Bile Acid Regulation of Hepatic Physiology
IV. Bile acids and death receptors

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Toxic bile acids facilitate Fas and tumor necrosis factor-associated apoptosis-inducing ligand (TRAIL)-death-receptor oligomerization and activation. Bile acid modulation of death-receptor signaling is multifactorial and includes trafficking of Fas to the cell surface, enhancing TRAIL-R2/DR5 expression, and suppression of function of cFLIP, an antiapoptotic protein modulating death-receptor function. Because bile acid-associated death receptor-mediated apoptosis is a common mechanism for cholestatic hepatocyte injury, inhibition of death receptors and their cascades may prove useful in attenuating liver injury during cholestasis.

Fas; tumor necrosis factor-associated apoptosis-inducing ligand; Bid

Bile Acids Are Amphipatic molecules synthesized from cholesterol within hepatocytes. After their synthesis, these compounds are secreted into bile, enter the small intestinal lumen, are reabsorbed in the terminal ileum, and are efficiently transported from the portal vein back into the hepatocyte. Bile acid homeostasis is tightly regulated in health, and their cellular and tissue concentrations are restricted. However, when their biliary secretion is impaired by pathophysiological processes, hepatocytes are exposed to elevated concentrations of bile acids. The elevated hepatic bile acid concentrations trigger cell death and subsequent fibrosis. Thus the mechanisms responsible for bile acid-induced liver injury are of biomedical importance. Current information suggests that bile acids induce hepatic injury by death receptor-mediated processes.

Death-Receptor Signaling in Hepatocytes

Apoptosis can be initiated by triggering cell surface death receptors, which belong to the TNF or nerve growth factor receptor superfamily. Currently, six death receptors are known, including Fas (also called CD95/APO-1), which binds Fas ligand; TNF receptor 1 (TNF-R1; also called p55/CD120a), which binds TNF-α; tumor necrosis factor-associated apoptosis-inducing ligand (TRAIL-R1; also called death receptor (DR) 4) and TRAIL-R2 [also called DR5/apolipoprotein (APO)-2/KILLERTRICK2], which bind TRAIL; DR3 (also called APO-3/TRAMP/WSL-1/LARD), which binds Apo-3 ligand/TWEAK; and DR6, which contains a death domain (DD) but has no identified ligand. Of these receptors, Fas, TRAIL-R1 and -R2, and TNF-R1 are richly expressed in liver cells.

Ligation of the death receptors with their cognate ligands facilitates receptor oligomerization; however, death-receptor oligomerization may also occur by a ligand-independent mechanism (1, 2). Both ligand-dependent and -independent receptor oligomerization signal apoptosis by recruitment of Fas-associated DD (FADD) protein to the oligomerized receptors via homotypic interactions between the DD of both proteins. FADD contains a death effector domain (DED) that promotes recruitment of the so-called initiator caspases (procaspase-8 and -10) by homotypic interactions between the DEDs, which are also present on procaspase-8/10, resulting in formation of the death-inducing signaling complex (DISC) (Fig. 1). The induced proximity of the initiator caspases is postulated to cause their activation by autocatalytic processes. The activation of initiator caspases at the DISC is believed to be a necessary step in death receptor-mediated apoptosis.

The cytotoxic signals downstream of the DISC differ between cell types and have been characterized as “type I” and “type II” cellular responses (19). In type I cells, activated initiator caspases cleave and thereby activate the effector caspases (caspase-3/6/7), which are responsible for the execution of the cell death program. Other cell types such as hepatocytes are so-called type II cells. In these cells, sufficient activation of the effector caspases requires a mitochondrial amplification pathway. Activated initiator caspases cleave Bid, a proapoptotic member of the Bcl-2 family, which, in turn, induces mitochondrial dysfunction (Fig. 1). Truncated Bid (tBid) translocates to mitochondria, where it induces release of proapoptotic mitochondrial factors including apoptosis-inducing factor, second mitochondria-derived activator of caspases/direct inhibitors of apoptosis (IAP) binding protein with low isoelectric point, Omi/HtrA2, and cytochrome c. Once in the cytosol, cytochrome c binds to the cofactor apoptosis-activating factor 1 facilitating its binding to procaspase-9, forming a complex referred to as the apoptosome. Through an energy-requiring reaction, procaspase-9 is processed to the mature enzyme, which, in turn, activates caspase-3, starting a caspase cascade downstream of the mitochondria. In contrast...
to type I cells, the mitochondrial pathway appears to be essential for the execution of the apoptotic program in type II cells. For example, hepatocytes from Bid-knockout mice are resistant to Fas-induced apoptosis (25).

Biliary acid-mediated death-receptor activation

Current concepts suggest that bile acid-associated hepatocyte apoptosis is death-receptor dependent (5, 15, 20). Bile acid cytotoxicity, to date, has been shown to occur by Fas and TRAIL-R2/DR5 death receptors (5, 9). In contrast, bile acids do not appear to enhance TNF-α/TNF-R1 cytotoxicity (unpublished observation).

Micromolar concentrations of glycochenodeoxycholate induce Fas aggregation on the plasma membrane via a Fas ligand-independent mechanism (5, 17).

Observations in bile duct-ligated mice, a model of extrahepatic cholestasis with elevated serum and hepatic bile acid concentration, also demonstrate that hepatocellular apoptosis and fibrosis are diminished in bile duct-ligated Fas-deficient lpr mice compared with wild-type animals (4, 15). Thus Fas ligand-independent Fas death-receptor activation appears to be a critical pathway for bile acid-associated hepatocyte apoptosis.

Toxic bile acids appear to promote Fas activation by altering cellular trafficking of this death receptor (Fig. 2). A mechanism for the posttranscriptional regulation of Fas receptors is to sequester this death receptor within intracellular pools, especially vesicles associated with the Golgi complex and the trans-Golgi network (3, 20). On stimulation, these Fas-containing vesicles can be trafficked to the plasma membrane, presumably by a microtubule transport pathway, to initiate cell death signals. During bile acid-induced hepatocyte apoptosis, a Golgi-associated and microtubule-dependent pathway has been implicated in the trafficking of Fas to the cell surface (20). Both PKC and JNK kinase have been implicated in this vesicle trafficking (8). The increased density of cell surface Fas promotes their oligomerization, initiating a death-signaling pathway in a ligand-independent mechanism. The increased density of cell surface Fas also sensitizes hepatocytes to

Fig. 1. Toxic bile acids promote death receptor-mediated cell death signaling. Toxic bile acids induce oligomerization of cell surface death receptors [i.e., Fas and tumor necrosis factor-associated apoptosis-inducing ligand (TRAIL)-R2/death receptor (DR) 5] by ligand-independent or -dependent mechanisms. On oligomerization and activation of the death receptors, apoptosis is initiated by the recruitment of Fas-associated death domain (FADD) to the oligomerized death receptors via homotypic interactions between the death domains on both proteins. FADD contains a death effector domain that promotes recruitment of procaspase-8/10 to the death-inducing signaling complex (DISC), again by the homotypic interactions between the death effector domains present on FADD and procaspase-8/10. Recruitment and accumulation of procaspase-8/10 at the DISC results in spontaneous activation of these caspases via autoproteolytic cleavage. In hepatocytes, activated caspase-8/10 leads to Bid cleavage, and the truncated form of Bid (tBid) transmigrates to mitochondria. tBid and Bax may cooperate to induce mitochondrial dysfunction including cytochrome c release. Cytosolic cytochrome c then binds to apoptosis-activating factor-1 (Apaf-1), resulting in activation of caspase-9. Caspase-9 can activate effector caspases such as caspases-3, -6, and -7 via a caspase cascade, which results in execution of the cell death program. tBid/Bax-dependent mitochondrial dysfunction is also accompanied by superoxide (O$_2^-$) formation. As a result of excess production, oxidative stress may exaggerate cell death, either enhancing apoptosis or inducing necrosis.

Fig. 2. Bile acids promote both Fas and TRAIL-R2/DR5 oligomerization by distinct mechanisms. Bile acids enhance TRAIL-R2/DR5 mRNA transcription and protein expression. In contrast, cellular Fas expression level is not altered by bile acids; however, bile acids stimulate Golgi-associated and microtubule-dependent Fas trafficking to the plasma membrane by JNK- and PKC-dependent processes, resulting in an increased density of cell surface Fas. The increased cell surface density of these death receptors likely promotes death-receptor oligomerization, initiating a DISC formation.
Fas agonist-induced cell death (20). Thus toxic bile acids promote both ligand-independent and -dependent hepatocyte apoptosis by Fas.

In vivo, genetic deletion of Fas only reduces hepatocyte apoptosis by 50%, suggesting alternative pathway(s) for bile acid-triggered cytotoxicity (15). This Fas-independent apoptosis also appears to be death-receptor dependent, because it is prevented by inhibiting death-receptor signaling. Indeed dominant-negative FADD, selective caspase-8 inhibitors such as IETD-cho or cytokine response-modifier A (CrmA), and depletion of Bid using specific antisense oligodeoxynucleotides all prevent both Fas-dependent and -independent apoptosis (9, 11). In a Fas-deficient cell line, enhanced expression and oligomerization of TRAIL-R2/DR5 have been observed following treatment with a toxic bile acid (9). Importantly, these observations have also been confirmed in the in vivo bile duct-ligated mouse model (10). Enhanced TRAIL-R2 expression in this model sensitizes the animal to hepatotoxicity by exogenous TRAIL administration. Because TRAIL is synthesized by activated Kupffer cells (6), it is likely that TRAIL also potentiates cholestatic liver injury due to Kupffer cell-generated TRAIL, which causes the hepatocyte apoptosis. Thus, although the role of TRAIL-mediated apoptosis in health is controversial (7), TRAIL is hepatotoxic in cholestasis.

BILE ACID MODULATION OF DEATH-RECEPTOR SIGNALING

Bile acids not only initiate ligand-independent death-receptor oligomerization but also modulate the signaling pathway, resulting in a strong sensitization of the cells to death receptor-mediated apoptosis. Current information suggests that death receptor-mediated apoptosis is regulated at the cell surface by the level of the receptor expression, at the DISC by expression of procaspase-8, FADD, or apoptosis inhibitor cellular FLICE inhibitory protein (cFLIP; also called Flame-1/I-FLICE/Casper/CASH/MRIT/CLARP/Usurpin), and at mitochondria by the expression of proapoptotic or antiapoptotic Bcl-2 family proteins such as Bcl-2, Bcl-xL, Bid, or Mcl-1 and promotes cell survival. The proapoptotic subfamily can be divided into those proteins containing three Bcl-2 homology domains (BH3) such as Bax, Bak, and Bok and those consisting of only one BH3 region like Bad, Bid, Bik, Bim, Bmf, and Hrk. The BH3-only protein, such as Bid, promotes translocation to and/or membrane insertion into mitochondria of Bax and Bak. Bid activation of Bax and/or Bak at the level of the mitochondria is essential for cytochrome c release in hepatocytes following Fas activation. Bax has been implicated as an upstream mediator of bile acid-induced mitochondrial cytochrome c release (18). Rodrigues et al. (18) demonstrated that Bax translocates to mitochondria during deoxycholate-induced apoptosis in rat hepatocytes. In these studies, mitochondrial Bax was accompanied by the mitochondrial permeability transition (MPT) and cytochrome c release. Bile acid-mediated death-receptor signaling likely promotes Bax-dependent mitochondrial dysfunction via Bid-induced activation of Bax (Fig. 1).

Apoptotic mitochondrial dysfunction is accompanied by impairment of the electron transport chain and secondary oxidative stress. Oxidative stress is thought to occur when a cell, tissue, or organism is exposed to excess oxidant generation, in particular to superoxide anion (O₂⁻) and its metabolites (Fig. 1). It has been postulated that hydrophobic bile acids retained in the hepatocyte during cholestasis initiate the generation of reactive oxygen metabolites from mitochondria, leading to lipid peroxidation and loss of cell viability (21).

The mitochondrial oxidative stress can trigger the MPT, resulting in exaggerated mitochondrial cytochrome c release and apoptosis. Yerushalmi et al. (24) reported that relatively low concentrations of bile acids induced oxidative stress in hepatocytes before the on-
set of apoptosis, and a significant linear correlation between apoptosis and the magnitude of oxidative stress was observed (24). These alterations were attenuated by caspase-8 inhibitor IETD-cho, suggesting that mitochondrial oxidative stress is death-receptor dependent. When the mitochondrial dysfunction is severe, sustained mitochondrial permeabilization and massive cytochrome c release may cause loss of ATP synthesis, likely causing necrosis rather than apoptosis. Indeed, Sokol et al. (21) have reported that bile acids induce oxidative stress in rat hepatic mitochondria, leading to hepatocyte necrosis. Nonetheless, it is likely that Bid/Bax activation following death-receptor signaling contributes to these observations.

**POTENTIAL MOLECULAR TARGETS TO PREVENT BILE ACID-MEDIATED DEATH-RECEPTOR SIGNALING**

Because hepatocytes express multiple death receptors, targeting a certain death receptor or ligand appears to be insufficient to prevent liver injury. Indeed, bile acid-induced activation of death-receptor signaling is observed by alternative activation of TRAIL-R2/DR5 in the absence of Fas (9, 11). Therefore, proteins that participate in common pathways during death-receptor signaling would be the best therapeutic targets.

**FADD**

Because FADD appears to be essential in death-receptor signaling, prevention of FADD expression or use of a dominant-negative FADD should block bile acid-induced death-receptor signaling. Indeed, dominant-negative FADD prevents bile acid-induced hepatocyte apoptosis in vitro by both Fas and TRAIL-R2 pathways (5, 9). A recent preliminary report also suggests that dominant-negative FADD attenuates liver injury in the bile duct-ligated mouse (16). Selective gene silencing, using RNA interference technology, may be a potential therapeutic approach to decrease hepatic FADD expression.

**Initiator Caspases**

Initiator caspases may also be potential molecular targets, because the selective caspase 8 inhibitors, IETD-cho, or the pox virus protein CrmA prevent bile acid-associated hepatocyte injury (5, 9). Although caspase-10 is implicated in death-receptor signaling in the absence of caspase-8, the ability of caspase-10 to substitute for caspase-8 in mammalian cells remains controversial (22). A pan-caspase inhibitor, IDN-6536, has been developed and shown to attenuate elevated serum transaminases in humans (23). Although the long-term safety of inhibiting caspases remains to be determined, such a drug may prove useful in human cholestatic liver diseases (23).

**Bid**

Because Bid is essential for death-receptor-mediated apoptosis in hepatocytes, we examined the role of Bid in bile acid-mediated apoptosis in primary mouse hepatocytes and bile duct-ligated mice. Inhibition of Bid expression using antisense technology but not scrambled antisense oligonucleotides markedly attenuated bile acid-induced hepatocyte apoptosis (10). More importantly, Bid antisense treatment of bile duct-ligated mice also reduced liver injury as assessed by serum alanine aminotransferase values. Thus Bid would also appear to be an attractive pharmacological target for the treatment of cholestatic liver diseases.

**PKC**

Certain isoforms of PKC have been shown to be activated by bile acids and participate in bile acid cytotoxicity (13). Bile acid-stimulated PKC activity appears to be instrumental in shuttling of Fas-containing vesicles to the plasma membrane and in cFLIP phosphorylation (12, 20). Indeed, PKC inhibition reduces bile acid-associated death receptor-mediated hepatocyte apoptosis (12, 20). The role and safety of PKC inhibition in cholestatic conditions require further studies.

**CONCLUSION**

Current studies demonstrate the importance of death receptor-mediated apoptosis in bile acid cytotoxicity. Bile acids promote death-receptor oligomerization by either ligand-dependent or -independent mechanisms. Toxic bile acids increase cell surface TRAIL-R2/DR5 or Fas by induction of their mRNA and/or enhancing cell surface trafficking to plasma membrane, respectively. The increased death-receptor protein levels on the plasma membrane result in ligand-independent death-receptor oligomerization or sensitization to ligand-mediated apoptosis. Oligomerized death receptors lead to DISC formation composed of the receptors, FADD, the initiator caspase (caspase-8/10), and the apoptosis inhibitor cFLIP. Bile acids stimulate cFLIP phosphorylation by a PKC-dependent mechanism. The phosphorylation reduces cFLIP recruitment to the DISC, resulting in enhanced caspase-8/10 activation. Initiator caspase-dependent Bid cleavage is also crucial for hepatocyte apoptosis. Thus inhibition of the death-receptor signaling would appear to be crucial for the treatment of cholestatic liver diseases. Selection of the most effective molecular targets (e.g., caspase-8/10, FADD, Bid, or PKC) will require further studies and will ultimately be based on effectiveness and safety.

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Bile acids and apoptosis

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