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A non-apoptotic role for BAX and BAK in eicosanoid metabolism

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Abstract

BCL-2 proteins are key regulators of programmed cell death. The interplay between pro- and anti-apoptotic BCL-2 members has important roles in many cancers. In addition to their apoptotic function, recent evidence supports key non-apoptotic roles for several BCL-2 proteins. We used an unbiased lipidomics strategy to reveal that the pro-apoptotic proteins BAX, and to a lesser extent BAK, regulate the cellular inflammatory response by mediating COX-2 expression and prostaglandin biosynthesis. COX-2 upregulation in response to the bacterial endotoxin lipopolysaccharide is blunted in the absence of BAX, and *Bax*^{-/-} mouse embryonic fibroblasts display altered kinetics of NFκB and MAPK signaling following endotoxin treatment. Our approach uncovers a novel, non-apoptotic function for BAX in regulation of the cellular inflammatory response and suggests that inflammation and apoptosis are more tightly connected than previously anticipated.

The balance between apoptosis and survival is essential for maintenance of homeostasis and health in living systems.¹ Apoptosis is an intricately regulated mechanism that proceeds through different cell type and signal-dependent pathways.^{1–2} The mitochondrial apoptotic pathway is modulated by pro- and anti-apoptotic BCL-2 family members whose interactions in response to a diverse set of cellular signals regulate cell fate.^{1, 3–4} BCL-2 members are classified based on structure and function into multidomain pro-apoptotic, multidomain anti-apoptotic and BH3 domain-only pro-apoptotic proteins.^{1, 3–4}

In addition to their roles in cell death, recent evidences point to novel, non-apoptotic functions for several BCL-2 members in metabolism⁵, immunity⁶ and mitochondrial morphogenesis.⁷ The BH3 domain-only BAD resides in a functional holoenzyme complex

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Notes

The authors declare no competing financial interest.

Supporting Information

Supplementary methods, table and figures are described in text. These materials are free of charge via the Internet at <http://pubs.acs.org>.

that includes glucokinase, and *Bad*^{-/-} mice display reduced glucose tolerance and abnormal glucose homeostasis.⁵ Genome-wide siRNA screen identified the BH3 domain-only BID as an *in vivo* regulator of innate immunity through the nucleotide-binding and oligomerization domain (NOD) pathway.⁶ These examples highlight the potential for individual BCL-2 proteins to partake in unexpected biology, and suggest that other BCL-2 members may also have non-apoptotic functions.

BAX and BAK are key multidomain pro-apoptotic proteins required for the mitochondrial apoptotic pathway.¹ BAX and BAK oligomerization in the outer mitochondrial membrane leads to mitochondrial outer membrane permeabilization, a crucial step in apoptosis^{1, 8}. In addition to their apoptotic function, BAX and BAK also regulate mitochondrial morphogenesis; BAX and BAK double knockout (DKO) mouse embryonic fibroblasts (MEFs) display a short, fragmented mitochondrial morphology relative to wildtype (WT).⁷ Given evidence supporting metabolic functions for BCL-2 proteins and dependence of the mitochondrial apoptotic pathway on lipid reorganization within the mitochondrial bilayer,⁹ we hypothesized that BAX and BAK could participate in mitochondrial morphogenesis and apoptosis through regulation of lipids.

To test our hypothesis, we used an unbiased, global lipidomics approach to identify differences in lipid profiles between WT and DKO MEFs. Lipids from WT and DKO MEF mitochondria were subjected to liquid chromatography–mass spectrometry (LC–MS) (Figure 1a). Analysis of major lipid classes yielded no significant changes based on our criteria (3-fold change, p-value < 0.05) (Table S1). While major lipid classes were unaltered, an unidentified metabolite (m/z: 369.2255, calculated formula: C₂₀H₃₄O₆) was consistently down-regulated in DKO MEF mitochondria relative to WT (Figure 1b, c). Perturbation in the level of a single metabolite in a background of unchanging lipids supports specificity in the pathway regulated by BAX and BAK.

Database searches (Lipid Maps¹⁰ and KEGG¹¹) uncovered six known eicosanoids (6-keto-prostaglandin F_{1α}, 6, 15-diketo-13, 14-dihydro-prostaglandin F_{1α}, 20-hydroxy-prostaglandin F_{2α}, thromboxane B₂, 19(R)-hydroxy-prostaglandin F_{2α} and 19(R)-hydroxy-prostaglandin E₁) that matched the calculated molecular formula (Figure 1d). Structural similarities among these candidates presented a challenge toward characterizing the unidentified metabolite. While our global lipidomics method enables simultaneous quantification of a wide range of lipid classes, this method is not suited to more sensitive resolution of the structurally similar eicosanoids, leaving open the possibility that the unidentified metabolite is a mixture of multiple species. To address this problem we performed tandem MS and chromatography optimization.

Comparisons between tandem MS spectra of candidate eicosanoid standards and the endogenous m/z 369.23 metabolite strongly suggest that the latter is predominantly 6-keto-prostaglandin F_{1α}, (6-keto-PGF_{1α}) (Figures 1e, S1). Based on tandem MS data we optimized our existing chromatographic method to allow baseline separation of 6-keto-PGF_{1α} from additional eicosanoids (Figure 1f). Co-injection of 6-keto-PGF_{1α} standard with endogenous sample using the optimized method yielded a single peak corresponding to the

m/z of 6-keto-PGF_{1α} (Figure 1g). The combination of tandem MS and co-injection provides strong evidence that the unidentified metabolite is 6-keto-PGF_{1α}.

Prostaglandins (PGs) are synthesized *de novo* in most cell types from arachidonic (C20:4) acid (AA) in response to mechanical trauma or inflammatory stimuli¹². Upon release from membrane phospholipids by phospholipases, AA is converted to the intermediate PGH₂ and further processed into downstream eicosanoids by cell type-specific enzymes (Figure 2a).¹² Within this pathway, 6-keto-PGF_{1α} is the hydrolysis breakdown product of the vasodilator and platelet activation inhibitor PGI₂ (prostacyclin) (Figure 2a).^{13–14} Relative stability of 6-keto-PGF_{1α} has allowed its measurement as a marker for eicosanoid metabolism.¹⁵

Reduced 6-keto-PGF_{1α} levels in DKO MEFs could be the result of decreased 6-keto-PGF_{1α} production or increased catabolism. In agreement with previous literature,^{16–17} we did not observe significant amount of additional PGs, a possible consequence of the short half-lives of these lipids.^{12, 18–19} Instead, we measured AA levels in WT and DKO MEF mitochondria to determine if absence of BAX and BAK could influence AA metabolism. Our analysis revealed a two-fold ($p < 0.05$) elevation in AA level in DKO MEF mitochondria relative to WT, with no changes in additional common free fatty acids (Figure 2b). Decreased 6-keto-PGF_{1α} and elevated AA levels in DKO MEFs support a reduction in the rate of PG biosynthesis in the absence of BAX and BAK.

To determine if BCL-2 regulation of eicosanoid metabolism requires both BAX and BAK, we extended our LC-MS analysis to include BAX and BAK single knockout (SKO) MEFs. 6-Keto-PGF_{1α} was reduced in DKO and *Bax*^{−/−} MEF mitochondria relative to WT (Figure 2c); a similar but non-statistically significant trend was observed for the *Bak*^{−/−} model (Figure 2c). AA was significantly elevated in DKO and SKO MEF mitochondria relative to WT, with more significant change present in the absence of BAX than BAK (Figure 2c). 6-Keto-PGF_{1α} and AA were further quantified in whole cell extracts to determine if the observed lipid differences were mitochondria-specific. 6-Keto-PGF_{1α} was reduced in DKO and *Bax*^{−/−} whole cell extracts while AA was elevated in SKO MEFs (Figure 2d). Taken together, our data demonstrate that BAX, and to a lesser extent BAK, regulate cellular eicosanoid metabolism, and the levels or activities of eicosanoid biosynthetic enzymes are decreased in the absence of these proteins. The inability of BAX and BAK to compensate for each other in this function stands in contrast to their capacity to subserve each other's pro-apoptotic and mitochondrial morphogenesis roles.^{7, 20} Thus, BAX and BAK could be modulating eicosanoid production through a separate pathway from their regulation of mitochondrial morphology or apoptosis, which are dependent on both proteins.

Conversion of AA to PGs is catalyzed by cyclooxygenase (COX) at the ER and nuclear membranes.¹² Two COX isoforms are known; COX-1 is responsible for basal, constitutive PG production while COX-2 is an inducible enzyme that is activated by stress signals.^{12, 21} COX-2 is the primary target of non-steroidal anti-inflammatory drugs due to its physiological role in inflammation.²¹ Elevated AA and decreased 6-keto-PGF_{1α} levels in the absence of BAX and/or BAK could be a consequence of COX down-regulation. To test this hypothesis, COX expression in WT, DKO and SKO whole cell lysates was determined by Western blot. While COX-1 level was unaltered, COX-2 expression was significantly

reduced in DKO and SKO lysates relative to WT (Figure 3a). The extent of BCL-2 regulation of COX-2 correlated well with BCL-2-induced changes at the metabolic level, with absence of BAX causing stronger changes in both PG and COX-2 levels in comparison to BAK (Figure 3a).

Given the most significant difference in COX-2 expression was observed between WT and *Bax*^{-/-} MEFs (Figure 3a), we focused on BAX for subsequent studies. *Cox-1* and *Cox-2* transcription was measured with qPCR in WT and *Bax*^{-/-} MEFs. Basal *Cox-1* transcription was reduced in the absence of BAX while COX-1 expression was unaltered (Figure 3a, b). While BAX regulates *Cox-1* transcriptionally, the role of the latter in basal PG biosynthesis implies significant change in the level of the COX-1 enzyme could be detrimental to the cell; consequently, comparable COX-1 expression between WT and *Bax*^{-/-} MEFs could be a translational or post-translational compensatory mechanism on the part of *Bax*^{-/-} cells to restore COX-1 production to WT levels. A similar, but non-statistically significant decrease was also observed for *Cox-2* in the absence of BAX (Figure 3b). Basal *Cox-2* transcript level was significantly lower than *Cox-1*, a potential consequence of the inducible nature of COX-2, suggesting that *Cox-2* transcription should be further examined under inflammatory conditions.

COX-2 induction is a key step of the inflammatory cascade, as demonstrated by the success of COX-2 inhibitors toward preventing inflammation.²¹ A variety of agents stimulate COX-2 expression and inflammation, including bacterial lipopolysaccharide (LPS), a bacterial endotoxin and ligand for Toll-like receptor 4 (TLR4).²² While our data in MEFs clearly demonstrate BAX regulation of basal COX-2 expression, we also wished to address the question of whether BAX regulates the cellular COX-2 response to LPS, which would more accurately mimic an endogenous inflammatory response. WT and *Bax*^{-/-} MEFs were harvested 0, 3 and 6 hours post-LPS treatment, and *Cox* mRNA and protein levels were measured. LPS increased *Cox-2*, but not *Cox-1*, transcription and expression in WT MEFs; however, the COX-2 response in *Bax*^{-/-} MEFs was significantly blunted (Figure 3b, c). Our data strongly support a diminished cellular inflammatory response *via* marked COX-2 reduction in the absence of BAX. To our knowledge, this is the first report linking BAX and BAK to the regulation of prostaglandins, COX-2 and inflammation; however, the BCL-2 family member BID was recently shown to have a role in innate immunity⁶, suggesting that there may exist a more general connection between inflammation and the BCL-2 family.

BID is downstream of NOD1 activation, which has been implicated in intestinal inflammation.⁶ Together with Toll-like receptors and two other receptor families, the NOD proteins belong to a group of pattern recognition receptors (PRRs) that recognize a variety of ligands associated with microbial infection or tissue damage.²³ BID mediates formation of the NOD signalome upon NOD activation; NOD activation in the absence of BID results in significantly blunted inflammatory response, as demonstrated by reduced NFκB and MAPK signaling, and cytokine production.⁶ To determine if loss of BAX could have similar effects on signal transduction, we measured time-dependent phosphorylation along NFκB and MAPK pathways (Figure 4a) in LPS-treated WT and *Bax*^{-/-} MEFs.

LPS activated both NF κ B and MAPK signaling in WT MEFs, as demonstrated by the time-dependent disappearance of I κ B α and phosphorylation of NF κ B and MAP kinases (Figures 4b, S2a). Surprisingly, LPS-treated *Bax*^{-/-} MEFs displayed altered kinetics in NF κ B and MAPK signaling; LPS addition to *Bax*^{-/-} MEFs induced significantly more rapid activation but also deactivation of both NF κ B and MAPK pathways relative to WT (Figures 4b, S2a). No significant differences in non-phosphorylated NF κ B, ERK, p38 and JNK levels were observed between WT and *Bax*^{-/-} MEFs (Figure S2a, b), suggesting that altered signal transduction in the absence of BAX was not a direct result of changes in the levels of these proteins. These experiments reveal that BAX, like BID, can influence signal transduction downstream of PRRs and upstream of NF κ B (Figure 4c). Similar to BID modification of the NOD signalome,⁶ our findings imply that BAX could also be facilitating the assembly of downstream inflammatory complex upon LPS binding to TLR4, and future work will focus on elucidation of this complex. Our lipidomics profiling of BCL-2 proteins revealed a novel connection among BAX, BAK and eicosanoid mechanism, and subsequent analysis identified BAX as a regulator of cellular signal transduction.

METHODS

Materials

Ammonium formate (516961), ammonium acetate (372331), ammonium hydroxide (338818) and lipopolysaccharides from *E. coli* 0111:B4 (L4391) were from Sigma-Aldrich. Formic acid (06440) was from Fluka. Water (365-4), methanol (230-4), isopropanol (323-4), acetonitrile (017-4) and chloroform (049-1L) for lipid extraction and LC-MS were from Honeywell Burdick & Jackson. C4 silica packing material for precolumn was from Western Analytical Products (214TPB1520) and C18 silica packing material for precolumn was from VWR (53501-270). 6-keto-PGF_{1 α} (10007219), 6, 15-diketo-13, 14-dihydro-PGF_{1 α} (15270), 20-hydroxy-PGF_{2 α} (16950), thromboxane B₂ (19030), 19(R)-hydroxy-PGF_{2 α} (16910) and 19(R)-hydroxy-PGE₁ (13910) were from Cayman Chemical. Mouse anti-COX-1 (ab695), rabbit anti-COX-2 (ab15191) and mouse anti- β -actin (ab8226) antibodies were from Abcam. NF- κ B Pathway Sampler Kit (9936), MAPK Family Antibody Sampler Kit (9926) and phospho-MAPK Family Antibody Sampler Kit (9910) were from Cell Signaling Technology. Rabbit anti-BAX (sc-493) and rabbit anti-JNK1/3 (sc-474) antibodies were from Santa Cruz Biotechnology. IRDye 680LT donkey anti-rabbit (926-68023), IRDye 800CW goat anti-rabbit (926-32211) and IRDye 800CW goat anti-mouse (926-32210) antibodies were from LI-COR Biosciences.

Tissue culture

MEFs were maintained in 5 % CO₂ at 37 °C in Dulbecco's Modified Eagle's Medium (Life Technologies, 11965) supplemented with 10 % fetal bovine serum (Hyclone, SH300070.03), penicillin (70 μ g/mL), streptomycin (110 μ g/mL), non-essential amino acids (1X) and L-glutamine (final concentration 6 mM). All cells were passaged (1:4–1:6) at least three times by trypsinization and harvested 36–40 h after the last passage by scraping.

Mitochondria isolation and lipid extraction

Mitochondria were isolated from $\sim 2.5 \times 10^8$ cells immediately upon cell harvest based on known protocol²⁴ using a sucrose isolation buffer (250 mM sucrose, 10 mM Tris-HCl, 0.1 mM EGTA · 4 Na, pH 7.4). Lipids were extracted from mitochondria (from $\sim 2.5 \times 10^8$ cells) or whole cell ($\sim 10^7$ cells) pellets with modifications on known protocol^{25–26}. Cells were either Dounce homogenized 30–40 times on ice or shaken for 30 s at RT in 2: 1: 1 chloroform: methanol: citric acid buffer (100 mM trisodium citrate, 1 M NaCl, pH 3.6), vortexed (in glass vials) for 15–30 s and centrifuged at 2200 g, 4 °C for 6 min to allow phase separation. The chloroform (bottom) layer was transferred to a new glass vial and concentrated under nitrogen. Dried lipids were stored at –80 °C and reconstituted in chloroform for LC–MS.

LC–MS

Global profiling was performed on an Agilent 6220 ESI-TOF online with an Agilent 1200 Series LC in positive and negative modes based on known protocol;²⁷ co-injection of 6-keto-PGF_{1 α} was performed on identical instrument in negative mode. Tandem MS and quantification of 6-keto-PGF_{1 α} and AA in whole cell lysates were performed on an Agilent 6410 Triple Quad MS online with an Agilent 1200 Series LC. Additional details are in Supporting Information.

qPCR

Total RNA was isolated from MEFs with the RNeasy Mini Kit (Qiagen) and reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen) following manufacturer's instructions. Primers for *Cox-1* (NM_008969) and *Cox-2* (NM_011198) were from Qiagen. Quantification for genes of interest was performed with the QuantiTect SYBR Green PCR Kit (Qiagen) on a MX3000p thermal cycler (Stratagene). Relative mRNA levels were calculated using the comparative Ct method with *Gapdh* (forward: 5'-GTG AGG CCG GTG CTG AGT AT-3', reverse: 5'-TCA TGA GCC CTT CCA CAA TG-3', Integrated DNA Technologies) as reference gene.²⁸

LPS treatment and Western blot

MEFs were passaged (1:4–1:6) at least three times by trypsinization and treated with LPS at 1 μ g/mL 24–40 h after the last passage. Cells ($\sim 10^7$ cells per sample) were harvested on ice by scraping into cold PBS 0, 3 and 6 h post-LPS addition and stored at –80 °C prior to qPCR and Western blot against COX-1/2. For signal transduction measurements, MEFs were treated with LPS at either 1 μ g/mL (Figure S2a) or 2 μ g/mL (Figures 4, S2b) to yield comparable results. Treated cells were harvested on ice by scraping into cold PBS either 15, 30, 45, 60 and 80 min or 15, 30, 60, 120 and 240 min post-LPS addition. Cells for the 0 min time point were harvested by scraping immediately prior to LPS addition to the other samples. For Western blot, cell pellets were thawed on ice and diluted in PBS with protease inhibitors (1.5X inhibitor cocktail [87786, Thermo Scientific] or 1X inhibitor tablet [11 836 170 001, Roche], 10 mM sodium fluoride, 10 mM sodium orthovanadate), mixed with 3X Laemmli buffer, sonicated at 4 °C and boiled at 100 °C for 5 min. SDS-PAGE and Western

blots were performed following standard protocol. PVDF membranes were imaged on an Odyssey Infrared Imager (LI-COR Biosciences).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparative lipidomics reveals reduction in eicosanoid level in DKO MEF mitochondria relative to WT. (a) Schematic of unbiased, comparative global lipidomic analysis of WT and DKO MEF mitochondrial lipid pools, allowing identification of metabolite changes between WT and DKO samples. (b) Volcano plot of all detected ions from WT and DKO MEF mitochondria. The DKO-down-regulated m/z 369.23 ion ($C_{20}H_{33}O_6^-$) is in green. Two-tailed Student's t test, $n=3$. (c) Extracted chromatograms of the m/z 369.23 metabolite in WT and DKO MEF mitochondria. (d) Structures of eicosanoid candidates with formula $C_{20}H_{34}O_6$. (e) Tandem MS of endogenous m/z 369.23 metabolite and 6-keto-PGF $_{1\alpha}$ standard. (f) Optimized chromatographic resolution of the six candidate eicosanoids,

enabling baseline separation of 6-keto-PGF_{1α} from additional species. TBX₂: thromboxane B₂. (g) Co-injection of 6-keto-PGF_{1α} standard with endogenous sample.

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Figure 2.

Absence of BAX and/or BAK reduces eicosanoid metabolism. (a) Prostacyclin biosynthesis from AA. (b) Levels of common free fatty acids (FFAs) in WT and DKO MEF mitochondria. (c) 6-keto-PGF_{1α} and AA levels in WT, DKO and SKO MEF mitochondria. (d) 6-keto-PGF_{1α} and AA levels in WT, DKO and SKO MEF whole cell lysates. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Two-tailed Student's *t* test, SEM, $n=3$ for b), c) and d).

Figure 3.

BAX and/or BAK regulate cellular inflammatory response *via* COX-2. (a) Western blots against COX-1 and 2 in WT, DKO and SKO MEFs. (b) qPCR of *Cox-1* and 2 in LPS or PBS-treated WT and *Bax*^{-/-} MEFs; cells were treated with LPS (1 μg/mL) or PBS for 6 h. (c) Western blots against COX-1 and 2 in LPS or PBS-treated WT and *Bax*^{-/-} MEFs; cells were treated with LPS (1 μg/mL) or PBS for 0, 3 or 6 h.

Figure 4.

BAX modulates kinetics of TLR4 signaling. (a) Schematic of NFκB and MAPK signaling in response to LPS stimulation of TLR4. (b) Western blots demonstrating time-dependent activation of NFκB and MAPK pathways in response to LPS treatment. Cells were treated with LPS (2 μg/mL) at the indicated time points. (c) Model for BAX modulation of inflammatory signaling downstream of TLR4 and upstream of NFκB/MAPK.