Mechanisms of Hepatic Toxicity
III. Intracellular signaling in response to toxic liver injury

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Jones, Brett E., and Mark J. Czaja. Mechanisms of Hepatic Toxicity. III. Intracellular signaling in response to toxic liver injury. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G874–G878, 1998.—Toxin-induced liver injury was formerly considered a passive biochemical event, but recent evidence has demonstrated that signal transduction pathways actively regulate the hepatocyte's response to this form of injury. Investigations have examined the effects of a variety of toxins on the activation of receptor-coupled signal transduction, mitogen-activated protein kinases, and Fas signaling, as well as the generation of second messengers such as ceramide and nitric oxide. Many of these pathways culminate in the activation of transcription factors such as activator protein-1, c-Myc; nuclear factor-κB; and Fas signaling, as well as the generation of second messengers such as ceramide and nitric oxide. These signaling events may play a crucial role in regulating hepatocyte proliferation and death following injury.

MEMBRANE-ASSOCIATED SIGNAL TRANSDUCTION

Adenylate cyclase. A significant focus of investigations into the effects of hepatotoxic injury on cell signaling has been on receptor-activated, membrane-bound proteins such as adenylate cyclase. Most of these investigations have employed the toxin ethanol, since it physically interacts with lipid membranes, potentially altering the activity of membrane-bound proteins. When activated, adenylate cyclase generates cAMP, causing protein kinase A activation and phosphorylation of cellular proteins. In vivo ethanol administration in rats inhibits hepatic cAMP accumulation (9). The failure of ethanol-fed rats to accumulate cAMP after the proliferative stimulus of partial hepatectomy is associated with decreased inactivation of the stimulatory G protein Gsα and increased expression of the inhibiting G protein Gia, which control adenylate cyclase through coupling with various receptors (9). Ethanol-associated alterations in G proteins may therefore decrease cAMP-dependent signaling and desensitize hepatocytes to hormones or growth factors, partially accounting for the known decreased regenerative capacity of the liver following chronic ethanol treatment.

Phospholipase C and Ca2+. A second receptor-coupled signal transduction pathway affected by ethanol is phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate to the distinct second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 induces Ca2+ release from intracellular stores, leading to an increased cytosolic free Ca2+ concentration, which may mediate many physiological responses in the cell, including proliferation. DAG activates protein kinase C (PKC). In normal, isolated hepatocytes, ethanol alone causes a rapid but transient activation of PLC and increase in Ca2+ levels (17). However, ethanol inhibits Ca2+ mobilization induced by hormonal activation of PLC due in part to an activation of PKC (16). Other investigations have demonstrated that hepatocytes from ethanol-fed rats exhibit major reductions in IP3 generation and decreased Ca2+ mobilization in response to epidermal growth factor, vasopressin, and phenylephrine (39). These alterations in PLC-
induced signaling may also contribute to the ability of alcohol to inhibit hepatocellular proliferation.

In addition to influencing proliferation, increases in intracellular Ca^{2+} concentrations are associated with cytotoxicity from a number of toxins such as CCl_4 and acetaminophen (for review, see Ref. 26). These Ca^{2+} changes can result from extracellular influx, release from intracellular stores, or reduced Ca^{2+} extrusion across the cell membrane. A causal role of Ca^{2+} flux in cell death is suggested by the fact that a variety of manipulations blocking this Ca^{2+} flux inhibits toxicity induced cell death. It remains to be determined whether these pathological changes in Ca^{2+} levels mediate toxicity through second messenger effects or by direct damage to cellular DNA, membranes, or cytoskeleton.

MITOGEN-ACTIVATED PROTEIN KINASES

The mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases that include extracellular signal-regulated kinase (ERK), c-jun NH_2-terminal kinase (J NK), and p38. Extracellular stimuli trigger cytoplasmic kinase cascades that phosphorylate and activate MAPK. Activated MAPK phosphorylate transcription factors, including c-jun, activating transcription factor-2 (ATF-2), and Elk-1, with individual MAPK having distinct substrate specificities. Activation of MAPK occurs in nonhepatic cells in response to a variety of stimuli, and these enzymes are potential regulators of both pathways of proliferation and cell death from apoptosis.

One of the known stimuli of MAPK is oxidative stress, and the reactive oxygen intermediate H_2O_2 has been shown to induce J NK activity in a hepatoma cell line (36). In vivo oxidative injury induced by CCl_4 in mice also increased J NK activity in whole liver (24). Mouse liver p38 activity decreased rather than increased following CCl_4 treatment, providing one of the first demonstrations of independent regulation of these two MAPK (24). This response is not unique to CCl_4-induced injury, since J NK induction in the absence of increases in ERK or p38 also occurred after cold ischemia and retransplantation in rats (4). In CCl_4-induced injury, decreased p38 activity was associated with increased mRNA expression of MAP kinase phosphatase-1 (MKP-1) (24), a known regulator of p38 and ERK. Thus injury from CCl_4 may result in the induction of selective MAPK through oxidative stress-induced concomitant upregulation of a phosphatase.

SPHINGOLIPIDS

Sphingolipids, long considered as only structural membrane elements, have recently been recognized as intracellular second messengers (for review, see Ref. 14). A number of extracellular stimuli, including TNF-α, lipopolysaccharides, Fas ligand (FasL), and chemotherapeutic agents, induce the hydrolysis of sphingomyelin to ceramide. Potential downstream targets of ceramide activation include phosphatases and kinases. The known involvement of several of these inducers of ceramide in hepatic injury and the fact that ceramide generation has been implicated in the regulation of cell proliferation and apoptosis in nonhepatic cells suggest that ceramide signaling may be important during liver injury. The effects of toxic injury on hepatic ceramide levels are unknown; however, increases in ceramide in vivo have been reported after cold storage of livers and retransplantation (4). In cultured hepatocytes, TNF-α treatment was shown to induce mitochondrial ceramide generation and H_2O_2 production, which may then contribute to TNF-α toxicity (11). Ceramide administration has been reported to induce necrotic rather than apoptotic cell death in hepatocytes in suspension (1). These initial investigations suggest that further studies into the possible contribution of ceramide signaling to hepatocellular toxicity are needed.

NITRIC OXIDE

Nitric oxide (NO) is constitutively generated in normal liver, and levels increase markedly in response to liver injury from diverse insults, including hepatotoxins, endotoxemia, and ischemia-reperfusion (22, 27). Increased NO production results from enhanced expression of inducible nitric oxide synthase (iNOS) in hepatocytes and Kupffer cells. Upregulation of iNOS mRNA in hepatocytes is mediated by inflammatory cytokines including interleukin-1β, TNF-α, and interferon-γ (27) and may result from activation of nuclear factor-κB (NF-κB) (7).

Increased levels of NO may beneficially modulate the hepatocyte’s response to injury through a variety of mechanisms. NO can directly or indirectly affect the transcription of genes that modulate postinjury proliferation or cell death. In hepatocyte cultures, NO prevented apoptosis induced by combined TNF-α and actinomycin D treatment through induction of heat shock protein 70 expression (20). In addition to direct transcriptional activation, NO may exert effects by activating guanylate cyclase, thereby generating the second messenger cGMP. NO effects may also be unrelated to cell signaling but be the result of direct nitrosylation of cellular enzymes (21). Both cGMP and nitrosylation are involved in the inhibition of activation of the proapoptotic enzyme caspase-3 by NO in hepatocytes (21). The hepatoprotective effects of NO are not confined to apoptosis inhibition, but may also prevent necrotic cell death following endotoxemia-induced liver injury (29), although the mechanism of this effect is unknown.

In contrast to evidence that NO is hepatoprotective, other studies have demonstrated that NO may exert deleterious effects following liver injury (12). NO may promote injury not only by second messenger effects but also by increasing injury directly through its oxidant properties. Alternatively, there is evidence in nonhepatic cells that NO can inhibit NF-κB activation by induction and stabilization of IκBα (30). In liver cells, NF-κB activation may be a protective hepatocyte response (see NF-κB), and therefore NO could be expected to enhance hepatocyte injury by this mechanism. However, the existence of this interaction within hepatocytes remains speculative. It is likely that the net effect of NO in various forms of liver injury may
differ with the overall effect depending on a complex interaction between the nature, intensity, and duration of the initial insult, the antioxidant reserves of the hepatocyte, and the inflammatory milieu surrounding the hepatocyte.

**TRANSCRIPTION FACTOR ACTIVATION**

Activator protein-1 and c-Myc. Transcriptional regulation is thought to play an important role in the cellular response to environmental stresses, presumably including that created in hepatocytes by toxic injury. The final target of the signaling pathways reviewed above is frequently the activation of a transcription factor. A prominent subset of transcription factors considered important in the cellular response to injury are the activator protein-1 (AP-1) family genes (c-fos, c-jun, junB, junD) and c-myc. After acute CCl₄-induced injury in rats, rapid increases in mRNA levels for c-fos, c-jun, junB, junD, and c-myc have been demonstrated by in situ hybridization or Northern blot analysis (15, 24, 31). Other studies have demonstrated increased AP-1 DNA binding in CCl₄-treated mice with the AP-1 proteins c-fos, c-jun, J unB, J unD, and ATF-2, all present in the activated complex (24). In protein studies during the first 4 h after injury, only J unD levels were increased, indicating that the other AP-1 components were activated by phosphorylation of constitutively expressed proteins. The finding of J NK activation before the increase in AP-1 binding supports this conclusion. Interestingly, J unD expression was localized to the pericentral hepatocytes, which undergo death following CCl₄. It has been suggested that this overexpression of the growth-inhibiting J unD along with that of MKP-1 in these damaged cells may block their replication (24).

Whereas the rapid increases in AP-1 and c-myc mRNAs occurring after CCl₄ administration coincided with the elevations in these mRNAs found after the purely proliferative stimulus of partial hepatectomy, a different pattern of gene expression has been reported after treatment with the toxin galactosamine. After galactosamine administration, increased expression of the AP-1 genes and c-myc did not occur until 6 h after injury and a prolonged elevation of these mRNAs ensued (15, 31). These results suggested that postinjury liver regeneration can occur in the absence of immediate AP-1 and c-myc gene expression. However, galactosamine-induced regeneration contrasted with that occurring after CCl₄ administration in that the proliferative response after galactosamine was delayed, of less magnitude, and marked by oval cell proliferation rather than the proliferation of differentiated hepatocytes. These findings suggest that the absence of early AP-1 and c-myc gene expression may have altered the nature of the subsequent regenerative response (31). Studies in alcohol models have also examined AP-1 and c-myc gene expression in an attempt to explain alcohol’s ability to inhibit hepatocyte regeneration. Whereas single-dose ethanol inhibited the posthepatectomy rise in c-myc (23), no alterations in c-fos or c-myc expression occurred in rats fed ethanol for 6 wk before partial heptatectomy despite a delayed regenerative response in the ethanol-fed animals (8).

Little is known about the roles that injury-induced activation of AP-1 and c-myc play in modulating hepato-cellular injury and cell death rather than proliferation. Recent investigations in nonhepatic cells have indicated that c-jun and c-myc regulate cell death as well as proliferation in these models. AP-1 activation and c-myc activation have been reported to lead to cell death in liver cells. AP-1 activation following H₂O₂-induced oxidative stress promoted cell death in the HuH-7 hepatoma cell line (36). Similar to findings in nonhepatic cells, c-myc overexpression in these cells triggered apoptosis under serum-free culture conditions (38). In contrast to nonhepatic cells, this death in liver cells was not prevented by specific growth factors but resulted from oxidative stress and decrease in glutathione content (37). Further investigations are needed to define the roles that AP-1 and c-myc activation have in both the proliferative and death responses of hepatocytes following injury. Additional attention must also be paid to the role of transcriptional repressors as the transcriptional repressor ATF-3 is induced during rat CCl₄-induced injury (5).

NF-κB. Another transcription factor commonly activated in cells in response to environmental stress or inflammatory cytokines is NF-κB (33). The first intimation that NF-κB activation may modulate hepatocyte responses relevant to liver injury was the finding that knockout mice deficient in the p65/Rel-A subunit of NF-κB were nonviable because of massive hepatocyte apoptosis during embryogenesis (2). Further evidence of a cytoprotective role for NF-κB emerged from reports in nonhepatic cells that NF-κB activation prevented apoptosis induced by TNF-α and chemotherapeutic agents (34). Recent reports from several laboratories have now demonstrated that NF-κB activation regulates hepatocyte proliferation and apoptosis in vivo and in vitro. In rats subjected to partial hepatectomy, inhibition of NF-κB activation impaired subsequent liver regeneration and triggered hepatocyte apoptosis (19). These findings suggest a critical role for NF-κB activation in hepatocytes following a mitogenic stimulus, although the mechanism by which inhibition of NF-κB activity blocks proliferation is unclear. Apoptosis may have resulted from a cell cycle block or from sensitization to TNF-α produced following partial hepatectomy. An essential role for NF-κB activation during hepatocyte proliferation is also supported by the finding that inhibition of NF-κB activity resulted in apoptosis in an exponentially growing murine hepatocyte cell line (3). However, other studies in confluent rat hepatocyte cultures have demonstrated that NF-κB inhibition by itself did not result in cell death (35). In these cells, NF-κB inhibition did convert the hepatocellular response to the mitogenic stimulus of TNF-α to proliferation to one of apoptosis (35). The mechanism by which NF-κB inactivation triggered TNF-α-induced apoptosis in these studies involved activation of the caspase cascade, and cell death could be prevented by caspase inhibition or NO (35).
The importance of NF-κB activation in mediating hepatocellular resistance to TNF-α cytotoxicity has stimulated interest in the potential ability of toxins to interfere with NF-κB activation, thereby sensitizing hepatocytes to toxicity from TNF-α. Chronic ethanol consumption in rats has been reported to inhibit the normal activation of NF-κB that occurs after partial hepatectomy (40). Hepatotoxins frequently block macro-molecular synthesis and alternatively they may not inhibit NF-κB activation but rather the subsequent synthesis of NF-κB-dependent gene products. In rat hepatocytes sensitized to TNF-α toxicity by addition of actinomycin D, this toxin failed to block TNF-α-induced NF-κB activation as determined by DNA gel shift, but actinomycin D did inhibit expression of an NF-κB-driven reporter gene (25).

The NF-κB-dependent gene product(s) that protects hepatocytes against TNF-α-induced injury remains to be identified. Possible candidate genes are iNOS and interleukin-6, since they are regulated by NF-κB and their gene products may have hepatoprotective effects. It also remains to be determined whether NF-κB activation inhibits hepatotoxicity from injurious agents other than TNF-α. In the hepatoma cell line Hep G2, treatment with a nontoxic concentration of the superoxide generator menadione protected against subsequent toxic doses of menadione or H₂O₂ by an NF-κB-dependent mechanism (36). However, studies in a rat hepatocyte cell line demonstrated that, although H₂O₂ and copper induced NF-κB activation and caused apoptosis at toxic concentrations, inhibition of NF-κB activity did not sensitize the cells to death from H₂O₂ or copper (35). NF-κB activation may therefore stimulate a defense mechanism specific for the TNF-α death pathway. The possibility that NF-κB activation in hepatocytes is protective following liver injury points to the complexity of events following global activation of NF-κB in all cell types in the liver. After a toxic stimulus, it is known that activation of NF-κB in hepatic macrophages results in the production of injurious products such as cytokines and reactive oxygen intermediates. Inhibition of hepatic NF-κB activation was therefore viewed as a potential therapy for liver injury. It now appears that NF-κB signaling represents a problematic therapeutic target, since blanket inhibition of hepatic NF-κB activation may lead to both beneficial and detrimental effects.

FAS SIGNALING PATHWAY

Hepatocytes constitutively express the receptor CD95 (APO-1/Fas), which when activated by its ligand (FasL) or by cross-linking anti-Fas antibodies induces apoptosis. CD95 binding causes rapid apoptosis in hepatocytes (28). The fact that FasL is primarily expressed by activated T cells initially suggested that this signaling pathway would have an important role in immune-mediated liver injury. However, several studies have indicated that Fas signaling may function in a variety of injuries, including that from toxins. First, in the hepatoma cell line Hep G2, death from chemotherapeutic agents was associated with p53-dependent increases in CD95 and FasL expression (25). Fas signaling was causally involved in this cell death, since the death from bleomycin was inhibited by blocking the CD95-FasL interaction. Induction of FasL by this toxin resulted from oxidant generation (18), suggesting that other toxins that induce oxidative stress may similarly activate the Fas pathway. Second, copper-induced Hep G2 toxicity was associated with upregulation of FasL, and death was inhibited by treatment with a neutralizing anti-FasL antibody or with the antioxidant N-acetylcysteine (32). Studies of a small number of human liver samples from patients with Wilson’s disease demonstrated increased CD95 expression and elevated levels of FasL mRNA (32). Similar investigations in human livers from patients with alcoholic cirrhosis revealed no increase in CD95 levels but elevated FasL mRNA expression (10). Further studies are needed to examine whether Fas-induced signaling of cell death is a common hepatocellular response to toxic injury, particularly that involving oxidative stress.

The mechanism by which CD95 receptor binding initiates hepatocyte apoptosis is unknown. CD95 receptor binding triggers the association of several cytoplasmic proteins, which lead to the activation of cysteine proteases or caspases that ultimately induce cell death. In a neuronal cell line, CD95 binding led to ERK and JNK activation, and dominant-negative interference with either pathway inhibited Fas-mediated apoptosis (13). It remains to be determined whether similar mechanisms mediate Fas-induced cell death in hepatocytes.

SUMMARY

A number of in vitro and in vivo investigations have demonstrated that a complex series of signaling events occurs during toxic liver injury. Cellular injury normally activates a variety of signal transduction pathways that mediate cell repair and proliferation, and specific hepatotoxins may amplify, inhibit, or alter the nature of this response. Normal signaling allows successful hepatocyte repair and proliferation to occur with resultant organ recovery. Aberrant signaling may leave cells unable to repair themselves or divide, triggering apoptotic or necrotic cell death and potentially causing organ failure. The current challenge is to develop novel methods of selectively upregulating or inhibiting individual hepatocyte signaling pathways in vivo to determine their function during liver injury and to potentially improve the outcome following toxic liver injury.

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