Bax regulates primary necrosis through mitochondrial dynamics

Russell S. Whelan*,b,c,f, Klitos Konstantinidis*a,b,c,f, An-Chi Wei*d, Yun Chen*, Denis E. Reyna*c,f, Saurabh Jha*e,s, Ying Yang*a,b,c, John W. Calvert9, Tullia Lindsten*b, Craig B. Thompson*c, Evripidis Gavathiotis*c,f, Gerald W. Dorn ii*, Brian O’Rourke*, and Richard N. Kitis*a,b,c,j,k

Abstract

The defining event in apoptosis is mitochondrial outer membrane permeabilization (MOMP), allowing apoptogen release. In contrast, the triggering event in primary necrosis is early opening of the inner membrane mitochondrial permeability transition pore (mPTP), precipitating mitochondrial dysfunction and cessation of ATP synthesis. Bcl-2 proteins Bax and Bak are the principal activators of MOMP and apoptosis. Unexpectedly, we find that deletion of Bax and Bak dramatically reduces necrotic injury during myocardial infarction in vivo. Triple knockout mice lacking Bax/Bak and cyclophilin D, a key regulator of necrosis, fail to show further reduction in infarct size over those deficient in Bax/Bak. Absence of Bax/Bak renders cells resistant to mPTP opening and necrosis, effects confirmed in isolated mitochondria. Reconstitution of these cells or mitochondria with wild-type Bax, or an oligomerization-deficient mutant that cannot support MOMP and apoptosis, restores mPTP opening and necrosis, implicating distinct mechanisms for Bax-regulated necrosis and apoptosis. Both forms of Bax restore mitochondrial fusion in Bax/Bak-null cells, which otherwise exhibit fragmented mitochondria. Cells lacking mitofusin 2 (Mfn2), which exhibit similar fusion defects, are protected to the same extent as Bax/Bak-null cells. Conversely, restoration of fused mitochondria through inhibition of fusion potentiates mPTP opening in the absence of Bax/Bak or Mfn2, indicating that the fused state itself is critical. These data demonstrate that Bax-driven fusion lowers the threshold for mPTP opening and necrosis. Thus, Bax and Bak play major roles in both processes (1, 2). Apoptosis is characterized by cell shrinkage, fragmentation, and phagocytosis, maintenance of plasma membrane integrity and ATP levels, and absence of an inflammatory response. In contrast, central features of necrosis include cellular and organelle swelling, marked depletion of ATP, disruption of membranes, and inflammation. Apoptosis has long been recognized as a highly regulated, genetically directed process, whereas, until recently, necrosis was considered an unregulated form of cell death. Studies over the past decade have challenged this view and demonstrated that a significant portion of necrotic deaths also occur through highly regulated mechanisms (3, 4).

Apoptosis and necrosis are mediated by distinct but overlapping pathways involving cell surface death receptors and mitochondria/endoplasmic reticulum (1,3). The critical mitochondrial event in apoptosis is mitochondrial outer membrane permeabilization (MOMP), which permits release of cytochrome c and other apoptogens leading to caspase activation. In contrast, the key mitochondrial event in primary necrosis is early opening of the mitochondrial permeability transition pore (mPTP) in the inner membrane, which occurs in the absence of cytochrome c release. Opening of the mPTP causes immediate dissipation of the electrical potential difference across the inner membrane (Δψm) leading to cessation of ATP synthesis and massive inflow of water into the solute-rich matrix causing severe mitochondrial swelling. In contrast to primary necrosis, secondary necrosis follows apoptosis if the removal of apoptotic bodies is delayed or nonexistent as in cell culture (5). In this case, necrotic events, such as loss of Δψm, occur coincident or after cytochrome c release (6).

Mitochondrial morphology is determined by a dynamic equilibrium between fusion and fission, repeated cycles of which redistribute mitochondrial constituents, including DNA, to maintain mitochondrial structure and function (7). Fission is mediated by dynamin-related protein 1 (Drp1), a GTPase that translocates from cytosol to mitochondria, and Fis1, an outer mitochondrial membrane protein. Fusion is controlled by three dynamin-related GTPases: Mfn1 and Mfn2 in the outer mitochondrial membrane and Opa1 in the inner mitochondrial membrane. The relationship between mitochondrial dynamics and cell death is poorly understood.

The Bcl-2 family consists of pro- and antiapoptotic members that engage in a complex set of interactions to regulate apoptosis (1). Apoptotic signals ultimately converge on Bax and Bak, multidomain proapoptotic proteins to promote MOMP, subsequent caspase activation, and apoptotic cell death. An additional function of Bax and Bak is to promote fusion in healthy cells, and cells deficient in these proteins contain fragmented mitochondria (8,9).

Prior studies have provided hints that Bcl-2 proteins may regulate cell death in situations where necrosis was thought to be involved (10–12), but molecular events and mechanisms have not yet been elucidated. Here we demonstrate that Bax regulates the sensitivity of cells to undergo primary necrosis. This effect of Bax occurs through a mechanism that is distinct from the role of Bax in apoptosis. Unexpectedly, Bax-driven fusion is critical for these effects.

Results

Absence of Bax and Bak Decreases Necrosis in Vivo. To assess the role of Bax and Bak in regulating necrosis in vivo, we used a mouse model of myocardial infarction, a disease process characterized by a mixture of necrotic and apoptotic cardiac myocyte death (13).
Combined deletion of Bax and Bak significantly reduced infarct size, a measure of total cell death (Fig. 1A and Fig. S1). Consistent with the known roles of Bax and Bak in apoptosis (14), this was accompanied by a decrease in the percentage of TUNEL-positive cardiac myocytes (Fig. 1B). Unexpectedly, absence of Bax and Bak markedly diminished characteristic features of necrosis, including amorphous mitochondrial densities, poorly defined cristae, swollen and ruptured mitochondria, and sarcomeric disorganization (Fig. 1C). Strikingly, triple knockout mice lacking Bax/Bak and cyclophilin D, a key regulator of mPTP opening and necrosis (15–17), failed to show any further reduction in infarct size over those deficient in Bax/Bak (Fig. 1A). These data indicate that Bax and Bak regulate necrosis in vivo and suggest a connection between mitochondrial events that mediate apoptosis and necrosis.

**Bax/Bak-Null Cells Are Protected from Necrosis, and Susceptibility Is Restored by Reconstitution with Bax.** To determine whether the defining events of necrosis are abrogated by the absence of Bax and Bak, we modeled necrosis and apoptosis in mouse embryo fibroblasts (MEFs). As Ca$^{2+}$ is a primary regulator of mPTP opening, the Ca$^{2+}$ ionophore ionomycin, was used to induce necrosis. Treatment with ionomycin triggered mPTP opening as early as 1 h, as assessed by loss of Δψm. Moreover, this occurred in the absence of cytochrome c release, indicative of primary necrosis (Fig. 2A and B). In contrast, the initial event following treatment with the apoptosis inducer staurosporine was cytochrome c release, minimally detectable at 4 h and becoming maximal at 8 h (Fig. 2B), and only then was loss of Δψm observed, consistent with apoptosis (6) (Fig. 2A). Ionomycin, but not staurosporine, resulted in loss of plasma membrane integrity, a hallmark of necrosis (Fig. 2C). Thus, early mPTP opening without cytochrome c release indicates primary necrosis, whereas later release of cytochrome c coincident with mPTP opening demarcates apoptosis.

Consistent with previous studies (14), cells lacking Bax and Bak did not undergo apoptosis, as assessed by cytochrome c release in response to staurosporine (Fig. 2B). Importantly, absence of Bax and Bak abrogated ionomycin-induced necrosis, as assessed by early mPTP opening and loss of plasma membrane integrity (Fig. 2A and C). Similarly, isolated mitochondria from Bax/Bak-null hearts required a greater Ca$^{2+}$ load to induce mPTP opening and swelling compared with wild-type mitochondria (Fig. 2D).

We focused on the role that Bax may play in this process. Reconstitution of Bax/Bak-null MEFs with Bax restored ionomycin-induced mPTP opening (Fig. 3B). Similarly, recombinant Bax restored Ca$^{2+}$-induced mPTP opening in Bax/Bak-null isolated cardiac mitochondria (Fig. 3C). These results demonstrate that absence of Bax and Bak abrogates necrosis, and sensitivity is restored by reconstitution with Bax.

---

**Fig. 1.** Deletion of Bax and Bak markedly reduces necrotic injury during myocardial infarction in vivo. (A) Infarct size following 45 min of left coronary artery occlusion followed by 24 h of reperfusion (IR). AAR/LV, area at risk/left ventricle; INF/AAR, infarct size normalized to AAR; (Left graph) WT, wild-type mice; DKO, double knockout mice lacking Bax and Bak; TKO, triple knockout mice lacking Bax, Bak, and cyclophilin D. (Right graph) WT, wild-type mice; Ppif KO, mice lacking cyclophilin D. Numbers of animals indicated in circles. Confirmation of knockouts in Fig. S1. (B) Apoptosis assessed within the AAR of heart sections from mice subjected to sham operation or 45 min ischemia/10 h reperfusion using TUNEL and costaining with troponin I to identify cardiac myocytes and DAPI. (C) Transmission electron microscopy of infarct zone and remote myocardium following 45 min ischemia/24 h reperfusion. Key features of myocardial necrosis including amorphous mitochondrial densities (red arrow), poorly defined cristae (black arrow), mitochondria swelling, and rupture, sarcomeric disorganization (on lower power images). Representative of at least 10 randomly selected fields for each genotype. Data mean ± SEM. ***P < 0.001, *P < 0.05, compared with WT. †No significant difference compared with DKO.
Oligomerization-Deficient Bax Restores mPTP Opening in Bax/Bak-Null Cells and Mitochondria. The precise roles of Bax and Bak in MOMP are incompletely understood, but homo- and/or hetero-oligomerization of these proteins is involved (18–20), and oligomerization-deficient Bax mutants cannot support MOMP and apoptosis (21, 22). Consistent with these observations, treatment of cells with staurosporine shifted Bax into high molecular weight complexes (Fig. 3A). In contrast, treatment of cells with ionomycin did not induce Bax oligomerization (Fig. 3A). Accordingly, we tested whether oligomerized Bax is needed for mPTP opening in response to ionomycin. Bax(63-65)A, harboring L63A, R64A, and R65A mutations in the BH3 domain, is unable to oligomerize (21). Equivalent reconstitution of Bax/Bak-null cells or isolated cardiac mitochondria with this mutant or wild-type Bax restored Ca\(^{2+}\)-induced mPTP opening to the same extent (Fig. 3B and C). These experiments demonstrate that nonoligomerized Bax, although unable to mediate apoptosis, is sufficient to mediate necrosis.

Mitochondrial Shape Regulates Sensitivity of mPTP Opening. Both wild-type and oligomerization-deficient Bax restore mitochondrial fusion in Bax/Bak-null cells (9), which contain fragmented mitochondria (Fig. 4A). Similarly, cells lacking Mfn2 also exhibit fragmented mitochondria (8, 9) (Fig. 4A), and these cells were protected from Ca\(^{2+}\)-induced mPTP opening to a similar extent as Bax/Bak-null cells (Fig. 4B). Given that Bax/Bak-null and Mfn2-null cells exhibit a common fragmented mitochondrial morphology, we tested whether inhibition of mPTP opening is mediated by deficiencies in these specific proteins or, more generally, by changes in mitochondrial morphology. Attenuation of mitochondrial fission with the small molecule Mdivi-1 (23), an inhibitor of the fission protein Drp1, restored both the fused state and sensitivity to ionomycin-induced mPTP opening in Bax/Bak-null and Mfn2-null cells (Fig. 4A and B). These results indicate that the shift to the fused state per se potentiates Ca\(^{2+}\)-induced mPTP opening.

Discussion

Using an in vivo model of myocardial infarction, MEFs, and isolated cardiac mitochondria, these data demonstrate that the absence of Bax and Bak confers resistance to necrotic cell death, and sensitivity can be restored by reconstitution with Bax. Cell death in these studies occurred by primary necrosis—not necrosis secondary to apoptosis—because the events that define necrosis (mPTP opening, loss of plasma membrane integrity, and cell death) take place within a few hours, without Bax.
oligomerization, in the absence of cytochrome c release, and can be reconstituted with oligomerization-deficient Bax(63-65)A, which cannot support MOMP and apoptosis (21). Moreover, the ability of this mutant to restore sensitization to necrosis, but not apoptosis, indicates that Bax modulates necrosis through mechanisms distinct from apoptosis.

In addition to their abilities to rescue sensitivity to necrosis in Bax/Bak-deficient cells, both wild-type Bax and Bax(63-65)A restore mitochondrial fusion in these cells, which exhibit baseline mitochondrial fragmentation (8, 9). These observations suggest the hypothesis that Bax-regulated fusion mediates sensitivity to necrosis, but not apoptosis, indicates that Bax modulates necrosis through mechanisms distinct from apoptosis.

In addition to their abilities to rescue sensitivity to necrosis in Bax/Bak-deficient cells, both wild-type Bax and Bax(63-65)A restore mitochondrial fusion in these cells, which exhibit baseline mitochondrial fragmentation (8, 9). These observations suggest the hypothesis that Bax-regulated fusion mediates sensitivity to necrotic cell death. Consistent with this possibility, cells lacking the fusion protein Mfn2, which also show fragmented mitochondria (8, 9), phenocopy the resistance to necrosis observed in Bax/Bak-null cells (Fig. 4B). To test the causality of these observations, and whether the fusion process itself or the fused mitochondrial morphology is critical, we restored the fused morphology in Bax/Bak-deficient cells through an independent means, by opposing Drp1-mediated fission with Mdivi-1. Restoration of the fused morphology reestablishes susceptibility to necrosis. Moreover, the same results were observed with inhibition of fission in Mfn2-null cells. These data strongly suggest that the fused morphology, regardless of how it is achieved, mediates sensitivity to necrosis.

The mechanistic connections between mitochondrial morphology and cell death remain unclear. Previous work has linked mitochondrial fission with apoptosis (24). Inhibition of fission genetically or pharmacologically with Mdivi-1 decreases or delays apoptosis. Moreover, during apoptosis, Bax oligomers in the outer mitochondrial membrane are associated with fission, although a causal connection has not been demonstrated. In contrast, nonoligomerized Bax is known to drive fusion in cells that are considered healthy—defined as not having been subjected to an apoptotic stimulus (9). The current study introduces the concept that the fused mitochondrial state renders these cells poised to undergo necrosis, if presented with an appropriate stimulus.
This model is consonant with previous observations showing that diffusion of Ca\(^{2+}\) waves is more efficient in fused versus fragmented mitochondria (25). In addition, it is consistent with observations that absence of Mfn2 inhibits cardiac myocyte death (26). Our findings conflict, however, with those of Ong et al. (27) who reported that inhibition of fission with Mdivi-1 delays mPTP opening and protects cardiac myocytes against ischemia/reperfusion injury. Although the reasons for this discrepancy are not clear, cell death in this other study was scored using plasma membrane integrity at late time points, raising the possibility of necrosis secondary to apoptosis. In addition, Mdivi-1 was used to treat wild-type cells, in which a substantial proportion of mitochondria are already fused. In contrast, the present study uses genetic models of defective mitochondrial fusion to delineate the role of the fused state in setting the baseline susceptibility of wild-type cells to undergo necrosis. Elucidation of the molecular and biophysical mechanisms by which the fused state sensitizes cells to mPTP opening and necrosis will likely require identification of the components of this pore, none of which have yet been determined with certainty (28).

Cell death in ischemic syndromes, such as myocardial infarction and stroke, is characterized by a spatially and temporally complex pattern of necrosis and apoptosis (4, 29). Whereas inhibition of either form of cell death reduces infarct size, optimal amelioration of both the acute injury in the central infarct zone and the subsequent cell death in immediately surrounding areas requires inhibition of both necrosis and apoptosis. Bax and Bak may provide especially potent therapeutic targets to achieve this goal in these common and lethal syndromes.

**Materials and Methods**

**Myocardial Infarction Model and Analysis.** Bax\(^{\text{fl}}\)/Bak\(^{\text{fl}}\) mice (30) were crossed with \(\alpha\)-myosin heavy chain-Cre transgenic mice (31) to generate mice with cardiac myocyte-specific deletion of Bax and generalized deletion of Bak. These mice were crossed with Ppif\(^{-/-}\) mice (15) to generate mice with cardiac myocyte-specific deletion of Bax and generalized deletion of Bak and Ppif. Ischemia/reperfusion was induced in 8- to 12-wk-old male mice by

---

**Fig. 4.** Cells with fragmented mitochondria are resistant to mPTP opening, which can be reversed by restoration of fused morphology. (A) Analysis of mitochondrial morphology by confocal microscopy of WT, Bax/Bak DKO, and Mfn2 KO MEFs not treated or treated for 6 h with Mdivi-1 (50 \(\mu\)M). Cells stained with MitoTracker Red and DAPI. Quantification of fused mitochondrial morphology by flow cytometry of mitochondria. FSC-A, forward scatter area. (B) Ionomycin-induced mPTP opening in the same groups except ionomycin (10 \(\mu\)M) added 2 h before analysis. \(n=3\) independent experiments. ***P < 0.001, compared with wild-type cells that were treated with ionomycin but not Mdivi-1. §§§P < 0.001 compared with cells of the same genotype that were treated with ionomycin but not Mdivi-1.
ligating the left coronary artery for 45 min followed by 24-h reperfusion. The area at risk (AAR) was assessed by Evan's blue dye, and the area of infarct (INF) was determined by staining with 2,3,5-triphenyltetrazolium chloride as described (32). TUNEL was performed as described (33) and sections were counterstained for troponin I (Santa Cruz) and DAPI (Vector Laboratories). Transmission electron microscopy was performed on samples fixed with 2% (vol/vol) paraformaldehyde, 2.5% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate, postfixed with 1% (wt/vol) osmium tetroxide, followed by 1% (wt/vol) uranyl acetate, dehydrated, and embedded in LX112 resin. Ultrathin sections stained with uranyl acetate followed by lead citrate were viewed on a JEOL 1200EX transmission electron microscope at 80 kV.

**Immunoblotting.** Bak and Bak antisera were from Cell Signaling. Cyclophilin D, mitochondrial membrane) markers.

**Subcellular Fractionation.** Cells were harvested and resuspended in 10 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 20 mM HEPES pH 7.2, 0.025% (wt/vol) digitonin, and protease inhibitors. Following 5 min incubation on ice, the lysate was spun down at 15,000 × g for 10 min at 4 °C, and the supernatant containing the cytosol was stored. The pellet was lysed with 1% (vol/vol) Triton X-100 in PBS for 1 h at 4 °C. Fractional purity was determined by blotting with GAPDH (cytosolic) and complex Vα (inner mitochondrial membrane) markers.


**LDH Release.** This was quantified using CytoTOX-One homogeneous membrane integrity assay (Promega).

**Ca2+ Loading Assay.** Cardiac mitochondria were isolated from adult mice and incubated with Ca-Green and TMRE (Invitrogen) to determine the Ca2+ load that triggers Δψm loss and swelling (34). For reconstitution experiments, recombinant WT Bax or Bax(63-65)A were added to the mitochondria at a final concentration of 100 nM and measurements made 10 min later.

**FPLC.** Total cellular protein was size fractionated on a Superose 6 sizing column using the AKTA FPLC system (S5), and fractions immunooblotted for Bax.

**Transfection.** Cells were transfected using TransIT-LT1 (Mirus Bio).

**Recombinant Bax Production.** WT Bax and Bax(63-65)A were generated using the CBP-intein system (36). Following purification, monomeric Bax in detergent-free buffer was isolated by size-exclusion chromatography.

**Mitochondrial FACS.** This procedure was performed as described (37).

**ACKNOWLEDGMENTS.** We thank Drs. Emily H.-Y. Cheng and Xu Luo for constructs and cell lines, Drs. Nina Kaludercic and Fabio DiIusa for advice regarding mitochondrial assays, Drs. Charles J. Steenbergen and Stephen M. Factor for advice regarding cardiac pathology, and Chad K. Nicholson for technical assistance. We also thank the Wilf family for their ongoing generosity and support. This work was supported by Grants SR01HL06665-13 (to R.N.K.), 5P03CA013330-39 (to R.N.K. and E.G.), 5P60DK020541-34 (to R.N.K.), 5R37HL054598-16 (to B.O.), 4R01HL095929-02 (to E.G.), American Heart Association Grant GRNT2290168 (to M.T.C.), 5T32AG023475-08 (to R.S.W.), and the A. G. Leventis Foundation (K.K.). R.N.K. is supported by The Dr. Gerald and Myra Dorros Chair in Cardiovascular Disease.

**Whelan et al.** PNAS | April 22, 2012 | vol. 109 | no. 17 | 6571