Mechanism of Mitochondrial Glutathione-Dependent Hepatocellular Susceptibility to TNF Despite NF-κB Activation

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Background & Aims: **Nuclear factor κB (NF-κB) is the master regulator of tumor necrosis factor (TNF) susceptibility. Although mitochondrial glutathione (mGSH) depletion was shown to sensitize hepatocytes to TNF despite NF-κB activation, the mechanisms involved, particularly the role of Bax oligomerization and mitochondrial outer membrane (MOM) permeabilization, 2 critical steps in cell death, remained unexplored.** ***Methods:*** **TNF signaling at the premitochondrial and mitochondrial levels was analyzed in primary mouse hepatocytes with or without mGSH depletion.** ***Results:*** **Unexpectedly, we observed that TNF activates caspase-8 independently of NF-κB inactivation, causing Bid cleavage and mitochondrial Bax oligomerization. However, their predicted consequences on MOM permeabilization, cytochrome *c* release, caspase-3 activation, and hepatocellular death occurred only on mGSH depletion. These events were preceded by stimulated mitochondrial reactive oxygen species that predominantly oxidized cardiolipin, changes not observed in acidic sphingomyelinase (ASMase)$^{-/-}$ hepatocytes. Oxidized cardiolipin potentiated oligomerized Bax-induced MOM-like liposome permeabilization by restructuring the lipid bilayer, without effect on membrane Bax insertion or oligomerization. ASMase$^{-/-}$ mice with mGSH depletion by cholesterol loading were resistant to TNF-induced liver injury in vivo.** ***Conclusions:*** **Thus, MOM-localized oligomeric Bax is not sufficient for TNF-induced MOM permeabilization and cell death requiring mGSH-controlled ASMase-mediated mitochondrial membrane remodeling by oxidized cardiolipin generation.**

Tumor necrosis factor α (TNF) is a pleiotropic inflammatory cytokine that induces different cellular responses, including inflammation, proliferation, or cell death. Given its key role in disease pathogenesis, a better understanding of the signaling events involved in TNF-induced cell death may be of clinical relevance. A significant advance in this regard was the description that the protein-protein interactions underlying TNF signaling by TNF receptor 1 segregate in 2 sequential complexes, complex I and complex II, responsible for the generation of survival or death signals through activation of nuclear factor κB (NF-κB) and caspase-8, respectively.[1,2] The early activation of NF-κB by complex I determines whether death signals are generated from complex II and hence whether cell death ensues. Indeed, NF-κB prevents caspase-8 activation through induction of cellular FLICE (FADD [Fas-associated death domain]–like interleukin-1 β-converting enzyme)–inhibitory protein (FLIP).[3,4] Moreover, NF-κB up-regulates inhibitors of apoptosis (IAPs), antiapoptotic Bcl-2 members,[5,6] and antioxidant enzymes that down-regulate the generation of reactive oxygen species (ROS),[7–9] thought to play a crucial role in TNF-induced cell death through sustained c-Jun N-terminal kinase (JNK) activation.[7,10,11]

In type II cells (eg, hepatocytes) the activation of caspase-8 by complex II is weak for activation of downstream caspases (eg, caspase-3), needing an amplification loop through mitochondria,[12] which are recruited through caspase-8–mediated Bid cleavage. The resulting truncated Bid fragment (tBid) translocates to mitochondria where it activates other proapoptotic Bcl-2 proteins, Bax/Bak. Whereas Bak resides in the mitochondrial outer membrane (MOM), Bax translocates to mitochondria

Abbreviations used in this paper: ASMase, acidic sphingomyelinase; FD-70, fluorescein-isothiocyanate–labeled dextrans of 70 kDa; FLIP, FLICE (FADD [Fas-associated death domain]–like interleukin-1 β-converting enzyme)–inhibitory protein; HP, 3-hydroxy-4-pentenoate; IAP, inhibitor of apoptosis; JNK, c-Jun N-terminal kinase; LUV, large unilamellar vesicle; mGSH, mitochondrial glutathione; MnSOD, manganese superoxide dismutase; MOM, mitochondrial outer membrane; mROS, mitochondrial reactive oxygen species; NF-κB, nuclear factor κB; siRNA, small interfering RNA; tBid, truncated Bid; TNF, tumor necrosis factor.

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and inserts in MOM.[13] Bax/Bak then uncover N-terminal epitopes and oligomerize, which is considered a central step in MOM permeabilization and cell death because it allows the release of cytochrome *c* into the cytosol to assemble the apoptosome.[14–16] However, it remains unclear how Bax/Bak conformational changes and oligomerization lead to MOM permeabilization.

Cardiolipin, an anionic phospholipid mainly found in the mitochondrial inner membrane, plays a key role in mitochondrial physiology and cell death regulation. Using synthetic liposomes, Kuwana et al[15] showed that cardiolipin cooperates with Bax to form large-scale openings independently of the formation of large (>100 kDa) Bax aggregates. Moreover, oxidized cardiolipin lessens the binding of cytochrome *c* to the mitochondrial inner membrane.[17,18] In addition, oxidized cardiolipin was reported to facilitate the permeabilization of isolated liver mitochondria.[18] However, the mechanisms underlying these observations were not addressed,[18] specifically whether oxidized cardiolipin regulated MOM biophysical properties and/or Bax oligomerization/insertion into the bilayer.

Most studies addressing the signaling of TNF at the level of mitochondria were performed in sensitized cells by prior inactivation or silencing of NF-κB,[1,2,4,7,19,20] and recent reports underscored a key role for NF-κB in the susceptibility of adult hepatocytes to TNF.[21,22] We have previously shown that selective mitochondrial GSH (mGSH) depletion sensitized hepatocytes to TNF without interfering with NF-κB activation.[23–25] Moreover, we observed that acidic sphingomyelinase (ASMase)–induced ceramide generation plays a key role in TNF-induced hepatocellular death and liver injury through stimulation of mitochondrial ROS (mROS).[23,24] Because of the relevance of the hepatocellular susceptibility to TNF in liver injury and diseases such as alcoholic and nonalcoholic steatohepatitis,[25–27] a better understanding of the mechanisms and signaling events underlying the NF-κB–independent mGSH-controlled hepatocellular sensitization to TNF may be of significance for disease pathogenesis and therapy. Because mitochondrial Bax oligomerization and MOM permeabilization are thought to be causally related and critical determinants of cell death and because oxidized cardiolipin regulates cytochrome *c* mobilization, we examined their role in the mGSH-dependent sensitization to TNF-induced hepatocellular death. The major findings we observed include the following: (1) TNF receptor 1 activation of caspase-8 occurs without the need of NF-κB inactivation; (2) MOM permeabilization, cytochrome *c* release, and procaspase-3 activation take place only after mGSH depletion; (3) oxidized cardiolipin potentiates Bax-induced MOM-like liposome permeabilization; and (4) ROS-induced oxidized cardiolipin formation is not detected in ASMase-null hepatocytes.

Materials and Methods

Materials

DMEM:F12 media, fetal bovine serum, penicillin-streptomycin, Trypsin-EDTA, Lipofectamine 2000, 4-acetoamido-4′-maleimidylstilbene-2-2′-disulfonic acid, TRIzol, MitoTracker, dihydrorhodamine 123, tetramethylrhodamine methylester, Calcein-AM, 2′-7′-dihydro-dichlorofluorescein-diacetate, and Höechst-33258 were from Invitrogen (Carlsbad, CA). BAY11-7085 and PhiPhiLix G_1D_2 were from Calbiochem (San Diego, CA). Recombinant human TNF-α was from PeproTech EC (London, United Kingdom), and Fas agonist antibody Jo2 was from BD Pharmingen (San Diego, CA). Nitrocellulose, polyvinylidene difluoride membranes, and enhanced chemiluminescence reagent were from Amersham (Little Chalfont, Buckinghamshire, United Kingdom). Unless otherwise stated, all other materials were from Sigma-Aldrich (Indianapolis, IN).

Recombinant Bcl-2 Proteins

Recombinant full-length monomeric Bax with an N-terminal His6 tag (Bax), caspase-8–cleaved Bid with an N-terminal His6 tag (cBid), and Bcl-2 devoid of the carboxyl-terminal hydrophobic domain (Bcl-2), were obtained as described previously.[28] All proteins were >90% pure electrophoretically. Octylglucoside-activated Bax was obtained by incubating Bax in 100 mmol/L KCl, 10 mmol/L HEPES, 0.1 mmol/L EDTA, pH 7.0 buffer (KHE buffer) containing octylglucoside (2% wt/vol) for 1 hour at 4°C as described.[28,29]

Animals, Cell Culture, and mGSH Depletion

Male C57BL/6 mice, 8–10 weeks old, were purchased from Charles River Laboratories (Wilmington, MA). $ASMase^{-/-}$ mice, C57BL/6 background, were maintained and used as previously described.[23,24] The experimental protocols used met the guidelines of and were approved by the Animal Care Committee of the Hospital Clinic-Universidad de Barcelona. Mouse hepatocytes were isolated and cultured as described[23,24]; they were incubated with TNF-α (100 ng/mL) and in some cases with the Fas agonist antibody Jo2 (0.1 μg/mL). To deplete mitochondrial GSH stores, cells were incubated in the presence of 0.5 mmol/L 3-hydroxy-4-pentenoate (HP) as described previously in detail.[23,24]

Subcellular Fractionation and Mitoplast Preparation

Hepatocytes washed with phosphate-buffered saline and scrapped off with a rubber policeman in isotonic HIM buffer (200 mmol/L mannitol, 70 mmol/L sucrose, 1 mmol/L EGTA, 10 mmol/L HEPES, pH 7.4) were homogenized to isolate mitochondria as described.[23–25] Mitoplasts were prepared, using digitonin as described previously,[30] monitoring monoamine oxidase activity for efficiency.