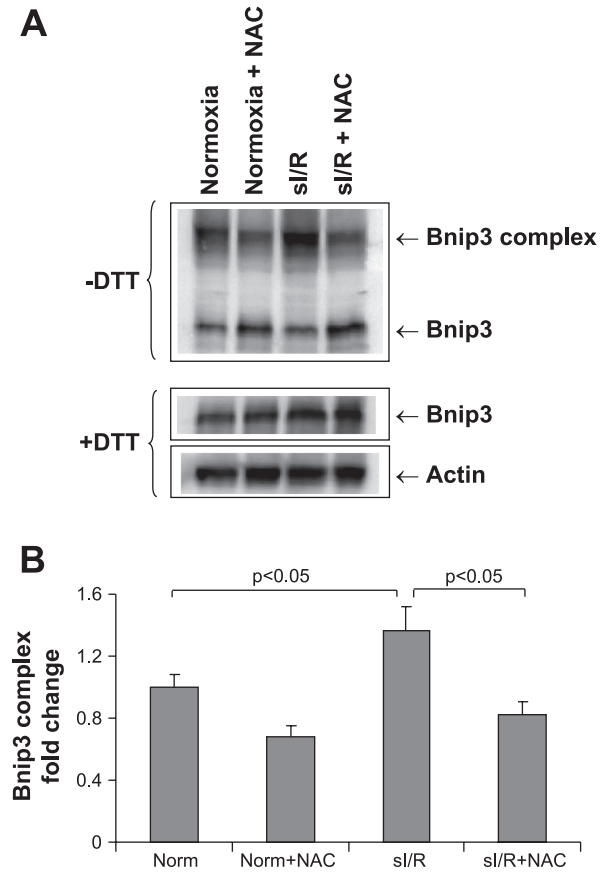

Fig. 3. Simulated I/R (sI/R) increases the Bnip3 complex in isolated adult myocytes. *A*: cells were subjected to 60 min of simulated ischemia followed by 60 min of reperfusion with or without the presence of 5 mM NAC. Bnip3 expression was analyzed by WB analysis in whole cell lysates. *B*: quantitation of the Bnip3 complex in isolated adult myocytes. The Bnip3 complex was normalized against actin, and data are presented as fold increases over normoxic cells ($n = 3$).

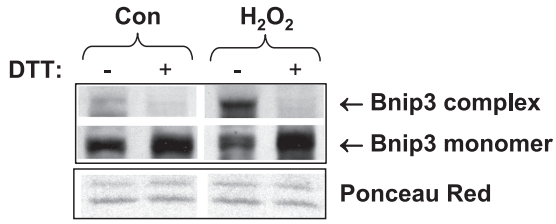

Fig. 4. H_2O_2 treatment increases the DTT-sensitive Bnip3 complex in isolated cardiac myocytes. Adult cardiac myocytes were treated with 100 μM H_2O_2 for 30 min. Cell lysates were prepared as described in MATERIALS AND METHODS, and Bnip3 was analyzed by WB analysis under nonreduced and reduced conditions. Membranes were stained with Ponceau red to confirm equal loading of proteins.

DNA constructs. Bnip3C64A and Bnip3H173A mutants were generated by overlap extension using PCR (19) with human Bnip3 in pcDNA3.1 as a template (18). pcDNA3.1-Bnip3 was also used as a template to amplify the deletion mutant Bnip3ΔN(1-64). Flag- and Myc-tagged Bnip3 constructs were generated by PCR-based cloning into the *Bam*HI and *Eco*RI sites of pCMV-Tag2A (Flage) and pCMV-Tag3A (c-*myc*) vectors (Stratagene, San Diego, CA).

Recombinant protein expression and purification. Bnip3 was produced as a fusion protein containing a polyhistidine tag in the expression vector pRSET and purified as previously described (18). Briefly, His-tagged Bnip3 was grown in BL21(DE3) cells, and expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. Bacteria were resuspended in native buffer [50 mM NaH_2PO_4 (pH 8.0) and 300 mM NaCl] followed by sonication. After centrifugation at 20,000 *g* for 20 min, the supernatant was added to columns containing Ni-nitrilotriacetic acid (Qiagen, Valencia, CA). The column was washed with native buffer plus 20 mM imidazole, and His-Bnip3 was eluted with 250 mM imidazole in the same buffer followed by desalting on PD-10 columns and Q-Sepharose chromatography. The recombinant protein was >95% pure as determined by SDS-PAGE and Coomassie blue staining.

Western blot analysis. Whole heart lysates were prepared as previously described (17). Briefly, hearts were minced and homogenized by polytron in ice-cold *buffer A*, which contained 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and complete protease inhibitor cocktail (Roche Applied Bioscience, Indianapolis, IN). To prepare whole cell lysates, cells were harvested by scraping and centrifugation at 550 *g* for 5 min at 4°C. Cell pellets were resuspended in ice-cold *buffer A*, incubated for 30 min on ice, and then cleared by centrifugation at 20,000 *g* for 20 min. The protein concentration of the supernatant was determined by Commassie blue binding assay (Pierce Chemicals, Rockford, IL) using BSA as the standard. Heart lysates (80 μg protein/well) or cell lysates (50 μg protein/well) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and stained with Ponceau red to confirm equal loading. The membrane was immunoblotted with mouse anti-Bnip3 (1:1,000), mouse anti-actin (1:1,000), mouse anti-Myc (1:1,000), or rabbit anti-Flag (1:1,000) antibodies (all from Sigma-Aldrich, St. Louis, MO). Visualization was done by ECL using the Supersignal West Dura Extended Duration Substrate (Pierce), and densitometric analysis was performed using ImageJ.

Cell culture and transient transfection. Atrium-derived HL-1 mouse cardiac myocytes (8) were cultured on gelatin-fibronectin-coated cell culture dishes in Claycomb medium (JRH Bioscience, Lenexa, KS) supplemented with 10% FBS, 0.1 mM norepinephrine, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 μg/ml amphotericin B. HeLa cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Immunoprecipitation. HeLa cells cultured in 60-mm dishes were cotransfected with Myc- and Flag-tagged Bnip3 using Lipofectamine 2000 for 6 h; cells were rinsed and then incubated in cell culture media containing 50 μM zVAD-FMK to prevent cell death (18). After 24 h, cells were lysed in *buffer A* as described above, and the concentrations were adjusted to 1 mg/ml. Before immunoprecipita-

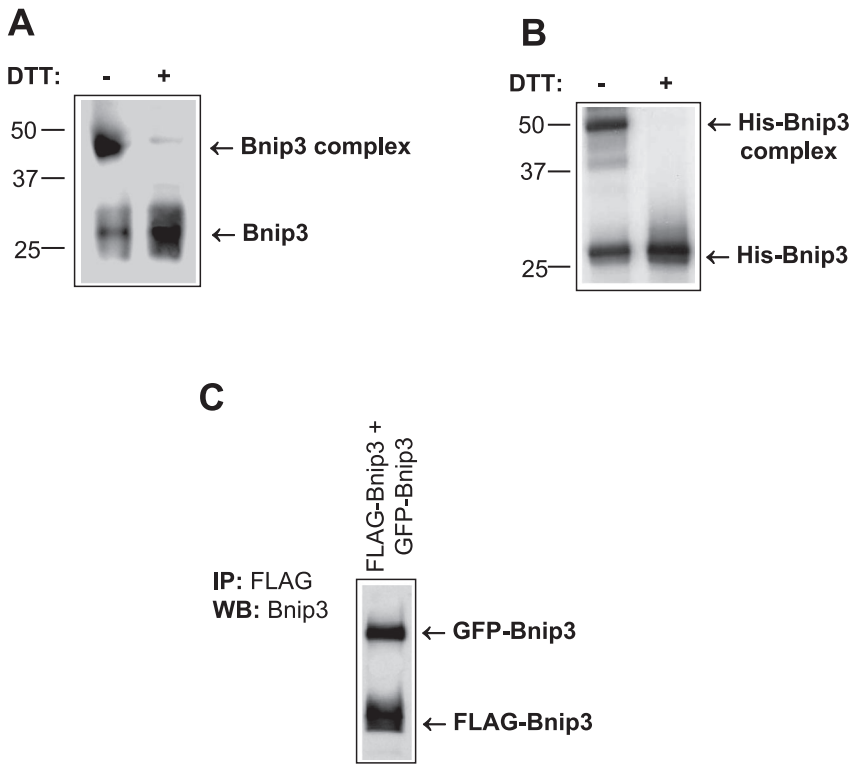

Fig. 5. The DTT-sensitive Bnip3 complex is a homodimer. *A*: HeLa cells were transfected with Bnip3 and then analyzed by WB analysis for Bnip3 under nonreduced and reduced conditions. *B*: WB analysis of purified His-Bnip3 showing the formation of a DTT-sensitive complex in the absence of other proteins. *C*: an antibody against Flag was used to immunoprecipitate Flag-Bnip3 from HeLa cells overexpressing Flag-Bnip3 and green fluorescent protein (GFP)-Bnip3. Subsequent WB analysis of the immunoprecipitates with an antibody against Bnip3 confirmed that GFP-Bnip3 coimmunoprecipitated with Flag-Bnip3. IP, immunoprecipitation.

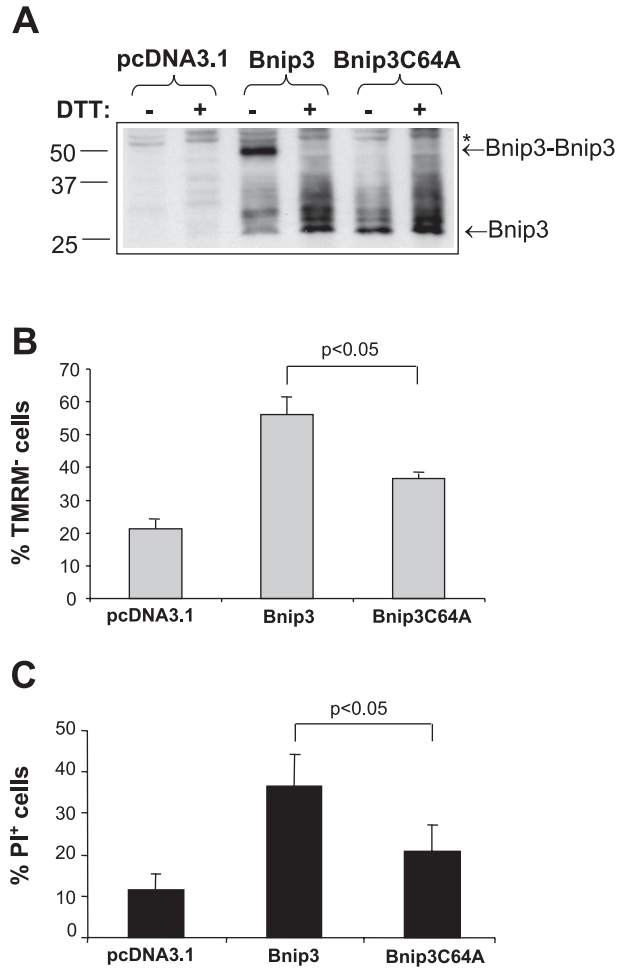

Fig. 6. Mutation of the conserved cysteine residue reduces the homodimerization and cell death activity of Bnip3. *A*: WB analysis under reduced and nonreduced conditions for Bnip3 in HL-1 myocytes transfected with pcDNA3.1, Bnip3, or Bnip3C64A (*nonspecific band). Cell death was assessed in HL-1 cells overexpressing pcDNA3.1, Bnip3, or Bnip3C64A by measuring mitochondrial membrane potential by tetramethylrhodamine methyl ester (TMRM) fluorescence (*B*) and plasma membrane permeability to propidium iodide (PI; *C*) ($n = 3$).

tion, cell lysates were analyzed for equal expression of Flag-Bnip3 and Myc-Bnip3 by Western blot analysis using anti-Flag or anti-Myc antibodies. Cell lysates were precleared with protein G PLUS agarose (Santa Cruz Biotechnology) for 1 h and then incubated with rabbit anti-Flag antibody (1 μg) to immunoprecipitate Flag-Bnip3. Immune complexes were captured with protein G PLUS agarose beads, centrifuged, washed four times in PBS, and solubilized in 2× SDS sample buffer. Proteins were analyzed by Western blot analysis with anti-Myc antibody to determine how much of Myc-Bnip3 coimmunoprecipitated with Flag-Bnip3 or an antibody to Flag to verify that equal amounts of Flag-Bnip3 were immunoprecipitated. Alternatively, anti-Flag was used for the immunoprecipitation of lysates prepared from HeLa cells overexpressing Flag-Bnip3 and green fluorescent protein (GFP)-Bnip3, and the immunoprecipitates were analyzed by Western blot analysis using anti-Bnip3 antibody.

Assessment of cell death. Bnip3 has been reported to cause apoptotic (18, 28), necrotic (31), and autophagic cell death (2, 20), and, to ensure the assessment of all types of cell death, cell viability was analyzed by assessing mitochondrial membrane potential and plasma membrane permeability. Cells transfected with pcDNA3.1 or pcDNA3.1-Bnip3 plus GFP were stained with 20 nM tetramethylrhodamine methyl ester (TMRM) or 1 μg/ml propidium iodide (PI) for 15 min at 37°C and then observed through a Nikon TE300 fluorescence microscope equipped with a cooled charge-coupled device camera (Orca-ER, Hamamatsu). TMRM accumulates in the mitochondrial matrix of viable cells, whereas PI accumulates in the nucleus of dying or dead cells. Ten images of random fields from two replicates were captured, and GFP-positive cells were scored for the uptake of TMRM or PI in three independent experiments.

Statistical analysis. All values are expressed as means ± SD. Multiple comparisons between experimental groups were made using ANOVA followed by the Dunnett's post test. $P < 0.05$ was considered significant.

RESULTS

During our investigation, we found that Bnip3 forms a ~48-kDa immunoreactive protein complex in heart lysates that was sensitive to reduction by DTT, suggesting that the complex is formed via disulfide bond(s) between cysteine residues (Fig. 1*A*). A scan of the human Bnip3 protein sequence revealed the presence of a single cysteine at residue 64, which was conserved in Bnip3 between different species as well as in the homolog Nix/Bnip3L (Fig. 1*B*). Since cysteine residues are sensitive to oxidation, and ischemia and reperfusion are both associated with increased oxidative stress, we investigated whether there would be a change in the DTT-sensitive Bnip3 complex after of 30 min of global ischemia and 15 min of reperfusion. Interestingly, I/R caused an increase in the Bnip3 complex (Fig. 2*A*), whereas the presence of the antioxidant NAC in the perfusion buffer prevented I/R-induced complex formation (Fig. 2*B*). Similarly, exposure of isolated adult myocytes to simulated I/R caused a significant increase in Bnip3 complex formation, which was reduced in the presence of NAC (Fig. 3, *A* and *B*). Direct treatment of cultured myocytes with hydrogen peroxide (H_2O_2) also promoted an increase in the Bnip3 complex without a change in Bnip3 protein levels (Fig. 4). These results demonstrate that increased oxidative stress induces the formation of a Bnip3 complex.

To verify that exogenous Bnip3 forms the same DTT-sensitive complex in cells, we overexpressed Bnip3 in HeLa cells for 24 h and then analyzed nonreduced and reduced cell lysates by Western blot analysis. Interestingly, almost all exoge-

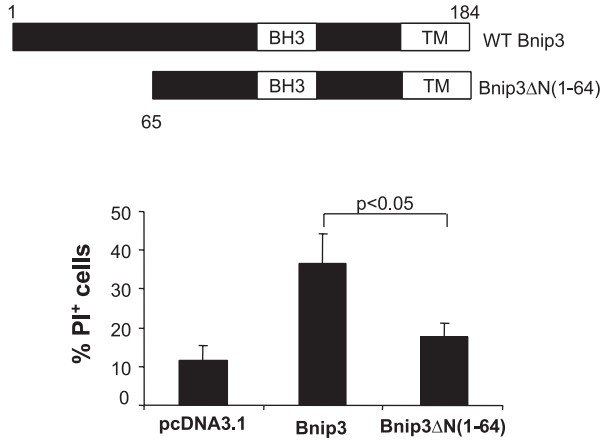

Fig. 7. The NH_2 terminus is important for the proapoptotic activity of Bnip3. pcDNA3.1, Bnip3, or Bnip3ΔN(1-64) plus GFP were overexpressed in HL-1 myocytes, and cell death was determined by measuring plasma membrane permeability to PI ($n = 3$). BH3, Bcl-2 homology domain 3; TM, transmembrane domain; WT, wild type.

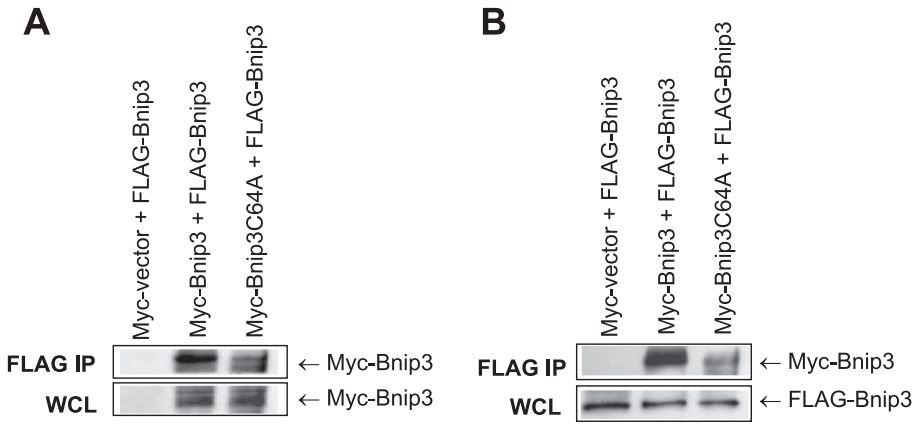

Fig. 8. Mutation of a cysteine residue reduces homodimerization with Bnip3. HeLa cells were transfected with vector, Myc-Bnip3, or Myc-Bnip3C64A plus Flag-Bnip3. Lysates prepared from transfected cells were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-Myc antibody. Before immunoprecipitation, whole cell lysates (WCL) were analyzed by immunoblot analysis with anti-Myc (*A*) or anti-Flag (*B*).

nous Bnip3 existed in the DTT-sensitive complex (Fig. 5*A*). Western blot analysis of recombinant Bnip3 in the absence of other proteins revealed the presence of the same immunoreactive DTT-sensitive complex, suggesting that this complex is a homodimer of Bnip3 (Fig. 5*B*). Coimmunoprecipitation and subsequent Western blot analysis for Flag-Bnip3 and GFP-Bnip3 confirmed that Bnip3 forms homodimers when overexpressed in HeLa cells (Fig. 5*C*).

To investigate the functional importance of the cysteine residue in Bnip3, we generated a Bnip3 mutant with the conserved cysteine residue mutated to alanine (Bnip3C64A). When overexpressed in HL-1 myocytes, wild-type Bnip3 was found mainly as a DTT-sensitive homodimer, whereas Bnip3C64A was unable to form this dimer (Fig. 6*A*). Although HL-1 myocytes express Bnip3 under normal conditions (18), endogenous Bnip3 was not detected in our Western blots, which is likely due to the strong (and quickly saturating) signal of exogenously expressed Bnip3. Overexpression of Bnip3 caused a significant increase in cell death as measured by loss of mitochondrial membrane potential (Fig. 6*B*) and increased plasma membrane permeability (Fig. 6*C*), whereas Bnip3C64A caused significantly less cell death. To further investigate the importance of the NH_2-terminal region for Bnip3 function, we generated a Bnip3 mutant that lacked NH_2-terminal residues 1-64 (Fig. 7). When overexpressed in HL-1 myocytes, we found that Bnip3ΔN(1-64) had reduced cell death activity compared with wild-type Bnip3.

Next, we investigated whether Bnip3C64A had a reduced capability of interacting with Bnip3 using immunoprecipitation coupled with Western blot analysis. Analysis of whole cell lysates before immunoprecipitation showed that both Myc-Bnip3 (Fig. 8*A*) and Flag-Bnip3 (Fig. 8*B*) were expressed at equal levels in the different samples. Immunoprecipitation of Flag-Bnip3 from cell lysates followed by Western blot analysis with an antibody to Myc showed that less of Myc-Bnip3C64A coimmunoprecipitated with Flag-Bnip3 compared with Myc-Bnip3 (Fig. 8). We found that Flag-Bnip3 and Myc-Bnip3 induced cell death to the same levels as pcDNA3.1-Bnip3 (data not shown).

In addition, a histidine residue at position 173 in the COOH-terminal transmembrane domain is important for Bnip3 homodimerization (4, 25, 29). Consistent with this, we confirmed that a mutation of the histidine to alanine (Bnip3H173A) prevented the formation of a homodimer with wild-type Bnip3 (Fig. 9*A*). Western blot analysis of cell lysates after immunoprecipitation showed that Bnip3H173A remained in this fraction. Interestingly, cell death was almost completely abrogated in cells overexpressing Bnip3H173A compared with Bnip3 (Fig. 9, *B* and *C*). We confirmed that mutation of the histidine residue does not affect localization of Bnip3 to the mitochondria (data not shown).

DISCUSSION

It is known that changes in the cellular environment can trigger the activation of cell death pathways. Bnip3 has previously been shown to contribute to cell death during I/R in ex vivo perfused hearts and in cell culture (13, 18). Bnip3 is not expressed in neonatal cardiac myocytes under normal conditions but is induced in these cells during hypoxia via the activation of cell cycle factor E2F-1 (36) and hypoxia inducible factor-1α (15). In contrast, Bnip3 is constitutively expressed in the adult heart and to a lower level in HL-1 myocytes, where it exists as an integral protein in the mitochondrial membrane (9, 18). This suggests that Bnip3 is maintained in an inactive state under normal conditions. We found that Bnip3 contains a single conserved cysteine residue in the NH_2 terminus, suggesting that it may be important for Bnip3 function. Many proteins, including PTEN, p53, and NF-κB, contain cysteine residues that are used as a "switch" to activate or inactivate these proteins (3). For instance, the phosphatase PTEN contains a critical cysteine residue in the active site that must be reduced for optimal catalytic activity, and the formation of a disulfide bond between this cysteine and another nearby cysteine in PTEN leads to the inactivation of the enzyme (23). In the present study, we provide evidence that Bnip3 acts as a sensor of oxidative stress where an increase in ROS induces homodimerization and activation of Bnip3 via a conserved cysteine residue in the exposed NH_2 terminus.

There are several lines of evidence that Bnip3 homodimerization via cysteine residues is an important part of the activation process. First, we found that most of endogenous Bnip3 does not exist in the DTT-sensitive homodimer under normal conditions in the heart and that the homodimer increases in hearts subjected to I/R and in isolated cardiac myocytes exposed to simulated I/R or hydrogen peroxide treatment. The fact that the antioxidant NAC prevents the increase in the Bnip3 dimer in response to I/R suggests that dimerization is dependent on increased oxidative stress. Bnip3 is anchored in the mitochondrial outer membrane via its COOH-terminal

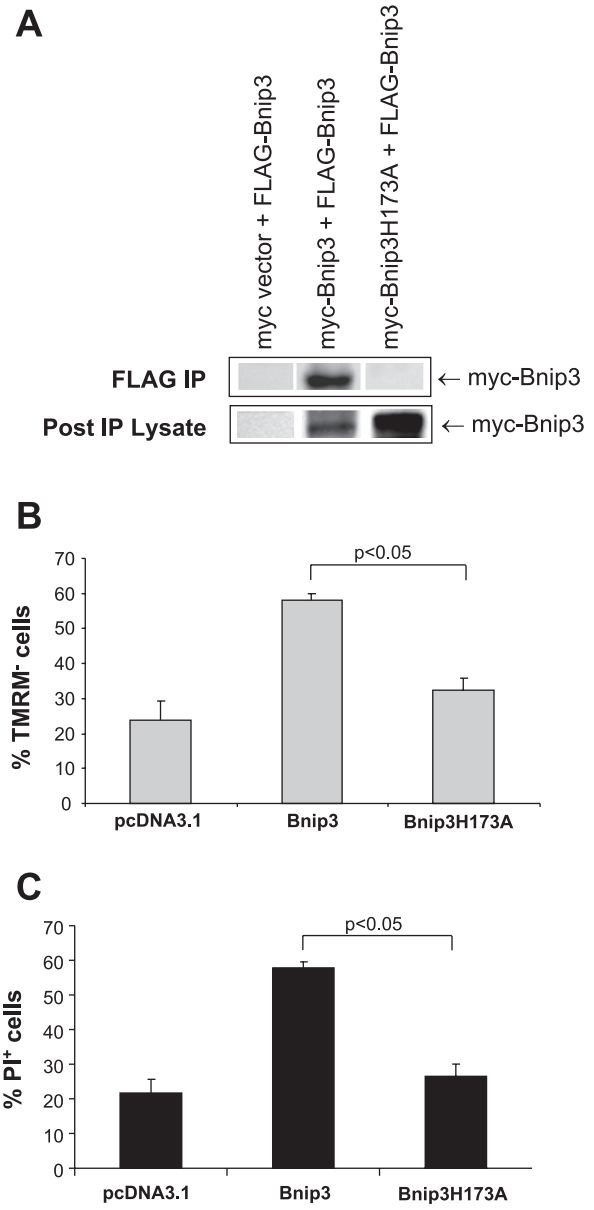

Fig. 9. A histidine residue in the core of the Bnip3 TM domain is essential for homodimerization and proapoptotic activity. *A*: Flag-Bnip3 coimmunoprecipitated with Myc-Bnip3 but not with Myc-Bnip3H173A in HL-1 myocytes. Very little Myc-Bnip3 was left in the cell lysate after immunoprecipitation with Flag-Bnip3, whereas all of Myc-Bnip3H173A remained in the cell lysate. A mutation in the TM domain also reduced the cell death activity of Bnip3, as measured by the loss of mitochondrial membrane potential (*B*) and increased plasma membrane permeability (*C*) ($n = 3$).

transmembrane domain, whereas the cysteine residue in the NH_2 terminus faces the cytosol, which makes it susceptible to oxidation (31). The cytosol is a highly reduced environment under normal conditions in which cysteines are maintained in their thiol (-SH) or thiolate ($-S^-$) state and where stable disulfide bonds rarely form. However, myocardial I/R is associated with a substantial increase in ROS, which results in the oxidation of cysteine residues and promotion of disulfide bonds between proteins (6). In contrast, we found that overexpressed Bnip3 exists mainly as a DTT-sensitive dimer and correlates with increased cell death, suggesting that this is the active form of Bnip3. This also suggests that another protein might be sequestering Bnip3 under normal conditions, preventing it from forming homodimers. When overexpressed, Bnip3 is in excess and there is not enough of the other protein to sequester all of Bnip3, allowing Bnip3 to homodimerize and activate cell death. Finally, we found that mutation of the cysteine residue or deletion of the NH_2-terminal region of Bnip3 significantly reduces homodimerization and the cell death activity of Bnip3, suggesting that homodimerization via the cysteine residue is important for full activation of Bnip3. Using deletion mapping of Bnip3, Ray et al. (27) reported that the NH_2-terminal residues 1-49 are important for heterodimerization with Bcl-2 and Bcl-x_L, but this group did not measure the cell death activity of their deletion mutants in their study. Our study clearly demonstrates that the NH_2 terminus is necessary for fully functional Bnip3.

There are conflicting reports in the literature regarding the functional significance of Bnip3 homodimerization. For instance, a mutation in the transmembrane domain of Bnip3 that disrupts SDS-resistant homodimerization has no effect on its cell death activity (10, 27). Depending on the antibody used, Bnip3 can be detected as an SDS-resistant 60-kDa dimer and/or as a 30-kDa monomer by Western blot analysis under reduced condition. It is important to note that these experiments only looked at the disruption of the SDS-resistant dimer by Western blot analysis and do not verify whether these mutants still form homodimers in vivo by coimmunoprecipitation experiments. In contrast, an inactive form of the Bnip3 homolog Nix inhibits Bnip3-mediated cell death by heterodimerization via the transmembrane domain, suggesting that homodimerization is required for Bnip3 function (26). Deletion of the transmembrane domain completely abrogates homodimerization and cell death (7), although this might be due to the fact that the transmembrane domain is also important for targeting Bnip3 to the mitochondria. Computational modeling and mutagenesis studies (4, 29, 33) have identified the histidine at residue 173 to be essential for homodimerization of Bnip3. Our study confirms the importance of the histidine at residue 173 in homodimerization and also demonstrates that this residue is essential for the cell death activity of Bnip3. Our results also suggest that the NH_2 and COOH termini of Bnip3 cooperate in the homodimerization and activation of Bnip3. Interestingly, based on structural analysis of the transmembrane domain using NMR and computational modeling, it has been proposed that the Bnip3 homodimer may be able to form a channel that is permeable to water (4, 33). However, it still remains to be determined whether the activated homodimer forms a channel in the mitochondrial membrane.

In summary, this study identifies Bnip3 as an important mitochondrial redox sensor that induces cell death in response to increased oxidative stress. An increase in ROS production such as during I/R induces the homodimerization and activation of Bnip3. Bnip3 forms a homodimer via the COOH-terminal transmembrane domain; however, our data suggest that to be fully active, the homodimer must be stabilized by covalently linking the two Bnip3 molecules via a disulfide bond. This new insight into Bnip3 function is important as Bnip3 may be an attractive therapeutic target to limit myocardial injury after myocardial infarction.

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REFERENCES

1. **Akao M, O'Rourke B, Teshima Y, Seharaseyon J, Marban E.** Mechanistically distinct steps in the mitochondrial death pathway triggered by oxidative stress in cardiac myocytes. *Circ Res* 92: 186–194, 2003.
2. **Azad MB, Chen Y, Henson ES, Cizeau J, McMillan-Ward E, Israels SJ, Gibson SB.** Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3. *Autophagy* 4: 195–204, 2008.
3. **Biswas S, Chida AS, Rahman I.** Redox modifications of protein-thiols: emerging roles in cell signaling. *Biochem Pharmacol* 71: 551–564, 2006.
4. **Bocharov EV, Pustovalova YE, Pavlov KV, Volynsky PE, Goncharuk MV, Ermolyuk YS, Karpunin DV, Schulga AA, Kirpichnikov MP, Efremov RG, Maslennikov IV, Arseniev AS.** Unique dimeric structure of BNip3 transmembrane domain suggests membrane permeabilization as a cell death trigger. *J Biol Chem* 282: 16256–16266, 2007.
5. **Byler RM, Sherman NA, Wallner JS, Horwitz LD.** Hydrogen peroxide cytotoxicity in cultured cardiac myocytes is iron dependent. *Am J Physiol Heart Circ Physiol* 266: H121–H127, 1994.
6. **Cargnoni A, Ceconi C, Gaia G, Agnoletti L, Ferrari R.** Cellular thiols redox status: a switch for NF-kappaB activation during myocardial post-ischaemic reperfusion. *J Mol Cell Cardiol* 34: 997–1005, 2002.
7. **Chen G, Ray R, Dubik D, Shi L, Cizeau J, Bleackley RC, Saxena S, Gietz RD, Greenberg AH.** The E1B 19K/Bcl-2-binding protein Nip3 is a dimeric mitochondrial protein that activates apoptosis. *J Exp Med* 186: 1975–1983, 1997.
8. **Claycomb WC, Lanson NA Jr, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ Jr.** HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci USA* 95: 2979–2984, 1998.
9. **Diwan A, Krenz M, Syed FM, Wansapura J, Ren X, Koesters AG, Li H, Kirshenbaum LA, Hahn HS, Robbins J, Jones WK, Dorn GW.** Inhibition of ischemic cardiomyocyte apoptosis through targeted ablation of Bnip3 restrains postinfarction remodeling in mice. *J Clin Invest* 117: 2825–2833, 2007.
10. **Frazier DP, Wilson A, Graham RM, Thompson JW, Bishopric NH, Webster KA.** Acidosis regulates the stability, hydrophobicity, and activity of the BH3-only protein Bnip3. *Antioxid Redox Signal* 8: 1625–1634, 2006.
11. **Gottlieb RA, Gruol DL, Zhu JY, Engler RL.** Preconditioning rabbit cardiomyocytes: role of pH, vacuolar proton ATPase, and apoptosis. *J Clin Invest* 97: 2391–2398, 1996.
12. **Graham RM, Frazier DP, Thompson JW, Haliko S, Li H, Wasserlauf BJ, Spiga MG, Bishopric NH, Webster KA.** A unique pathway of cardiac myocyte death caused by hypoxia-acidosis. *J Exp Biol* 207: 3189–3200, 2004.
13. **Graham RM, Thompson JW, Wei J, Bishopric NH, Webster KA.** Regulation of bnip3 death pathways by calcium, phosphorylation, and hypoxia-reoxygenation. *Antioxid Redox Signal* 9: 1309–1316, 2007.
14. **Granville DJ, Tashakkor B, Takeuchi C, Gustafsson AB, Huang C, Sayen MR, Wentworth P Jr, Yeager M, Gottlieb RA.** Reduction of ischemia and reperfusion-induced myocardial damage by cytochrome P450 inhibitors. *Proc Natl Acad Sci USA* 101: 1321–1326, 2004.
15. **Guo K, Searfoss G, Krolikowski D, Pagnoni M, Franks C, Clark K, Yu KT, Jaye M, Ivashchenko Y.** Hypoxia induces the expression of the pro-apoptotic gene BNIP3. *Cell Death Differ* 8: 367–376, 2001.
16. **Gustafsson AB, Gottlieb RA.** Bcl-2 family members and apoptosis, taken to heart. *Am J Physiol Cell Physiol* 292: C45–C51, 2007.
17. **Gustafsson AB, Tsai JG, Logue SE, Crow MT, Gottlieb RA.** Apoptosis repressor with caspase recruitment domain protects against cell death by interfering with Bax activation. *J Biol Chem* 279: 21233–21238, 2004.
18. **Hamacher-Brady A, Brady NR, Logue SE, Sayen MR, Jinno M, Kirshenbaum LA, Gottlieb RA, Gustafsson AB.** Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. *Cell Death Differ* 14: 146–157, 2007.
19. **Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR.** Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77: 51–59, 1989.
20. **Kanzawa T, Zhang L, Xiao L, Germano IM, Kondo Y, Kondo S.** Arsenic trioxide induces autophagic cell death in malignant glioma cells by upregulation of mitochondrial cell death protein BNIP3. *Oncogene* 24: 980–991, 2005.
21. **Kubasiak LA, Hernandez OM, Bishopric NH, Webster KA.** Hypoxia and acidosis activate cardiac myocyte death through the Bcl-2 family protein BNIP3. *Proc Natl Acad Sci USA* 99: 12825–12830, 2002.
22. **Kubli DA, Ycaza JE, Gustafsson AB.** Bnip3 mediates mitochondrial dysfunction and cell death through Bax and Bak. *Biochem J* 405: 407–415, 2007.
23. **Lee SR, Yang KS, Kwon J, Lee C, Jeong W, Rhee SG.** Reversible inactivation of the tumor suppressor PTEN by H_2O_2. *J Biol Chem* 277: 20336–20342, 2002.
24. **Long X, Goldenthal MJ, Wu GM, Marin-Garcia J.** Mitochondrial Ca^{2+} flux and respiratory enzyme activity decline are early events in cardiomyo cyte response to H_2O_2. *J Mol Cell Cardiol* 37: 63–70, 2004.
25. **Metcalf DG, Law PB, DeGrado WF.** Mutagenesis data in the automated prediction of transmembrane helix dimers. *Proteins* 67: 375–384, 2007.
26. **Ohi N, Tokunaga A, Tsunoda H, Nakano K, Haraguchi K, Oda K, Motoyama N, Nakajima T.** A novel adenovirus E1B19K-binding protein B5 inhibits apoptosis induced by Nip3 by forming a heterodimer through the C-terminal hydrophobic region. *Cell Death Differ* 6: 314–325, 1999.
27. **Ray R, Chen G, Vande VC, Cizeau J, Park JH, Reed JC, Gietz RD, Greenberg AH.** 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, 2000.
28. **Regula KM, Ens K, Kirshenbaum LA.** Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. *Circ Res* 91: 226–231, 2002.
29. **Sulistijo ES, Jaszewski TM, MacKenzie KR.** Sequence-specific dimerization of the transmembrane domain of the "BH3-only" protein BNIP3 in membranes and detergent. *J Biol Chem* 278: 51950–51956, 2003.
30. **Tsuchida A, Liu Y, Liu GS, Cohen MV, Downey JM.** α_1-Adrenergic agonists precondition rabbit ischemic myocardium independent of adenosine by direct activation of protein kinase C. *Circ Res* 75: 576–585, 1994.
31. **Vande Velde C, Cizeau J, Dubik D, Alimonti J, Brown T, Israels S, Hakem R, Greenberg AH.** BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol Cell Biol* 20: 5454–5468, 2000.
32. **Vanden Hoek TL, Li C, Shao Z, Schumacker PT, Becker LB.** Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. *J Mol Cell Cardiol* 29: 2571–2583, 1997.
33. **Vereshaga YA, Volynsky PE, Pustovalova JE, Nolde DE, Arseniev AS, Efremov RG.** Specificity of helix packing in transmembrane dimer of the cell death factor BNIP3: a molecular modeling study. *Proteins* 69: 309–325, 2007.
34. **Wu ML, Tsai KL, Wang SM, Wu JC, Wang BS, Lee YT.** Mechanism of hydrogen peroxide and hydroxyl free radical-induced intracellular acidification in cultured rat cardiac myoblasts. *Circ Res* 78: 564–572, 1996.
35. **Yasuda M, Theodorakis P, Subramanian T, Chinnadurai G.** Adenovirus E1B-19K/BCL-2 interacting protein BNIP3 contains a BH3 domain and a mitochondrial targeting sequence. *J Biol Chem* 273: 12415–12421, 1998.
36. **Yurkova N, Shaw J, Blackie K, Weidman D, Jayas R, Flynn B, Kirshenbaum LA.** The cell cycle factor E2F-1 activates Bnip3 and the intrinsic death pathway in ventricular myocytes. *Circ Res* 102: 472–479, 2008.