Hepatic Xbp1 Gene Deletion Promotes Endoplasmic Reticulum Stress-induced Liver Injury and Apoptosis*

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Background: The unfolded protein response (UPR) either restores homeostasis or promotes apoptosis in response to endoplasmic reticulum (ER) stress.

Results: ER stress causes prolonged UPR activation, severe liver injury, and enhanced apoptosis in mice lacking hepatic Xbp1.

Conclusion: Hepatic Xbp1 is critical for hepatic recovery from ER stress.

Significance: We implicate Xbp1 in mediating the pro-survival response of the UPR.

Endoplasmic reticulum (ER) stress activates the unfolded protein response (UPR), a highly conserved signaling cascade that functions to alleviate stress and promote cell survival. If, however, the cell is unable to adapt and restore homeostasis, then the UPR activates pathways that promote apoptotic cell death. The molecular mechanisms governing the critical transition from adaptation and survival to initiation of apoptosis remain poorly understood. We aim to determine the role of hepatic Xbp1, a key mediator of the UPR, in controlling the adaptive response to ER stress in the liver. Liver-specific Xbp1 knockout mice (Xbp1LKO) and Xbp1fl/fl control mice were subjected to varying levels and durations of pharmacologic ER stress. Xbp1LKO and Xbp1fl/fl mice showed robust and equal activation of the UPR acutely after induction of ER stress. By 24 h, Xbp1fl/fl controls showed complete resolution of UPR activation and no liver injury, indicating successful adaptation to the stress. Conversely, Xbp1LKO mice showed ongoing UPR activation associated with progressive liver injury, apoptosis, and, ultimately, fibrosis by day 7 after induction of ER stress. These data indicate that hepatic XBP1 controls the adaptive response of the UPR and is critical to restoring homeostasis in the liver in response to ER stress.

Endoplasmic reticulum (ER) stress is increasingly recognized as a salient feature of numerous chronic liver diseases, including hepatitis C virus infection, alcoholic liver disease, and nonalcoholic fatty liver disease (1–8). Under conditions of ER stress, normal ER function becomes compromised, leading to the accumulation of unfolded or misfolded proteins, triggering an evolutionarily conserved intracellular signal transduction pathway known as the unfolded protein response (UPR) (9, 10). The UPR is comprised of three branches, initiated by three distinct transmembrane proteins. Activation of the inositol-requiring enzyme 1α (IRE1α) pathway, the most highly conserved arm of the UPR, induces splicing of the mRNA encoding X-box binding protein 1 (Xbp1) (11). Spliced (activated) XBP1 is a transcriptional activator that has many targets, including ER chaperones and genes involved in ER-associated degradation (12). Low levels of hepatic XBP1 expression have been associated with more advanced liver disease in patients with non-alcoholic fatty liver disease, but the role of XBP1 in the progression of liver disease remains unknown (3).

The primary function of the UPR is to promote cell survival by activating genes and proteins that halt ER protein synthesis and promote protein degradation. If, however, the cell is unable to adapt to the stressor and restore homeostasis, then pathways leading to apoptosis are initiated (13–15). XBP1 has been implicated in the fine-tuning of the UPR and may mediate, in part, the process of recovery from ER stress (16, 17). However, a definitive role of XBP1 in mediating the hepatic response to ER stress has not been established.

Although the molecular signals within the UPR that initiate the transition from a pro-survival to a pro-apoptotic response remain incompletely understood, several key mediators of ER stress-induced apoptosis have been identified. C/EBP homologous protein (CHOP) is transcriptional activator of numerous pro-apoptotic genes, including death receptor 5 (DR5) (18–20). CHOP overexpression has been shown to sensitize cells to apoptosis, whereas CHOP depletion attenuates ER stress-induced apoptosis (21, 22). Caspase-12 is considered an ER stress-specific mediator of apoptosis (23). Under conditions of ER stress, but not other types of cellular stress, caspase-12 is cleaved to active caspase-12. JNK is a mitogen-activated protein kinase that is activated in response to ER stress and promotes inflammation and apoptosis in the liver (24, 25). BAK and BAX are Bcl2 family members that localize not only to the mitochondria but also to the ER, where they promote apoptosis in response to ER stress (26, 27).

The role of the IRE1α signaling in ER stress-induced apoptosis is complex and incompletely understood. Prolongation of IRE1α signaling has been shown to promote cell survival (28,
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A) Xbp1 fl/fl Xbp1 LKO

B) 300

Plasma ALT (IU/L)

fl/fl LKO

* p < 0.05

FIGURE 1. Hepatic injury in Xbp1LKO mice subjected to severe ER stress. A and B, H&E staining of liver sections (A) and plasma ALT levels (B) in Xbp1LKO and Xbp1floflo mice treated with a single dose of tunicamycin (1 mg/kg i.p.) and sacrificed 4 days later. Data are mean (n = 6) ± S.D. *, p < 0.05.

Results

Deletion of Hepatic Xbp1 Sensitizes Mice to Severe ER Stress—We began by determining the effects of hepatic Xbp1 deletion on the response to severe ER stress in the liver. Tunicamycin is a well established ER stress-inducing agent in mice (34–36). On the basis of work published previously and our preliminary dose-response data, we found that wild-type C57Bl6 mice treated with tunicamycin at doses higher than 1 mg/kg show mortality beginning on day 4 after a single injection (37). Therefore, we began by treating Xbp1LKO and Xbp1floflo control mice with 1 mg/kg of tunicamycin i.p., the maximum sublethal dose for a wild-type mouse. All Xbp1floflo mice survived and were...
grossly well appearing on day 7, when the experiment was terminated. Conversely, all Xbp1^{LKO} mice died between 5 and 6 days after induction of ER stress. To characterize the hepatic effects of severe ER stress in these mice, additional cohorts of Xbp1^{LKO} and Xbp1^{fl/fl} mice were treated with the same protocol (1 mg/kg tunicamycin i.p.) and sacrificed on day 4. H&E staining of liver sections of Xbp1^{LKO} mice showed necrosis and marked hepatocyte swelling consistent with severe hepatocyte injury (Fig. 1A). The plasma alanine aminotransferase level, an indirect plasma marker of liver injury, was elevated markedly in Xbp1^{LKO} mice compared with Xbp1^{fl/fl} mice (Fig. 1B).

Xbp1^{LKO} Mice Appropriately Activate but Fail to Normally Deactivate the UPR Over Time—We next lowered the dose of tunicamycin to 0.5 mg/kg, a dose at which both Xbp1^{LKO} and Xbp1^{fl/fl} mice survived for the duration of the experiment. Mice
were sacrificed either 6 h, 24 h, 3 days, or 7 days after treatment. 6 h after induction of ER stress, Xbp1LKO and Xbp1fl/fl mice demonstrated robust transcriptional activation of the UPR markers Chop, Grp78/Bip, and Atf4 (Fig. 2A). The degree of UPR activation was similar in Xbp1LKO and Xbp1fl/fl mice, indicating normal induction of the UPR in Xbp1LKO mice. As expected, Xbp1LKO mice showed a near absence of spliced Xbp1 expression in the liver and markedly attenuated transcription of Edem and Erdj4, direct downstream targets of XBP1 (Fig. 2A).

At all time points beyond 6 h, the expression of Chop, Grp78/Bip, and Atf4 in Xbp1fl/fl mice was at a baseline level similar to that of unstressed mice, indicating rapid resolution of ER stress. Conversely, Xbp1LKO mice showed a persistent elevation in Chop, Grp78/Bip, and Atf4 24 h, 3 days, and 7 days after induction of ER stress, consistent with ongoing UPR activation (Fig. 2A). Phosphorylation of eIF2α, a downstream consequence of PERK (protein kinase RNA-like endoplasmic reticulum kinase) activation, was greatly induced in Xbp1fl/fl mice at 6 h and showed a delayed course of deactivation relative to other UPR markers. The levels of phosphorylated eIF2α began to attenuate on day 7 after induction of ER stress (Fig. 2B). Unlike other UPR markers, eIF2α was constitutively active in Xbp1LKO mice.

Deletion of Xbp1 is associated with hyperactivation of IRE1α (38). As expected, we found that, in the unstressed state, Xbp1LKO mice had increased phosphorylation (activation) of IRE1α relative to Xbp1fl/fl mice (Fig. 2B). Xbp1LKO mice and Xbp1fl/fl mice showed similar expression of phosphorylated IRE1α 6 h after induction of ER stress. By day 3, Xbp1fl/fl mice showed resolution of IRE1α activation, whereas Xbp1LKO mice showed profoundly increased phosphorylation of IRE1α, which persisted on day 7 after induction of ER stress (Fig. 2B). Phosphorylation (activation) of JNK is a well established consequence of IRE1α activation. Consistent with the observed hyperactivation of IRE1α in Xbp1LKO mice, we found significant hyperphosphorylation of JNK on days 3 and 7 after induction of ER stress (Fig. 2B).

Xbp1LKO Mice Show Enhanced ER Stress-induced Liver Injury—We next assessed whether ongoing UPR activation in Xbp1LKO mice was associated with enhanced liver injury. Xbp1fl/fl mice showed no histologic evidence of liver injury at any time point after a single dose of tunicamycin (0.5 mg/kg i.p.) (Fig. 3A). Conversely, Xbp1LKO mice showed progressive hepatic injury from days 3 to 7 after induction of ER stress. Specifically, there was increased infiltration of inflammatory cells and early hepatocyte swelling evident on day 3. By day 7, there was markedly increased inflammatory infiltrate, worsening architectural distortion, and hepatocyte swelling, consistent with progressive liver damage. Plasma ALT levels were also markedly higher in Xbp1LKO mice compared with Xbp1fl/fl mice on days 3 and 7, consistent with enhanced liver injury (Fig. 3B).

Hepatic triglyceride accumulation is a well established consequence of chronic or severe ER stress (39). Therefore, one might hypothesize that tunicamycin-treated Xbp1LKO mice would show enhanced hepatic triglyceride accumulation as a consequence of unrelieved ER stress. However, XBP1 has been shown to transcriptionally activate hepatic triglyceride synth-
sis genes, raising the possibility that Xbp1LKO mice may be protected from ER stress-induced hepatic triglyceride accumulation. We found no overt hepatic steatosis present histologically in either Xbp1LKO or Xbp1fl/fl mice at the dose and duration of ER stress used in these experiments (Fig. 3A). We did, however, find that, 6 h after induction of ER stress, Xbp1fl/fl mice showed a modest increase in hepatic triglyceride content from which Xbp1LKO mice were protected (Fig. 3C). By day 3, Xbp1fl/fl mice showed normalization of the hepatic triglyceride level, whereas Xbp1LKO mice showed significantly increased hepatic triglyceride content (Fig. 3C).

Xbp1LKO Mice Show Enhanced ER Stress-induced Apoptosis—CHOP and JNK are critical mediators of ER stress-induced apoptosis (21, 22). Given the finding of persistent CHOP and JNK activation in Xbp1LKO mice, we hypothesized that loss of hepatic Xbp1 leads to enhanced ER stress-induced activation of pro-apoptotic pathways and apoptotic cell death. Although Xbp1fl/fl mice showed scant apoptotic cells in response to ER stress, we found a significant amount of apoptosis in the livers of Xbp1LKO mice 3 and 7 days after induction of ER stress (Fig. 4, A and B). Consistent with persistent CHOP activation and enhanced apoptosis, Xbp1LKO mice showed markedly increased hepatic expression of Dr5, a downstream target of CHOP (Fig. 4C). The hepatic levels of the proapoptotic protein BAX were increased greatly in Xbp1LKO mice compared with Xbp1fl/fl mice 3 and 7 days after induction of ER stress (Fig. 4D). Proteolytic cleavage of caspase-12 is considered a highly specific indicator of ER stress-induced apoptosis (23). Xbp1LKO mice showed enhanced cleavage of caspase-12 (Fig. 4D).
mice showed enhanced cleavage of caspase-12 in response to ER stress (Fig. 4D).

JNK activation has been shown to promote ER stress-induced apoptosis (24, 25). We considered whether hyperactivation of IRE1α and the resultant hyperactivation of JNK may mediate the development of apoptosis and injury in Xbp1LKO mice after prolonged ER stress. We therefore pretreated cohorts of Xbp1LKO mice and Xbp1^{fl/fl} mice with a JNK inhibitor, SP600125, followed by induction of ER stress for 3 days. As expected, pretreatment with SP600125 resulted in attenuated activation of JNK in Xbp1LKO mice in response to ER stress (Fig. 5A). JNK inhibition prevented ER stress-induced apoptosis in Xbp1LKO mice, as evidenced by a dramatic reduction in the number of positive TUNEL-stained nuclei on liver sections (Fig. 5, B and C). Xbp1LKO mice treated with SP600125 prior to induction of ER stress showed a similar degree of liver injury histologically and comparably elevated plasma ALT levels compared with Xbp1LKO mice treated with tunicamycin alone (Fig.
5, B and D). Inhibition of JNK did not attenuate the activation of the UPR markers Chop and Grp78/Bip in Xbp1LKO mice subjected to ER stress (Fig. 5E). Furthermore, treatment of Xbp1LKO mice with SP600125 + tunicamycin increased IRE1α activation to an even greater degree than tunicamycin alone, indicating that the observed protection from ER stress-induced apoptosis among JNK-inhibited Xbp1LKO mice is not due to attenuated UPR activation (Fig. 5A). Treatment of Xbp1fl/fl mice with SP600125 + tunicamycin also enhanced phosphorylation of IRE1α relative to Xbp1fl/fl mice treated with tunicamycin alone, indicating a compensatory hyperactivation of IRE1α in the absence of active JNK. Consistent with increased activation of IRE1α, Xbp1fl/fl mice treated with SP600125 + tunicamycin also showed increased hepatic expression of spliced Xbp1 in response to ER stress (Fig. 5E).

5, B and D). Inhibition of JNK did not attenuate the activation of the UPR markers Chop and Grp78/Bip in Xbp1LKO mice subjected to ER stress (Fig. 5E). Furthermore, treatment of Xbp1LKO mice with SP600125 + tunicamycin increased IRE1α activation to an even greater degree than tunicamycin alone, indicating that the observed protection from ER stress-induced apoptosis among JNK-inhibited Xbp1LKO mice is not due to attenuated UPR activation (Fig. 5A). Treatment of Xbp1fl/fl mice with SP600125 + tunicamycin also enhanced phosphorylation of IRE1α relative to Xbp1fl/fl mice treated with tunicamycin alone, indicating a compensatory hyperactivation of IRE1α in the absence of active JNK. Consistent with increased activation of IRE1α, Xbp1fl/fl mice treated with SP600125 + tunicamycin also showed increased hepatic expression of spliced Xbp1 in response to ER stress (Fig. 5E).

**Xbp1LKO Mice Show Enhanced Hepatocyte Proliferation**—Severe liver damage triggers a compensatory response in which hepatocyte proliferation occurs (40). We next assessed whether a proliferative response to the observed hepatic injury is present in Xbp1LKO mice. Hepatocyte proliferation, as assessed by proliferating cell nuclear antigen and Ki67 staining, was unchanged in Xbp1LKO and Xbp1fl/fl mice at baseline and 6 h after induction of ER stress (Fig. 6A). However, by day 3, hepatocyte proliferation was increased markedly among Xbp1LKO mice compared with Xbp1fl/fl mice (Fig. 6A). Induction of hepatic tumor necrosis factor α (Tnfa) is a critical mediator of the proliferative response after severe liver injury (40–42). The expression of hepatic Tnfa was increased to a greater degree in Xbp1LKO mice compared with Xbp1fl/fl mice 3 days after induction of ER stress (Fig. 6B).
Deletion of Hepatic Xbp1 Promotes Hepatic Fibrosis—The ultimate consequence of severe liver injury and attempted repair is the development of hepatic fibrosis. 7 days after induction of ER stress, Xbp1 LKO mice demonstrated pericellular collagen deposition on trichrome staining (Fig. 7A). Consistent with the finding of enhanced fibrosis, Xbp1 LKO mice showed a marked elevation in hepatic expression of the fibrosis markers α-Sma, Timp-1, and Collagen I in response to ER stress (Fig. 7, B–D).

We have shown that inhibiting JNK attenuates ER stress-induced apoptosis in Xbp1 LKO mice. We next assessed the effect of JNK-inhibition on the development of hepatic fibrosis in Xbp1 LKO mice subjected to ER stress. On day 7 after induction of ER stress, we found that Xbp1 LKO mice pretreated with SP600125 showed significantly reduced hepatic fibrosis compared with Xbp1 LKO mice treated with tunicamycin alone (Fig. 7A). The expression of fibrosis markers was also attenuated in JNK-inhibited Xbp1 LKO mice.

Discussion

The unfolded protein response is a highly complex and tightly orchestrated signaling cascade that can function either as a protective or injurious response depending on the severity and duration of ER stress. The precise molecular mechanisms that control the shift from the adaptive to apoptotic phase of the UPR have remained elusive. In this work, we demonstrate that loss of hepatic Xbp1 shifts the pattern of UPR activation in the liver to preferentially activate pro-apoptotic signals, sensitizing mice to ER stress-induced liver injury and apoptosis. These data firmly establish that hepatic Xbp1 promotes the pro-survival response of the UPR in the liver. Moreover, these data strongly implicate Xbp1 in mediating the critical transition from cell survival to apoptotic cell death in response to ER stress.

It has been reported previously Lee et al. (38) that mice bearing a liver-specific deletion of Xbp1 show normal induction of the UPR, as evidenced by a similar induction of Grp78/Bip and Chop and no evidence of hepatic injury 8 h after a standard dose of tunicamycin. We demonstrate findings consistent with Lee et al. (38) in that Xbp1 LKO mice show normal induction of Grp78/Bip, Chop, and Atf4 6 h after treatment with tunicamycin. However, when followed for up to 7 days after induction of ER stress, we show that Xbp1 LKO mice exhibit persistent UPR activation, whereas Xbp1 fl/fl mice show rapid and complete resolution of UPR activation. Furthermore, we demonstrate that ongoing UPR activation in Xbp1 LKO mice is associated with progressive liver injury, apoptosis, and, ultimately, fibrosis.

The role of the IRE1α branch in mediating cell fate in response to ER stress is controversial. Activation of IRE1α has been shown to generate either pro- or anti-apoptotic effects.
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depending on the experimental conditions (28–32, 43). Among the downstream targets of activated IRE1α are XBP1 and JNK. Activated JNK is a well established mediator of the proapoptotic response to a variety of cellular stressors, including ER stress (24, 25). We hypothesized that the relative activation of XBP1 versus JNK in response to IRE1α activation may underlie the divergent effects of IRE1α activation with respect to apoptosis. This work provides substantial support for this hypothesis, in which deletion of hepatic Xbp1 leads to marked hyperactivation of IRE1α and JNK associated with enhanced ER stress-induced apoptosis. Furthermore, we demonstrated that inhibiting JNK attenuates apoptosis in Xbp1\(^{fl/fl}\) mice subjected to prolonged ER stress. These findings strongly suggest that, in the absence of hepatic Xbp1, preferential activation of JNK by activated IRE1α may be a dominant driver of enhanced apoptosis.

ER stress is known to induce hepatic triglyceride accumulation. As expected, we found that Xbp1\(^{fl/fl}\) mice showed a transient increase in hepatic triglyceride content when challenged with ER stress. Consistent with the known role of XBPI in the transcriptional regulation of hepatic lipogenesis, Xbp1\(^{LKO}\) mice showed reduced hepatic triglyceride accumulation 6 h after induction of ER stress. However, we found that Xbp1\(^{LKO}\) mice exhibit enhanced hepatic triglyceride accumulation in response to prolonged, severe ER stress. Our data strongly suggest that, in the absence of hepatic Xbp1, the pro-steatotic effects of prolonged, unrelieved ER stress outweigh the anti-steatotic effects of suppressed lipogenesis.

Severe liver injury triggers a compensatory repair process characterized by hepatocyte proliferation. We find that, in response to prolonged ER stress, Xbp1\(^{LKO}\) mice show enhanced hepatocyte proliferation associated with induction of Tnfa, a critical mediator of injury-related hepatocyte proliferation. Therefore, we may conclude that Xbp1 is not essential for ER stress-induced hepatocyte proliferation. However, the compensatory response is clearly inadequate to permit complete recovery from the injury and prevent the development of hepatic fibrosis. Therefore, we cannot exclude the possibility that loss of hepatic Xbp1 impedes ER stress-induced hepatocyte proliferation to some degree.

Activation of the UPR is a feature of many common chronic liver diseases. Dysregulation of the hepatic IRE1α-XBP1 axis is characteristic of nonalcoholic fatty liver disease, but the precise role of XBPI in the progression of liver disease has been poorly understood (3). On the basis of our findings, we speculate that failure to activate hepatic XBP1 in response to ER stress may promote liver disease progression. Furthermore, enhancing hepatic XBP1 levels may serve as a therapeutic strategy to prevent the progression of chronic liver disease.

Author Contributions—S. O. performed the majority of the technical work and participated in experimental design and data analysis. A. S. H. designed the study, performed technical work, analyzed the results, and wrote the manuscript. S. O. and A. S. H. approved the final version of the manuscript.

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