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Regulation of Bnip3 Death Pathways by Calcium, Phosphorylation, and Hypoxia–Reoxygenation

REGINA M. GRAHAM, JOHN W. THOMPSON, JIANQIN WEI, NANETTE H. BISHOPRIC, and KEITH A. WEBSTER

ABSTRACT

Bnip3 is a proapoptotic member of the Bcl-2 family of death-regulating proteins that promote the intrinsic pathway of programmed cell death. The Bnip3 death program requires membrane insertion through an N-terminal transmembrane domain that directs the protein to mitochondrial and endoplasmic reticular (ER) membranes. We have reported that simulated ischemia induces transcription of the Bnip3 gene, and Bnip3 protein is stabilized by acidosis. Bnip3 programmed death is atypical, with features of both apoptosis and necrosis. Here we demonstrate that hypoxia–reoxygenation and agents that activate protein kinase C, including calcium ionophore, phorbol 12-myristate 13-acetate, and okadaic acid, also induce Bnip3. The molecular size of Bnip3 predicted from the amino acid sequence is 21.5 kDa, but the protein typically migrates in SDS-PAGE as a 31-kDa monomer and 60-kDa dimer. Treatment of cell extracts containing Bnip3 with phosphatase yielded a series of rapidly migrating species, the smallest of which corresponded with the theoretic molecular size of Bnip3. Conversely, treatment of cells with okadaic acid eliminated the rapidly migrating species, suggesting that Bnip3 phosphorylation is a dynamic process. Elevated levels of the phosphoprotein correlated with initiation of Bnip3-dependent death, whereas the dephosphorylated species correlated with extreme acidosis. *Antioxid. Redox Signal.* 9, 1309–1315.

INTRODUCTION

Numerous clinical studies and animal experimental models have confirmed that rates of apoptosis and necrosis are increased in the myocardium under conditions of disease (14, 22, 24, 25). Death of cardiac myocytes through both programmed and nonprogrammed pathways is a central feature of ischemic heart disease, contributing to infarction after acute episodes of ischemia–reperfusion, and as a continuing process associated with coronary artery disease and heart failure [reviewed in (13, 14, 17, 21, 23)]. Distinct stress pathways are activated by ischemia and reperfusion; extended periods of severe ischemia are usually associated with necrotic cell death, whereas reperfusion is more often associated with apoptosis. Damage to the myocardium after coronary artery occlusion is closely related to the duration and severity of ischemia. Damage may continue to evolve for hours or days, even after successful reperfusion, because of ischemia-related injury to blood vessels and subsequent disruption of flow within and bordering the jeopardized tissue. Loss of cardiac myocytes may result from death pathways that are initiated by ischemia and become manifest during restoration of blood flow (4, 12, 18, 25, 28). The relative contribution of necrosis and apoptosis to cell death during and after coronary occlusion is unclear and may involve combinations of both processes (13, 23).

Hypoxia and acidosis are obligatory components of ischemia, and the combination of these may provide a critical signal for death (31, 32). We have shown that programmed death in hypoxic cardiac myocytes parallels the development of acidosis (33). We have shown that hypoxia induces the expression of Bnip3, a proapoptotic member of the Bcl-2 family, and that acidosis promotes the stabilization and membrane integration of Bnip3. This in turn initiates a cell-death pathway that requires both Bnip3 and mitochondrial permeability transition, but is

Department of Molecular and Cellular Pharmacology and the Vascular Biology Institute, University of Miami School of Medicine, Miami, Florida.

caspases independent (15). Here we show that a number of other stimuli in addition to acidosis can cause the accumulation of cellular Bnip3, including reoxygenation, calcium ionophore A23187, and the protein kinase C activator phorbol myristic acid (PMA). We also provide evidence that monomeric Bnip3 is a phosphoprotein, suggesting a novel layer of regulation. Finally, we show that Bnip3-specific siRNAs reduce DNA fragmentation and nuclear condensation associated with reoxygenation-mediated death. We propose that reoxygenation-mediated stabilization and phosphorylation of Bnip3 participate in hypoxia-reoxygenation–mediated cardiac myocyte death.

METHODS

Human subjects

Anonymized human myocardial tissue samples together with nonidentifying demographic and clinical information were obtained through the Cooperative Human Tissue Network (Eastern and Midwestern branches) and used according to a protocol approved by the University of Miami Human Subjects Research Office.

Materials

Cell-culture reagents were obtained from Invitrogen. Phorbol 12-myristate 13-acetate (PMA), calphostin C, and okadaic acid were obtained from Sigma. Calf intestinal alkaline phosphatase (CIP) and the appropriate reaction buffers were from New England Biolabs (Ipswich, MA). Anti-actin and anti-Bnip3 were from Sigma (St. Louis, MO) and Chemicon-Millipore (Billerica, MA), respectively. Protease inhibitors, secondary antibodies, and ECL were obtained from Pierce (Biotechnology, Rockford, IL). Calcium ionophore A-23187 and Hoechst 33342 were from Molecular Probes. Bnip3 and negative control siRNAs and transfection reagents were from Pharmacon (Tromsø, Norway). Block-It fluorescent oligonucleotides were from Invitrogen Corporation (Carlsbad, CA). All other reagents were obtained from Sigma.

Cardiac myocyte culture

Methods for primary culture of neonatal rat cardiac myocytes have been previously described (31, 32). In brief, enriched cultures of cardiac myocytes were obtained from 1- to 2-day-old neonatal rats by stepwise trypsin dissociation and plated at a density of 4×10^6/60-mm dish, in minimal essential medium supplemented with 5% fetal calf serum, 1% penicillin, and streptomycin (MEM + 5% FCS). After 3–5 days, cells were rinsed 3 times in MEM and transferred to a defined serum-free MEM supplemented with transferrin (5 μg/ml), vitamin B_{12} (0.17 n*M*), and insulin (5 μg/ml). The final cultures contained >97% cardiac myocytes contracting at >200 beats/min.

Hypoxia

Cells were exposed to hypoxia (0.5% oxygen) by using the Invivo 500 Hypoxic workstation (Biotrace Int.). Contractility was monitored by edge detection, as described previously (31, 32). Cultures were lysed under hypoxia by using ice-cold deoxygenated buffers for analyses.

Western blot analysis

Our procedures for Western blots have been described in detail elsewhere (15, 33). Equal amounts of protein were fractionated on 10–12% SDS-polyacrylamide gels and electroblotted to nitrocellulose (BioRad). Blots were stained with Ponceau Red to monitor the transfer of proteins. Membranes were

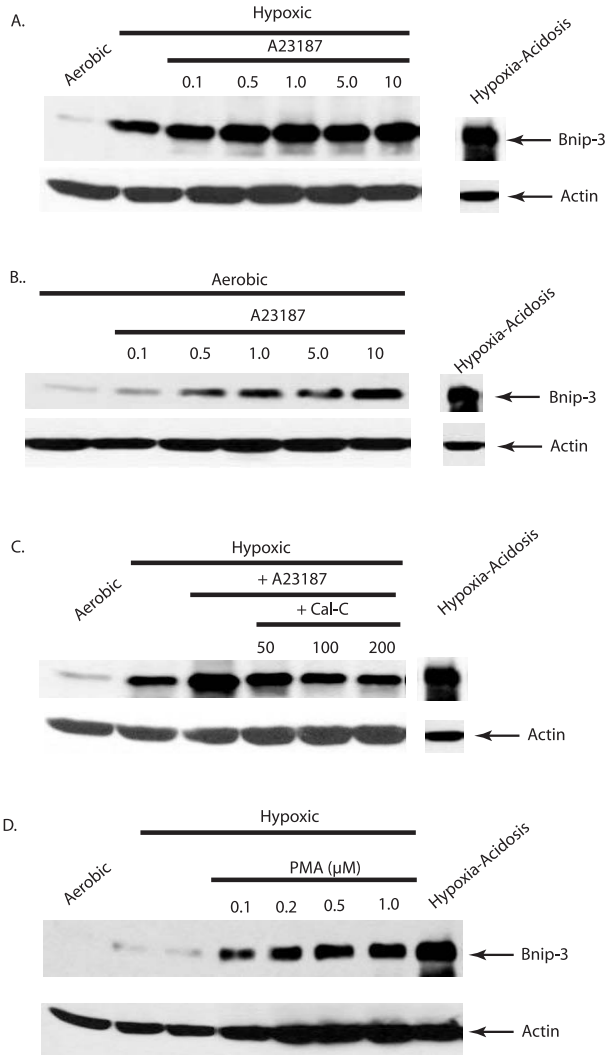

FIG. 1. Enhanced Bnip3 accumulation by calcium ionophore A23187 and PMA. (**a**) Cardiac myocytes were exposed to hypoxia-neutral conditions for 36 h with 0.9 m*M* calcium and treated with the indicated dose (μM) of A23187 for an additional 6 h. At each time point, protein extracts were analyzed by Western blots with anti-Bnip3 antibody. In panel (**b**), hypoxia was omitted, and in panel (**c**), myocytes were treated under hypoxia with 0.5 μM A23187 and the indicated dose of Calphostin-C (n*M*). In panel (**d**), hypoxic myocytes were treated with the indicated dose of PMA for an additional 6 h. For hypoxia–acidosis, myocytes were exposed to hypoxia for 48 h in high-glucose medium. Results are representative of three separate experiments.

blocked as described previously and incubated with specific antibodies for 1–2 h. After washing, the blots were reacted with horseradish peroxidase–conjugated secondary antibodies and visualized by enhanced chemiluminescence (ECL).

Alkaline phosphatase digestion of Bnip3

Hypoxic cardiac myocyte cell lysates (50 μg) were incubated with 30 units of CIP for 5, 15, 30, and 60 min in the presence of protease inhibitors. In some samples, sodium orthovanadate (10 m*M*) was added to the cell lysate before CIP incubation.

Bnip3 siRNA transfection

Cardiac myocytes were transfected for 24 h with a pool of Bnip3-specific siRNAs or negative control siRNA by using DharmaFECT 4 transfection reagent (5 μl/60-mm dish) at a final concentration of 10 n*M* according to manufacturers' instructions. After 24 h, the medium was replaced with MEM containing 10% FCS, and after a further 24 h, to serum-free media for 48 h before exposure to hypoxia. Block-It fluorescent oligonucleotides were transfected in parallel plates to monitor transfection efficiency.

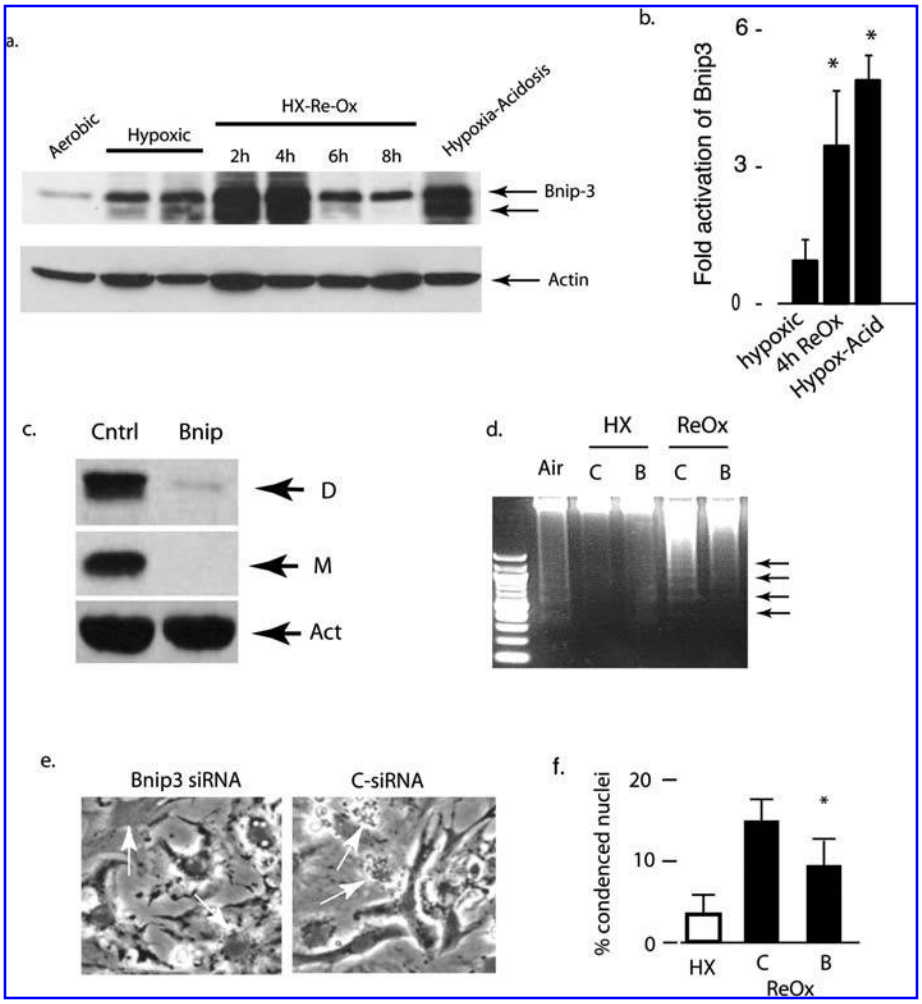

FIG. 2. Transient stimulation of Bnip3 by hypoxia–reoxygenation and protection with siRNA. (**a**) Cardiac myocytes were exposed to hypoxia for 24 h or hypoxia followed by reoxygenation for the indicated times. Fold inductions of Bnip3 are quantified in (**b**); induction of Bnip3 by reoxygenation and acidosis was significantly greater than the induction by hypoxia alone ($n = 3$). (**c**) Cardiac myocytes were transfected with control (contrl) or Bnip3-specific siRNA and exposed to hypoxia for 16 h and reoxygenation for 4 h. Proteins were separated on 12% SDS-polyacrylamide gels and analyzed for Bnip3 expression. *Arrows* indicate positions of the Bnip3 dimer (D) and monomer (M). In (**d**), cardiac myocytes were subjected to air, hypoxia (16 h), or hypoxia and reoxygenation for 16 h in the presence of control (C) or Bnip3-specific siRNA (B), and genomic DNA was analyzed by agarose gel electrophoresis as described in Methods; *arrows* indicate DNA fragments. In (**e**), cardiac myocytes were transfected with siRNAs and subjected to hypoxia–reoxygenation as in (**d**). Cultures were stained with Hoechst 33342, as described in Methods; *arrows* indicate typical round intact nuclei in Bnip3 siRNA-treated cultures and examples of condensed, fragmented nuclei in cultures with control siRNA. (**f**) Quantification of condensed nuclei from multiple fields and at least 200 nuclei per condition.

Analysis of apoptosis

Cells were examined for morphologic evidence of apoptosis after staining with the fluorescent DNA-binding dye Hoechst 33342. Cells were incubated with 2 μg/ml Hoechst 33342 for 15 min, visualized, and photographed by using the Zeiss Axiovert 200 inverted fluorescence microscope. Genomic DNA fragmentation (DNA ladders) was analyzed as described previously (15, 33). Samples (10 μg) were subjected to electrophoresis in 2% agarose gels and imaged by ethidium bromide staining and digital photography.

Coronary artery ligation

Chronic myocardial ischemia was generated by using coronary artery ligation, as described previously (35). In brief, thoracotomy was performed under gas anesthesia, the atrial appendage was elevated, and the left anterior descending coronary artery (LAD) located. A silk black braided suture (6-0) was inserted around the vessel near its origin, and the entire vessel was ligated by tightening the suture. Ischemia was monitored continuously by ECG. Rats were killed after 12 h, 2 days, and 5 days, and left ventricles dissected for protein analysis.

RESULTS

Bnip3 is dose-dependently induced by calcium ionophore A23187 and PMA

Elevated intracellular calcium is associated with ischemic myocardial injury, and calcium-dependent signals regulate the activity of multiple Bcl-2 family members [reviewed in (21)]. To determine whether intracellular calcium influences Bnip3, cardiac myocytes were incubated under aerobic or hypoxic conditions and treated for 6 h with increasing doses of the calcium ionophore A-23187 (Fig. 1A). Under hypoxia, Bnip3 levels were elevated at the lowest dose of ionophore and peaked between 0.5 and 1.0 μM A23187, achieving levels comparable to those induced by hypoxia–acidosis. The ionophore also induced Bnip3 in normoxia, but the concentration dependence was shifted to the right and peaked at 10 μM A-23187 (Fig. 1B). To investigate the mechanism of induction, we pretreated hypoxic myocytes with the PKC inhibitor calphostin C before adding ionophore (Fig. 1C). Calphostin C blocked the induction of Bnip3 by 5.0 μM A23187 in a dose-dependent manner, with an IC_{50} of 100 nM. PMA, an activator of PKC, increased Bnip3 accumulation under hypoxia (Fig. 1D). These findings together suggest that increased cytosolic calcium levels induced by A23187 lead to PKC activation, resulting in the accumulation of Bnip3.

Bnip3 is activated by hypoxia–reoxygenation and contributes to programmed death

Both hypoxia and reoxygenation are associated with elevated myocyte calcium levels. To determine whether hypoxia–reoxygenation induces Bnip3, cardiac myocytes were subjected to 16 h of hypoxia and reoxygenated for various intervals as described previously (32). As shown in Fig. 2A, Bnip3 was strongly but transiently induced at 2 and 4 h of reoxygenation. Peak levels of Bnip3 were again equivalent to those seen under hypoxia–acidosis (Fig. 2B). The rapid onset of Bnip3 accumulation during reoxygenation suggests that, like acidosis, the effect is probably posttranscriptional. We reported previously that antisense oligonucleotides directed against Bnip3 mRNA reduced Bnip3 protein by >70% and significantly reduced DNA fragmentation caused by hypoxia–acidosis (6). To determine whether knockdown of Bnip3 would also protect against hypoxia–reoxygenation damage, cardiac myocytes were transfected with siRNAs directed against Bnip3 or a scrambled sequence (control), as described in Methods. As shown in Fig. 2C, Bnip3-directed but not control siRNA was able to reduce both Bnip3 dimers (D) and monomers (M) by >90% in cardiac myocytes subjected to 16 h of hypoxia and 4 h of reoxygenation. We then treated cardiac myocytes with Bnip3 or control siRNA and subjected the cells to hypoxia for 16 h and reoxygenation for another 16 h. Reoxygenation-induced DNA fragmentation was significantly reduced by Bnip3 siRNA, but not by control siRNA (Fig. 2D). In parallel plates, treatment of

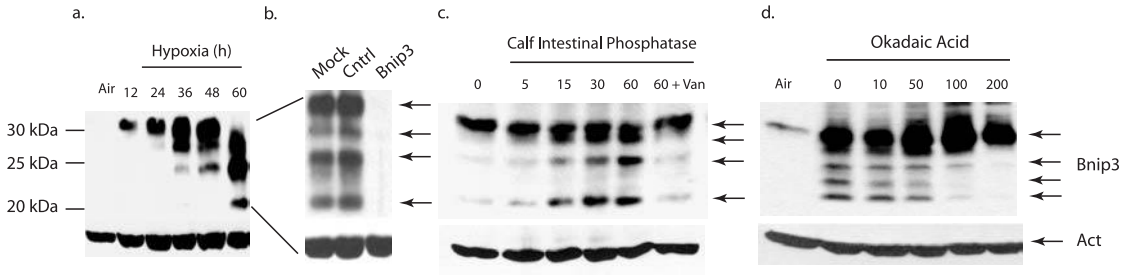

FIG. 3. Phosphatase-dependent induction of high-mobility Bnip3 species. (**a**) Cardiac myocytes were exposed to hypoxia for the indicated periods and cell extracts analyzed by Western blot with anti-Bnip3 antibody. (**b**) Cardiac myocytes were mock transfected or transfected with control (Cntrl) or Bnip3-specific (Bnip3) siRNA oligonucleotides and exposed to hypoxia for 48 h. *Arrows* indicate positions of four specific Bnip3 protein species, each of which was eliminated by Bnip3-specific siRNA. (**c**) Extracts of hypoxic cardiac myocytes were treated with CIP for the indicated times (min) in the presence or absence of sodium orthovanadate (Van, 10 mM), as indicated. (**d**) Cardiac myocytes were exposed to hypoxia for 48 h and then to okadaic acid at the concentration indicated (nM); *arrows* indicate dose-dependent reductions of the rapidly migrating Bnip3 species.

myocytes with Bnip3 siRNA oligonucleotides also reduced the numbers of condensed, fragmented nuclei visualized by Hoechst 33342 staining [15 ± 2.0% control; 9 ± 2.2% Bnip3 siRNA ($n = 3$; $p < 0.05$)] (Fig. 2E and F). However, despite the almost complete loss of Bnip3 protein, cardiac myocyte death was substantial, suggesting that Bnip3 is one of several alternative pathways for cell death under these conditions.

Bnip3 phosphorylation

Two major species of Bnip3 can be identified in 12% SDS-polyacrylamide gels, migrating at 31 and 60 kDa, and presumably represent monomeric and dimeric forms of the protein. We routinely observe faster-migrating Bnip3 species in extracts of cardiac myocytes exposed to hypoxia for extended periods. At least four Bnip3-immunoreactive species can be seen under these conditions, the most rapidly migrating of which has an apparent molecular size of ~21 kDa (Fig. 3A). The amino acid sequence of Bnip3 predicts a similar monomer size of 21.5 kDa. As shown in Fig. 3B, all of these rapidly migrating species were eliminated by treatment with Bnip3-directed siRNA, confirming that these species are related to Bnip3. These Bnip3 species may result from successive protein cleavage, or they may be products of posttranslational modification. To address the latter possibility, protein extracts from hypoxic cardiac myocytes were incubated with a CIP for various times and analyzed by immunoblot (Fig. 3C). CIP treatment resulted in the time-dependent appearance of progressively smaller Bnip3 species, an effect blocked by the phosphatase inhibitor sodium vanadate (10 m*M*). These results suggest that phosphorylation accounts for at least some of the electrophoretic variability of Bnip3. To investigate this possibility further, cardiac myocytes were exposed to hypoxia for 48 h in the presence of okadaic acid, a specific inhibitor of protein phosphatase 2a (PP2a). As shown in Fig. 3D, increasing concentrations of okadaic acid were associated with the progressive loss of rapidly migrating Bnip3 species. Bnip3 phosphorylation was also indicated by binding to a phosphoprotein affinity column (CalBiochem). Bnip3 eluted from the column in the same fraction as phosphorylated ERK (data not shown). These data do not rule out the possibility that a native 31-kDa Bnip3 undergoes phosphorylation-dependent proteolysis. However, the similarity between the apparent molecular size of the most rapidly migrating fragment and the calculated molecular size of Bnip3 suggests that successive phosphorylation of native 21-kDa Bnip3 produces a protein with an electrophoretic mobility equivalent to 31 kDa.

Elevated Bnip3 in ischemic myocardium from rat models and human biopsies

To determine whether Bnip3 levels are also induced in ischemic myocardium *in vivo*, rat hearts were subjected to permanent coronary artery occlusion, and Bnip3 levels were determined in the left ventricles at intervals thereafter. Ischemia induced myocardial Bnip3 beginning at 2 days and increasing significantly after 4 days of ischemia (Fig. 4A). Bnip3 levels were also measured in six samples of myocardial tissue obtained from patients undergoing cardiac transplantation. Two of these had nonischemic dilated cardiomyopathy (DCM), two had ischemic cardiomyopathy (ICM), and two had unspecified end-stage heart failure (ESHF) (Fig. 4B). Bnip3 levels were low or undetectable in DCM, but abundant in the remaining four samples, suggestive of an association between ischemia and augmented Bnip3 levels in the failing human heart. Rapid-migrating Bnip3 species were evident in the ischemic rat hearts but not in the human samples.

DISCUSSION

The data presented here show that Bnip3 is induced by calcium ionophore and phorbol ester, consistent with a role for a calcium- and diacylglycerol-dependent PKC in the regulation of Bnip3-dependent cell death. Bnip3 levels were also activated transiently by reoxygenation, a stress that also elevates calcium and PKC activity (9, 11). Our data illuminate a potentially novel pathway for control of Bnip3 through calcium and/or calcium-dependent kinases.

Our previous studies indicated that pathways for cell death induced by hypoxia–acidosis and hypoxia–reoxygenation are divergent, with acid-stabilized Bnip3 playing a critical role in the former (15). Our current findings that Bnip3 accumulates during reoxygenation, and that anti-Bnip3 siRNA protects cardiac myocytes during reoxygenation, support a previous report that Bnip3 contributes to reperfusion-mediated death in cell culture and *in vivo* (9). Although Hamacher-Brady *et al.* did not

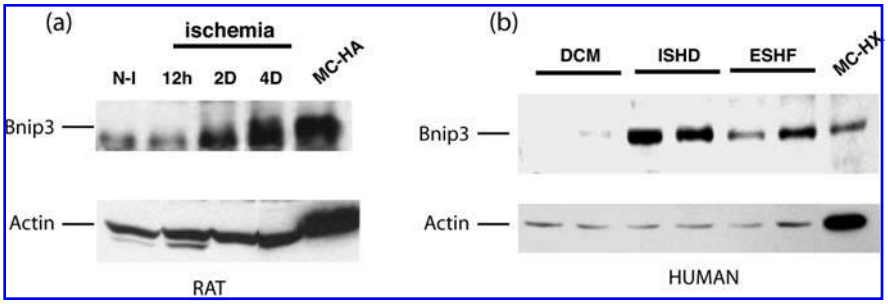

FIG. 4. Elevated levels of Bnip3 in ischemic rat and human hearts. (**a**) Coronary artery occlusions of rat hearts were implemented as described in Methods. Proteins extracted from the left ventricles of nonischemic hearts (NI) or hearts made ischemic for the indicated times were analyzed by Western blots. Control (MC-HA) is cardiac myocytes subjected to hypoxia–acidosis as previously described (17). (**b**) Human tissue samples were obtained as frozen pellets from the Human Tissue Network Corporation. DCM, dilated cardiomyopathy; ISHD, ischemic heart disease; ESHF, end-stage heart failure, MC-HX, control sample from hypoxic neonatal rat cardiac myocytes.

observe increased levels of Bnip3 in their models of ischemia–reperfusion, this could be due to the transient nature of Bnip3 induction by reoxygenation. Our data suggest that reoxygenation-associated increases in cytosolic calcium result in activation of PKC, which permits acid-independent stabilization of Bnip3 and initiation of Bnip3-mediated cell death.

This model is further supported by our finding that the 31-kDa Bnip3 monomer is a phosphoprotein, consistent with the presence of consensus phosphorylation sites for PKC (2). We have identified multiple rapidly migrating Bnip3-immunoreactive species that accumulate over 16 h of hypoxia in cardiac myocytes, and appear after exposure of myocyte Bnip3 to phosphatase. A study by Ray *et al.* (26) supports the concept that posttranscriptional modifications of Bnip3 affect its apparent molecular size. Bnip3 expressed in HEK293 cells had an apparent molecular mass of 31–32 kDa, but only 26–27 kDa when translated *in vitro* by reticulocyte extract, where extensive posttranslational modification is unlikely to occur. Our results do not definitively identify mature Bnip3 as a phosphoprotein; it is possible that phosphorylation marks Bnip3 for proteolytic cleavage, or that a protein kinase–dependent protease cleaves Bnip3 into the sub-31 kDa, phosphatase-sensitive Bnip3 species. However, these results support a potentially significant role for protein kinase(s) in regulating the expression or posttranslational modifications of Bnip3 or both.

It remains to be determined whether and how phosphorylation regulates Bnip3, and which protein kinases are responsible. However, many Bcl-2 family members are regulated by phosphorylation. Under basal conditions, phosphorylated Bad protein is bound to cytosolic 14-3-3; dephosphorylation of Bad promotes its dissociation from 14-3-3 and initiates apoptosis (36). Phosphorylation of the BH3-only proteins Bim and Bmf by JNK induces dissociation from dynein motor complexes, inducing Bax-dependent apoptosis (16). Similarly, phosphorylation of Bik is required for effective Bik-mediated cell death (30). Furthermore, TNF-α–induced dephosphorylation in of Bcl-2 on serine 87 promotes Bcl-2 ubiquitination and degradation (3). Although the appearance of the rapidly migrating Bnip3 species is temporally related to the development of cell death (15), the relation between the two events is unknown, and the rapidly migrating species may be destined for degradation rather than active participants in cell death.

Protein kinases and other factors potentially regulating the Bnip3-dependent death pathway may be cell-type specific. Transfection of Bnip3 was sufficient to induce death of HEK 293 cells, whereas Bnip3-transfected cardiac myocytes died only with coincident acidosis (8, 15, 26, 34). The mechanism of Bnip3-dependent death is also unclear; Hamacher-Brady *et al*. and Regula *et al*. reported caspase-dependent death pathways, whereas we do not find that Bnip3 requires caspases to induce death (9, 27). In our hands, reoxygenation-induced death of cardiac myocytes has a Bnip3-dependent component, as well as other pathway(s) that are indeed caspases dependent (33). Myocardial infarction caused by ischemia–reperfusion also has both caspase-dependent and -independent components (1, 10, 20). In either case, our findings of elevated Bnip3 levels in ischemic rat and human hearts establish Bnip3 as a strong candidate effector of cell death in multiple contexts of ischemic heart disease.

ACKNOWLEDGMENTS

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ABBREVIATIONS

CIP, calf intestinal alkaline phosphatase; DCM, dilated cardiomyopathy; ER, endoplasmic reticulum; ERK, extracellular regulated kinase; ESHF, end-stage heart failure; HEK, human embryonic kidney; ICM, ischemic cardiomyopathy; LAD, left anterior descending coronary artery; OA, okadaic acid; PKC, protein kinase C; PMA, phorbol myristic acid.

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Address reprint requests to:
Keith A. Webster
Department of Molecular and Cellular Pharmacology and Vascular Biology Institute
University of Miami School of Medicine
1600 NW 10th Ave., RMSB 6038
Miami, FL 33136

E-mail: kwebster@med.miami.edu

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