Original Article

Both Caspase and Calpain are Involved in Endoplasmic Reticulum-Targeted BNIP3-Induced Cell Death

(BNIP3 / cell death / endoplasmic reticulum / calpain / caspase)

J. ZENG[1,2], Y. Y. HUANG[1,2], X. M. XU[1,2], S. H. LI[2], D. C. ZUO[3]

[1]Department of Orthodontics, [2]Orofacial Reconstruction and Regeneration Laboratory, The Affiliated Stomatology Hospital of Southwest Medical University, Luzhou, Sichuan, China
[3]The Key Laboratory of Medical Electrophysiology, Ministry of Education, Collaborative Innovation Center for Prevention and Treatment of Cardiovascular Disease, Institute of Cardiovascular Research, Southwest Medical University, Luzhou, Sichuan, China

Abstract. Bcl-2/E1B-19K-interacting protein 3 (BNIP3) is a member of the apoptotic B-cell lymphoma-2 family that regulates cell death. Although BNIP3 targeted normally to the mitochondrial outer membrane by its transmembrane domain was originally considered to be essential for its pro-apoptotic activity, accumulating evidence has shown that BNIP3 is localized to endoplasmic reticulum at physiological conditions and that forced expression of BNIP3 can initiate cell death via multiple pathways depending on the subcellular compartment it targets. Targeting BNIP3 to endoplasmic reticulum has been shown to participate in cell death during endoplasmic reticulum stress. However, the molecular events responsible for BNIP3-induced cell death in the endoplasmic reticulum remain poorly understood. In the present study, the transmembrane domain of BNIP3 was replaced with a segment of cytochrome b5 that targets BNIP3 into endoplasmic reticulum, which induced cell death as effectively as its wild-type molecule in the SW480 cell line (colon carcinoma). Furthermore, a pan-caspase inhibitor, z-VAD-fmk, and PD150606, a specific calpain inhibitor, both significantly suppressed the endoplasmic reticulum-targeted BNIP3-induced cell death. These results suggest that endoplasmic reticulum-targeted BNIP3 induced a mixed mode of cell death requiring both caspases and calpains.

Received August 28, 2019. Accepted April 20, 2020.

This work was supported by funding from the Affiliated Stomatology Hospital of Southwest Medical University (#0800103009), Luzhou, Sichuan, China.

Corresponding author: Dongchuan Zuo, The Key Laboratory of Medical Electrophysiology, Ministry of Education, Collaborative Innovation Center for Prevention and Treatment of Cardiovascular Disease, Institute of Cardiovascular Research, Southwest Medical University, 319 Zhongshan Road, Luzhou 646000, Sichuan, China. Phone: (+86) 830-3160619; e-mail: zuodongchuan@swmu.edu.cn.

Abbreviations: Bcl-2 – B-cell lymphoma-2 protein family, BH – Bcl-2 homology, BNIP3 – Bcl-2/E1B-19K-interacting protein 3, ER – endoplasmic reticulum, PCR – polymerase chain reaction.

Introduction

Programmed cell death (apoptosis) is a physiological process shared by all multi-cellular organisms. It is critical for normal development and tissue homeostasis, and dysregulation of apoptosis has been implicated in a wide variety of diseases. It is tightly regulated by a number of apoptosis regulators. Among them, the B-cell lymphoma-2 protein family (Bcl-2) is unique in that it can be either pro-apoptotic or anti-apoptotic (Knight et al., 2019). The anti-apoptotic members possessing all four Bcl-2 homology (BH) domains include Bcl-2, Bcl-xl, Bcl-w, Mcl-1 and A1, while the pro-apoptotic proteins can be further divided into two distinct sub-families: the multi-domain proteins including Bax, Bak and Bok, and the BH3 only proteins including Bad, Bid, Bik/Nbk, Bim, Bmf, Nix, Hrk, Noxa and Puma, and BNIP3. BNIP3 comprises four main domains: a PEST domain that targets BNIP3 for degradation, a putative BH3 domain that is homologous to other Bcl-2 family members, a conserved domain that is conserved from *C. elegans* to humans, and a transmembrane domain (TM) that is responsible for dimerization and essential for the mitochondria-targeted BNIP3-induced apoptosis (Opferman and Kothari, 2017).

Endoplasmic reticulum (ER) has been recognized as the second most common intracellular organelle next to mitochondria for initiating the apoptosis process by Bcl-2 protein family (Rodriguez et al., 2010; Pihán et al., 2017). Bcl-2 family proteins in ER regulate apoptosis through both caspase-dependent and caspase-independent signalling pathways (Nutt et al., 2002; Scorrano et al., 2003). Conversely, Bcl-2 family proteins themselves

can be modulated by signals inside ER, where they play an essential role in apoptosis during ER stress (Foyouzi-Youssefi et al., 2000). Like other Bcl-2 family members, BNIP3 can localize to ER in both physiological and pathological conditions, indicating that BNIP3 may execute its apoptosis-related functions (Hanna et al., 2012). Recently, it was demonstrated that targeting BNIP3 to mitochondria or ER impairs the intracellular Ca^{2+} homeostasis and induces a mixed mode cell death in Mes23.5 dopaminergic cells (Zhang et al., 2009). This suggests that BNIP3 may induce cell death via different signalling pathways depending on the cell type and its intracellular localization. ER stress has been implicated in BNIP3-induced cell death. However, the molecular mechanisms involved in this event are not fully elucidated. Here, we demonstrated that the ER-targeted BNIP3-induced cell death is mediated by both calpains and caspases, but not caspase-3, a cytoplasmic protein activated with the help of cytochrome c released from mitochondria.

Material and Methods

Reagents

Mito-tracker Red CMXRos and ER-Tracker Red dye were purchased from Thermo Fisher Scientific (Waltham, MA). Pan-caspase inhibitor z-VAD-fmk, caspase-3-specific inhibitor AC-VAD-CHO and calpain inhibitor PD150606 were purchased from Calbiochem (La Jolla, CA). Anti-caspase-3 (ab4051) antibody was purchased from Abcam (Cambridge, UK). SW480 and MCF-7 cells were purchased from ATCC (Manassas, VA), Lipofectamine 2000, foetal bovine serum, penicillin/streptomycin, and cell culture media and additives were the products of Thermo Fisher Scientific. All other chemicals, unless specified otherwise, were from Sigma-Aldrich (Steinheim, Germany). Plasmid construction and transient transfection: the human sequence encoding BNIP3 (NM_004052.3) was synthesized and cloned into pcDNA3.0 (Invitrogen, San Diego, CA), generating pcDNA-BNIP3, and two mutants, BNIP3ΔTM where amino acid (AA) residues 164-184 were deleted, and BNIP3cyb5 where AA164-184 was replaced with AA100-134 of rat hepatic cytochrome b5, were generated by splice overlap polymerase chain reaction (PCR) as described (Ray et al., 2000). All plasmids were subcloned into pEGFP-N1 with forward primer (5‘-ACTAAGCTTAATGGGCGACGCGGCCG- CAG-3‘) containing a *Hind*III site, and reverse primer (5’-CATGGATCCGAAAGGTGCTGGTGGAGG-TTG-3’) containing a *Bam*HI site. All plasmids were sequenced to confirm their identities.

Cell culture and transient transfection

The SW480 cells, a human colon carcinoma cell line and MCF-7 cells, a human breast adenocarcinoma cell line, were cultured with RPMI-1640 medium containing 10% foetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and in humidified air with 5% CO_2 at 37 °C. Cells were seeded in 60 mm dishes containing coverslips at 0.8×10^6 the day before transfection. Cells were then transiently transfected with plasmids using Lipofectamine 2000 following vendor’s instructions. After overnight incubation, cells were loaded with Mitotracker Red CMXRos (mitochondria probe, Ex/Em = 579 nm/ 599 nm) or ER-Tracker Red dye (endoplasmic reticulum probe, Ex/Em = 587 nm/615 nm), respectively, and continued to incubate for another 12 h. After incubation, coverslips were washed with PBS and cells were fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature, permeabilized, and then counterstained with DAPI (solution at 0.5 μg/ml (CST, 1 : 50,000) for 15 min at room temperature. After rinsing with PBS, the coverslips were mounted with mounting medium.

Confocal microscopy

The intracellular localization was analysed by confocal microscopy using a Zeiss LSM 510 NLO Two-Photon Microscope (Jena, Germany) with a ×40 C-Apochromat objective. Lasers employed for excitation of fluorescence were Ar (458, 477,488, and 514 nm), green HeNe (543 nm), and red HeNe (633 nm) and images were analysed with Aim Image Browser (Zeiss, Jena, Germany).

Cell death analysis

Cells were fixed in 4% paraformaldehyde for 40 min at 24 or 48 h, respectively, post-transfection, and then stained with 1 μg/ml DAPI for 20 min. GFP-positive cells were counted under a fluorescence microscope (Nikon, Tokyo, Japan). The percentage of dying cells was calculated by assessing the number of blue cells with condensed or fragmented nuclear morphology in the total population of GFP-positive cells. For each group of cells, at least 3,000 cells were counted.

Caspase-3 activity assay

The activity of caspase-3 was measured using the Caspase-3 activity kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer’s instructions. Briefly, cell lysates were prepared after their respective transfection with BNIP3 and its mutants at indicated time points. Assays were performed in 96-well microtitre plates by incubating 10 μl protein of cell lysate per sample in 80 μl reaction buffer (1% NP-40, 20 mM Tris-HCl (PH 7.5), 137 mM Nad and 10% glycerol) containing 10 μl caspase-3 substrate (Ac-DEVD-pNA) (2 mM). Lysates were incubated at 37 °C for 4 h. Samples were measured with an ELISA reader at an absorbance of 405 nm.

Western blotting

After 24 h of transient transfection, cells were washed with ice-cold phosphate-buffered saline and lysed in a buffer containing 50 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, 20 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100,

3 mM benzamidine, 10 mM phenylmethylsulphonyl fluoride, 1 μM pepstatin, 10 μg/ml aprotinin, 5 mM iodoacetic acid, and 2 μg/ml leupeptin to prepare whole-cell lysates. Lysates were clarified by centrifugation at 14,000 × *g* for 5 min, and the supernatant was quantitated by BCA assay. Ten μg of lysate was resolved in 10% SDS-PAGE and transferred to PVDF membrane. The membrane was sequentially blocked with PBST containing 5% non-fat dry milk powder, probed with primary antibodies against caspase-3 and α-actin, respectively, and incubated with the corresponding HRP-conjugated secondary antibody. The membrane was then developed with Superglow ECL (Shenggong, Shanghai, China) and images were acquired with BioRad Gel Documentation systems (Hercules, CA).

Statistical analyses

All data are presented as mean ± SD from at least three independent experiments with each in triplicate. Statistical analysis between two groups was performed using the Student's *t*-test. $P < 0.05$ was considered significant and $P < 0.01$ was considered very significant.

Results

The transmembrane domain is required for BNIP3-mediated apoptosis

Although hypoxia had been regarded as the major pathological inducer of BNIP3-mediated apoptosis (Kubasiak et al., 2002; Regula et al., 2002), many studies have now shown that hypoxia is not indispensable, especially when BNIP3 was over-expressed (Zhang et al., 2009; Ray et al., 2000). When plasmids pEGFP-N1 (BNIP3Empty), pEGFP-N1-BNIP3 (BNIP3WT), and pEGFP-N1-BNIP3ΔTM (BNIP3ΔTM) were transiently transfected into SW480 cells, the respective plasmid expression in SW480 cells was confirmed by fluorescence microscopy (Fig. 1A). To evaluate the pro-apoptotic effects of BNIP3WT, the GFP-positive cells were counted under a fluorescence microscope, and the percentage of dying cells was calculated by assessing the number of blue cells with condensed or fragmented nuclear morphology in the total population of GFP-positive cells (Fig. 1B). As shown in Fig.1C, BNIP3WT resulted in significantly increased apoptosis at both 24 and 48 h compared to BNIP3Empty, whereas deletion of the transmembrane domain (BNIP3ΔTM) completely abolished its apoptotic effect in SW480 cells. Similar results were obtained in MCF-7 cells (Fig. 1D).

Targeting BNIP3 into ER by replacing the TM with a fragment of cytochrome b5

We generated BNIP3cyb5, where the COOH-terminal TM of BNIP3 was replaced with the COOH-terminal transmembrane segment of cytochrome b5 (AA100-134), a sequence previously shown to target ER (Fig. 2A). Transient transfection of SW480 cells showed that the vector expressing GFP only (BNIP3Empty) was al-

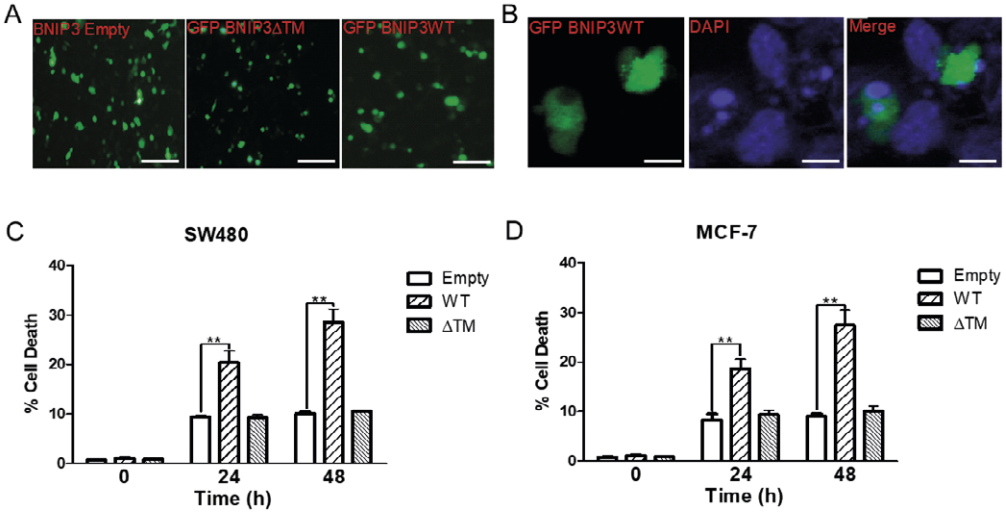

Fig. 1. Over-expression of BNIP3 induces cell death.
(**A**) Representative photomicrographs showing GFP-positive cells after transfection of SW480 cells with BNIP3Empty, BNIP3 Δ TM and BNIP3WT, respectively, at 24 h. (**B**) Representative images showing condensed or fragmented nuclear morphology stained by DAPI (blue) after transfection of SW480 cells with BNIP3WT at 24 h. (**C**) To evaluate the pro-apoptotic effects of BNIP3WT and its mutants on SW480 and MCF-7 cells, the GFP-positive cells were counted under a fluorescence microscope, and the percentage of dying cells was calculated by assessing the number of blue cells with condensed or fragmented nuclear morphology in the total population of GFP-positive cells. Forced expression of BNIP3 WT significantly increased cell death at both 24 and 48 h compared to that of BNIP3Empty, and forced expression of BNIP3 Δ TM abolished BNIP3WT-induced cell death in both SW480 (1C) and MCF-7 (1D) cells. Data are presented as means ± SD, with ** denoting $P < 0.01$. Scale bar in Fig. A, 200 μm, Scale bar in Fig. B, 10 μm.

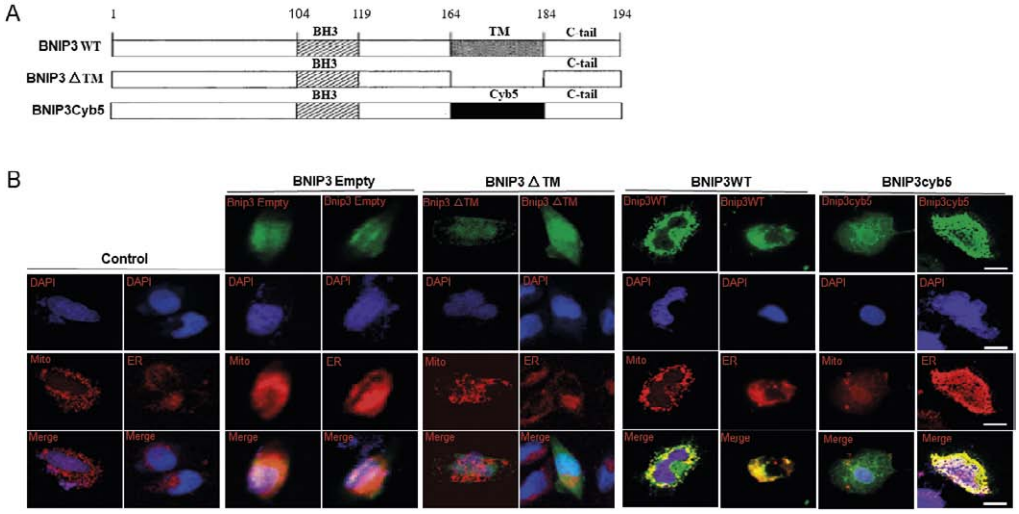

Fig. 2. Subcellular localization of BNIP3 and its mutants
(**A**) Schematic presentation of BNIP3WT, BNIP3ΔTM, and BNIP3cyb5 constructs. (**B**) Representative images showing that DAPI-stained nuclei are in blue, Mito-tracker Red CMXRos and ER-tracker Red Dye are in red, respectively, and GFP indicates the forced expression of BNIP3 and its mutants. Merged images showing that BNIP3WT localized to both mitochondria and ER, BNIP3cyb5 localized mainly to ER, and BNIP3ΔTM and GFPEmpty are localized neither to mitochondria nor to ER in SW480 cells. Scale bar, 10 μm.

most homogenously intracellular, BNIP3WT was localized to both mitochondria (co-localized with Mito-Tracker Red) and ER (co-localized with ER-Tracker Red), BNIP3cyb5 was largely localized to ER as expected, and BNIP3ΔTM was localized neither to mitochondria nor to ER, but dispersed throughout the cell (Fig. 2B).

ER-targeted BNIP3 induces a similar degree of cell death as mitochondria-localized BNIP3

To explore whether ER-localized BNIP3 has any pro-apoptotic effect, SW480 cells, seeded in 6-well plates with a coverslip in each well, were transiently transfected with BNIP3Empty, BNIP3WT, BNIP3cyb5, and BNIP3ΔTM, respectively. As shown in Fig. 3, BNIP3cyb5 localized to ER and induced cell death with condensed or fragmented nuclear morphology after transfection with BNIP3cyb5 at 24 h. Quantitated data showed that BNIP3cyb5 (Cyb5) induced more than 10% and 20% increased apoptosis at 24 and 48 h relative to BNIP3Empty (Empty), respectively, which was similar to that of BNIP3WT (WT). BNIP3ΔTM (ΔM) abolished the pro-apoptotic effect of BNIP3WT, showing no differences compared to that of the BNIP3Empty at both 24 and 48 h.

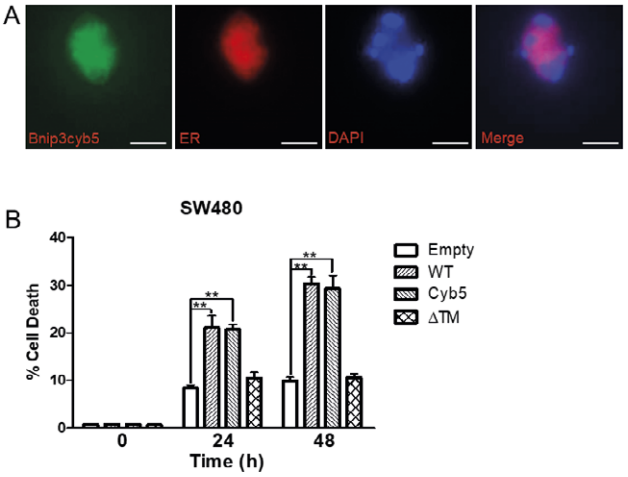

Fig. 3. BNIP3-mediated cell death in ER stress
(**A**) Representative photomicrographs showing that BNIP-3cyb5 localized to ER and induced cell death. (**B**) Quantitated data showing that BNIP3WT and BNIP3Cyb5 induced significant cell death compared to BNIP3Empty after transient transfection of SW480 cells at 24 and 48 h, respectively, whereas transfection of BNIP3ΔTM almost completely abrogated WT-induced cell death at both 24 and 48 h. Data are presented as means ± SD, with ** denoting $P < 0.01$. Scale bar, 10 μm.

ER-targeted BNIP3-induced apoptosis requires both calpains and caspases

To investigate the molecular mechanisms involved in BNIP3-induced apoptosis in ER, SW480 cells were transfected with the aforementioned plasmids in the absence or presence of 20 μM z-VAD-fmk, a pan-caspase inhibitor, or 30 μM of PD150606, a specific calpain inhibitor, 3 h prior to the transfection, respectively. The cells were continued to incubate for a total of 48 h. Treatment with z-VAD-fmk had no effect on BNIP3Empty

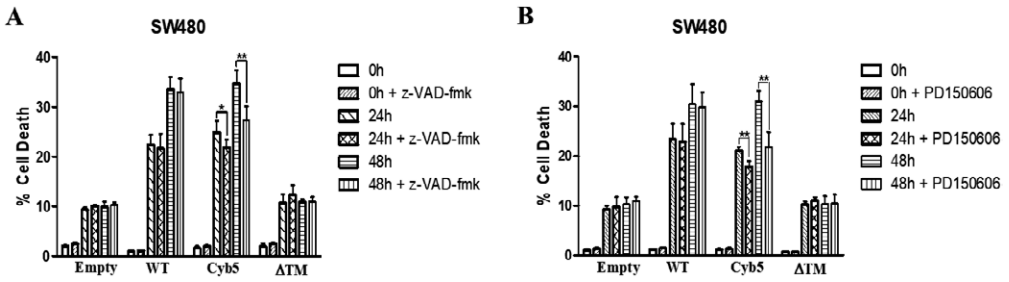

Fig. 4. Both caspases and calpains are required for BNIP3-induced cell death in ER.
(**A**) Quantitated data showing that both BNIP3WT and BNIP3Cyb5 induced significant cell death compared to BNIP3Empty and BNIP3ΔTM plasmid-transfected SW480 cells at both 24 and 48 h, respectively, and pre-treatment of cells with 20 μm z-VAD-fmk significantly inhibited BNIP3Cyb5-induced cell death at both 24 and 48 h, respectively. (**B**) Both BNIP3WT and BNIP3Cyb5 induced significant cell death compared to Empty and BNIP3ΔTM plasmid-transfected SW480 cells at both 24 and 48 h, respectively, and pre-treatment of cells with 30 μm PD150606 significantly inhibited cell death in BNIP3Cyb5-transfected cells at both 24 and 48 h, respectively. Data are presented as means ± SD, with * denoting $P < 0.05$ and ** denoting $P < 0.01$.

(Empty), BNIP3WT and BNIP3ΔTM (ΔTM)-induced apoptosis, whereas it significantly decreased BNIP3cyb5 (Cyb5)-induced apoptosis at both 24 and 48 h (Fig. 4A). Similarly, treatment with PD150606 had no effect on BNIP3Empty, BNIP3WT and BNIP3ΔTM-induced apoptosis, whereas it significantly decreased BNIP3cyb5-induced apoptosis at both 24 and 48 h (Fig. 4B). Caspase-3 is the most extensively studied apoptotic protein among the caspase family members. To investigate whether caspase-3 is involved in BNIP3-induced apoptosis due to ER stress in SW480 cells, we first examined the effect of AC-VAD-CHO, a caspase-3 specific inhibitor, on the apoptotic effects of BNIP3 and its mutants. As shown in Fig. 5A, treatment with 20 μM AC-VAD-

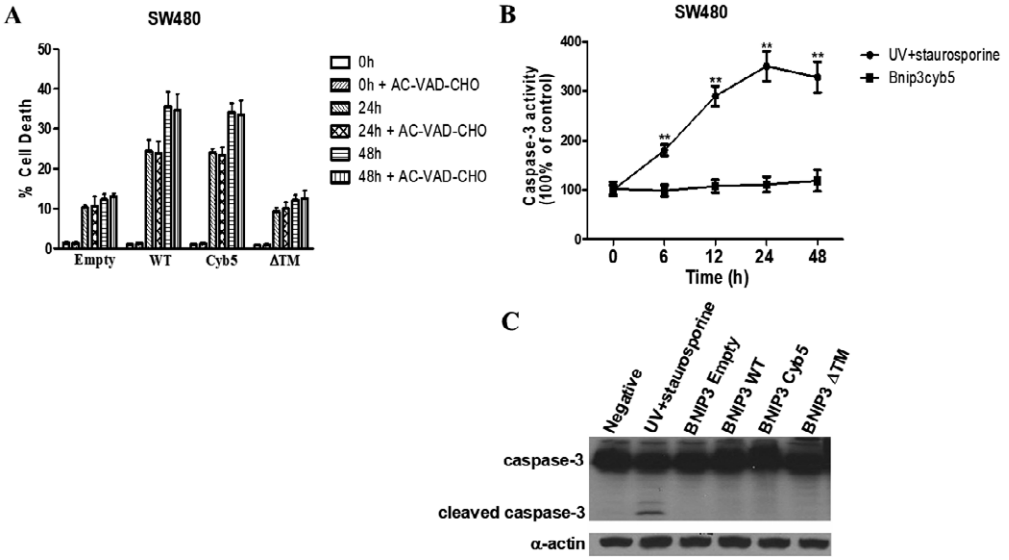

Fig. 5. Caspase-3 is not involved in BNIP3-induced cell death in ER.
(**A**) Quantitated data showing that both BNIP3WT and BNIP3cyb5 induced significant cell death compared to BNIP3Empty and BNIP3ΔTM plasmid-transfected SW480 cells at both 24 and 48 h, respectively, and pre-treatment of cells with 20 μm AC-VAD-CHO did not inhibit the cell death induced by BNIP3cyb5 at both 24 and 48 h, respectively. Data are presented as means ± SD. (**B**) The activity of caspase-3 was measured at indicated time points using the Caspase-3 activity kit. Cells were either treated with 1 μm staurosporine followed by UV exposure, or transfected with BNIP3cyb5. Each value was expressed as the ratio of caspase-3 activation level to control level (0 h), and the value of control was set to 100 %. Data are presented as means ± SD of five measurements, with ** denoting $P < 0.01$. (**C**) Representative image showing caspase-3 bands in all lanes and cleaved caspase-3 band in the only lane labelled as Positive (6 h UV irradiation in the presence of 1 μm staurosporine) (top panel), and their corresponding α-actin (bottom panel) in all lanes labelled after 24 h transfection.

CHO had no effects on both BNIP3WT (WT) and BNIP3cyb5 (Cyb5)-induced apoptosis. We also estimated the caspase-3 activity in SW480 cells after transfection of BNIP3cyb5. Positive control cells treated with 1 μm staurosporine followed by UV exposure showed increased levels of caspase-3 activity at indicated time points, whereas the levels of caspase-3 activity were not changed in the cells transfected with BNIP3cyb5 at indicated time points (Fig. 5B). In accordance with the time course data, forced expression of BNIP3Empty, BNIP3WT, BNIP3cyb5, and BNIP3ΔTM did not change much of the ratio of caspase-3 to α-actin, nor caused the cleavage of caspase-3. In contrast, a clear cleaved caspase-3 immunoreactive band was seen in the positive control (Positive) where cells were treated with 1 μm staurosporine followed by UV exposure, suggesting that caspase-3 was not activated by forced expression of those plasmids (Fig. 5C). Taken together, these results suggest that ER-targeted BNIP3-induced cell death requires both calpains and caspases, but not caspase-3.

Discussion

In the present study, we demonstrated that (1) the TM domain of BNIP3 that targets BNIP3 mainly to mitochondria is necessary for BNIP3-induced cell death, (2) targeting BNIP3 into ER induced cell death nearly as effectively as that of BNIP3WT, and (3) targeting BNIP3 into ER induced cell death in dependence on calpains and caspases, but not caspase-3.

It has been well established that the TM of BNIP3 mediating dimerization and mitochondrial localization plays important roles in BNIP-3-induced cell death. Consistently, deletion of the TM prevented BNIP3 from targeting to mitochondria in SW480 cells (Fig. 2B) and abrogated its death-inducing effect in both SW480 and MCF-7 cells (Fig. 1C-D).

Many members of the Bcl-2 family have been shown to induce cell death through their effects on ER (Singh et al., 2019; Yang et al., 2019). Like other Bcl-2 members, BNIP3 is also localized to ER under physiological conditions and increasingly during ER stress. Recently, it was demonstrated that selective targeting of BNIP3 to ER can induce autophagy in HeLa and uncharacterized cell death in Mes23.5 dopaminergic cells (Zhang et al., 2009; Hanna et al., 2012), suggesting that BNIP3 can also induce cell death in a mitochondria-independent pathway. In agreement, when the TM of BNIP3 was replaced with the ER-targeting fragment of cytochrome b, the resulting chimaeric protein, BNIP3cyb5, localized mainly to ER (Fig. 2B), where it induced cell death nearly as effectively as BNIP3WT in SW480 cells (Fig. 3).

Caspases belong to a family of proteases that are essential for programmed cell death (Mcilwain et al., 2013). However, it remains controvesial whether BNIP3-induced cell death is caspase dependent or not. Regula, et al. (2002) reported that BNIP3-induced cell death depends on caspase activation (Hamacher-Brady et al., 2007), whereas others showed that caspase inhibitors failed to block the BNIP3-induced cell death (Velde et al., 2000; Kubasiak et al., 2002; Azad et al., 2008). Although the discrepancy was attributed to differences in cell models and experimental conditions used (Regula et al., 2002; Hamacher-Brady et al., 2007), it remains possible that the caspase activation in BNIP3-mediated cell death resulted from the BNIP3 targeting to organelles other than mitochondria. Indeed, caspases localized in ER can be activated by ER stress, leading to the activation of the ER-specific apoptosis pathway (Nakagawa et al., 2000a,b). When BNIP3 was targeted to ER by replacing the TM with a fragment of cytochrome b5 (Fig. 2B), pre-incubation of SW480 cells with z-VAD-fmk, a specific caspase inhibitor, suppressed ER-targeted BNIP3-induced cell death (Fig. 4A). However, the most extensively studied pro-apoptotic member of the caspase family, caspase-3, was not activated in the cells over-expressing BNIP3WT and BNIP3cyb5 (Fig. 5B and C). Furthermore, AC-VAD-CHO, a caspase-3-specific inhibitor, had no effect on both the BNIP3WT- and BNIP3cyb5-induced apoptosis (Fig. 5A). Therefore, we speculate that caspase-3 may not be required in BNIP3-induced cell death in normal conditions, whereas other caspases are indispensable when BNIP3 was targeted into ER, a condition mimicking ER stress.

Calpains are a family of Ca^{2+}-dependent, non-lysosomal cysteine proteases expressed ubiquitously in many organisms. Like caspases, they are synthesized as inactive pro-enzymes that can be auto-processed to become active, participating in many physiological processes including apoptotic cell death (Storr et al., 2011; Chen et al., 2018). Treatment of SW480 cells with PD150606, a specific inhibitor of calpains, significantly suppressed BNIP3cyb5-induced cell death (Fig. 4B), suggesting that calpains are indispensable for the BNIP3-induced cell death due to the BNIP3 ER localization. This is in agreement with the discovery reported by Graham et al. (2015) that calpains were the downstream modulator of BNIP3-induced cardiomyocyte death under hypoxia condition.

In summary, BNIP3 initiates different cell death signalling events in the mitochondria and ER. The BNIP3 TM domain-mediated targeting of BNIP3 to mitochondria results in dysfunction of mitochondria, such as mitochondrial swelling, rupture of the outer mitochondrial membrane, release of small pro-apoptotic molecules, and these events finally lead to cell death (Ray et al., 2000; Zhang et al., 2009). In ER, BNIP3-induced cell death requires the coordinated action of caspases and calpains. Further studies are warranted to dissect the detailed molecular pathways in ER-targeted BNIP3-induced cell death.

Acknowledgements

We thank professor Jae-Hoon Park, Department of Pathology, Kyung Hee University, for providing BNIP3 cDNA.

References

Azad, M. B., Yongqiang, C., Henson, E. S., Jeannick, C., Eileen, M. M. W., Israels, S. J.,Gibson, S. B. (2008) Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3. *Autophagy* **4**, 195-204.

Chen, J.,Wu Y., Zhang, L., Fang X., Hu X. (2018) Evidence for calpains in cancer metastasis. *J. Cell. Physiol.* **234**, 8233-8240.

Foyouzi-Youssefi, R., Arnaudeau, S., Borner, C., Kelley, W. L., Tschopp, J., Lew, D. P., Demaurex, N., Krause, K. H. (2000) Bcl-2 decreases the free Ca^{2+} concentration within the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **97**, 5723-5728.

Graham, R. M., Thompson, J. W., Webster, K. A. (2015) BNIP3 promotes calcium and calpain-dependent cell death. *Life Sci.* **142**, 26-35.

Hamacher-Brady, A., Brady, N. R., Logue, S. E., Sayen, M. R., Jinno, M., Kirshenbaum, L. A., Gottlieb, R. A., Gustafsson, B. (2007) Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. *Cell Death Differ.* **14**, 146-157.

Hanna, R. A., Quinsay, M. N., Orogo, A. M., Kayla, G., Shivaji, R., Sa B, G. (2012) Microtubule-associated protein 1 light chain 3 (LC3) interacts with Bnip3 protein to selectively remove endoplasmic reticulum and mitochondria via autophagy. *J. Biol. Chem.* **287**, 190-194.

Knight, T., Luedtke, D., Edwards, H., Taub, J. W., Ge, Y. (2019) A delicate balance - the BCL-2 family and its role in apoptosis, oncogenesis, and cancer therapeutics. *Biochem. Pharmacol.* **162**, 250- 261.

Kubasiak, L. A., Hernandez, O. M., Bishopric, N. H., Webster, K. A. (2002) Hypoxia and acidosis activate cardiac myocyte death through the Bcl-2 family protein BNIP3. *Proc. Natl. Acad. Sci. USA* **99**, 12825-12830.

Mcilwain, D. R., Thorsten, B., Mak, T. W. (2013) Caspase functions in cell death and disease. *Cold Spring Harb. Perspect. Biol.* **5**, a008656.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A., Yuan, J. (2000a) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β. *Nature* **403**, 98-103.

Nakagawa, T., Yuan J. Y. (2000b) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J. Cell Biol.* **150**, 887-894.

Nutt, L. K., Abujiang, P., Jessica, P., Bingliang, F., Jack, R., Mcconkey, D. J., Swisher, S. G. (2002) Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca^{2+} stores. *J. Biol. Chem.* **277**, 9219-9225.

Opferman, J. T., Kothari, A. (2017) Anti-apoptotic BCL-2 family members in development. *Cell Death Differ.* **25**, 37-45.

Pihán, P., Carreras-Sureda, A., Hetz, C. (2017) BCL-2 family: integrating stress responses at the ER to control cell demise. *Cell Death Differ.* **24,** 1478-1487.

Ray, R., Chen, G., Velde, C. V., Cizeau, J., Park, J. H., Reed, J. C., Gietz, R. D., Greenberg, A. H. (2000) BNIP3 heterodimerizes with Bcl-2/Bcl-XL 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.

Regula, K. M., Karen, E., Kirshenbaum, L. A. (2002) Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. *Circ. Res.* **91**, 226-231.

Rodriguez, D., Rojas-Rivera, D., Hetz, C. (2010) Integrating stress signals at the endoplasmic reticulum: the BCL-2 protein family rheostat. *Biochim. Biophys. Acta* **1813**, 564-574.

Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., Korsmeyer, S. J. (2003) BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. *Science* **300**, 135-139.

Singh, R., Letai, A., Sarosiek, K. (2019) Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins. *Nat. Rev. Mol. Cell Biol.* **20**,175–93.

Storr, S. J., Carragher, N. O., Frame, M. C., Parr, T., Martin, S. G. (2011) The calpain system and cancer. *Nature Reviews Cancer* **11**, 364–374.

Velde, C., Vande, Cizeau, J., Dubik, D., Alimonti, J., Brown, T., Israels, S., Hakem, R., Greenberg, A. H. (2000) BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol. Cell. Biol.* **20**, 5454-5468.

Yang, B., Liu, Q., Bi, Y. (2019) Autophagy and apoptosis are regulated by stress on Bcl2 by AMBRA1 in the endoplasmic reticulum and mitochondria. *Theor. Biol. Med. Model.* **16**,18-27.

Zhang, L., Li, L., Liu H., Borowitz, J. L., Isom, G. E. (2009) BNIP3 mediates cell death by different pathways following localization to endoplasmic reticulum and mitochondrion. *FASEB J.* **23**, 3405-3414.