GASTROENTEROLOGY 2010;139:1730–1739

Stress Signaling in the Methionine-Choline–Deficient Model of Murine Fatty Liver Disease

RUSSELL K. SOON Jr,* JIM S. YAN,* JAMES P. GRENERT,‡ and JACQUELYN J. MAHER*

*Department of Medicine, ‡Department of Pathology, The Liver Center, University of California, San Francisco, California

BACKGROUND & AIMS: Stress signaling, both within and outside the endoplasmic reticulum, has been linked to metabolic dysregulation and hepatic steatosis. Methionine-choline–deficient (MCD) diets cause severe fatty liver disease and have the potential to cause many types of cellular stress. The purpose of this study was to characterize hepatic stress in MCD-fed mice and explore the relationship between MCD-mediated stress and liver injury. **METHODS:** Stress signaling was examined in mice fed MCD formulas for 4–21 days. Signaling also was evaluated in mice fed MCD formulas supplemented with clofibrate, which inhibits hepatic triglyceride accumulation. The role of the pro-apoptotic stress protein C/EBP homologous protein (CHOP) in MCD-mediated liver injury was assessed by comparing the responses of wild-type and CHOP-deficient mice to an MCD diet. **RESULTS:** MCD feeding caused steatohepatitis coincident with the activation of cJun N-terminal kinase and caspase-12. In contrast, MCD feeding did not activate inositol-requiring protein-1 and actually suppressed the expression of X-box protein-1s. MCD feeding caused weak stimulation of double-stranded RNA-activated protein kinase–like endoplasmic reticulum–resident kinase, but robust activation of general control nonderepressible-2, followed by the phosphorylation of eukaryotic initiating factor-2α and induction of CHOP. Clofibrate eliminated MCD-mediated hepatic steatosis but did not inhibit diet-induced stress. CHOP deficiency did not alleviate, and in fact worsened, MCD-mediated liver disease. **CONCLUSIONS: MCD feeding causes an integrated stress response in the liver rather than a classic unfolded protein response. This stress response does not by itself lead to liver injury. CHOP, despite its identity as a mediator of stress-related cell death, does not play a central role in the pathogenesis of MCD-mediated liver disease.**

Keywords: Steatosis; Steatohepatitis; Endoplasmic Reticulum Stress; Integrated Stress.

BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Endoplasmic reticulum (ER) *stress* is the term used to describe a situation in which the amount of unfolded protein entering the ER exceeds the processing capacity of the organelle. This imbalance is recognized by 3 signal transducers in the ER membrane: inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6), and double-stranded RNA-activated protein kinase–like ER-resident kinase (PERK). IRE1, ATF6, and PERK work together to provide a comprehensive response to ER stress that includes the suppression of further protein synthesis, the enhancement of protein folding capacity, and the degradation of unfolded or misfolded proteins. Collectively, this series of events is designated the *unfolded protein response* (UPR).[1]

Interestingly, ER stress often is observed in fatty livers, even though steatosis does not represent the type of abnormality that should induce a UPR. The connection lies in the ability of triglyceride to induce the synthesis of apolipoprotein B100 (apoB100) within hepatocytes.[2,3] If excessive, apoB100 synthesis can initiate a UPR; importantly, once activated in a fatty liver, the UPR can reduce apoB100 synthesis and up-regulate the expression of enzymes involved in hepatic lipogenesis, which can result in the exaggeration of hepatic steatosis.[2,4,5] This scenario predicts a vicious cycle in which steatosis and ER stress fuel each other to promote the progressive accumulation of hepatic triglyceride. Indeed, such cross-talk between ER stress and hepatic steatosis has been documented experimentally: manipulations that affect ER stress influence hepatic steatosis,[4–8] and maneuvers that affect steatosis influence ER stress.[2,9]

Whether ER stress contributes specifically to hepatocellular injury in a fatty liver is not completely understood. Such a question could be addressed directly in an animal model of severe steatohepatitis such as that induced by a methionine-choline–deficient (MCD) diet. The MCD model is attractive for this purpose because it progresses

Abbreviations used in this paper: apoB100, apolipoprotein B100; ATF6, activating transcription factor-6; Bcl-xL, B-cell lymphoma extra-large; BiP, immunoglobulin-binding protein; CHOP, C/EBP homologous protein; eIF2α, eukaryotic initiation factor-2α; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERO1, endoplasmic oxidoreductase protein-1; GCN2, general control nonderepressible-2; Grp94, glucose-regulated protein 94; HRI, heme-regulated inhibitor kinase; IRE1, inositol-requiring protein-1; ISR, integrated stress response; JNK, cJun N-terminal kinase; MCD, methionine-choline-deficient; MCS, methionine-choline-sufficient; NASH, nonalcoholic steatohepatitis; Pdia4, protein disulfide isomerase-associated-4; PERK, double-stranded RNA-activated protein kinase–like endoplasmic reticulum–resident kinase; TRB3, tribbles homolog 3; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UPR, unfolded protein response; WT, wild-type; XBP-1, X-box protein-1.

0016-5085/$36.00
doi:10.1053/j.gastro.2010.07.046

beyond hepatic steatosis to include significant hepatocellular injury and inflammation.[10,11] When investigating the impact of ER stress on the pathogenesis of fatty liver disease in MCD-fed mice, however, it is important to consider that hepatic triglyceride accumulation may not be the only event that perturbs the ER. For example, MCD feeding depletes cellular phospholipids, which can activate IRE1 in an effort to restore normal phospholipid levels.[12,13] In addition, MCD formulas are by definition amino acid–deficient, and amino acid deprivation can activate general control nonderepressible-2 (GCN2), a stress transducer similar to PERK.[14] GCN2 is one of a family of PERK-like signal transducers that includes heme-regulated inhibitor kinase (HRI) and double-stranded RNA-activated protein kinase (PKR). GCN2, HRI, and PKR are distinct from PERK in that they reside in the cytoplasm rather than the ER.[1] All 3, however, are functionally similar to PERK in that they activate a common signaling pathway beginning with eukaryotic initiating factor-2α (eIF2α). The ability of these diverse stress transducers to converge on eIF2α has led to the designation of eIF2α and its downstream targets as components of an integrated stress response (ISR).[15]

Whatever the origin of cellular stress in the livers of MCD-fed mice, the model offers an opportunity to investigate the role of stress signaling in the pathogenesis of steatohepatitis. In this context it is important to note that although the primary purpose of a UPR or ISR is to alleviate stress,[1] the UPR and ISR signaling cascades may commit cells to apoptosis if stress is particularly severe or prolonged. Specifically, stress signaling results in the activation of proapoptotic proteins such as cJun N-terminal kinase (JNK), C/EBP homologous protein (CHOP), and caspase-12.[16–18] In many experimental systems, ER stress-induced cell death is discernable by a robust or protracted increase in CHOP expression.[8,19] Although CHOP has been linked to fatty liver injury in response to excess alcohol consumption,[20] its role in nonalcoholic steatohepatitis (NASH) is unknown.

The objective of this study was 2-fold. First, we wished to determine the extent to which MCD feeding causes activation of a UPR, ISR, or both, and to assess whether the stress that leads to these responses is related to hepatic steatosis or some other diet-induced abnormality. Second, we wanted to explore whether the induction of CHOP in MCD-fed mice plays a causative role in MCD-mediated liver disease. The results indicate that MCD feeding activates a unique pattern of stress in the liver dominated by features of an ISR rather than a UPR. CHOP is induced in MCD-fed livers as part of this diet-related ISR, but its up-regulation is not central to the pathogenesis of MCD-mediated liver injury.

Materials and Methods

Animals and Diets

Adult male C3H mice were fed methionine-choline–sufficient (MCS) or MCD formulas (Dyets, Inc, Bethlehem, PA) for 4–21 days. The diets were matched in all nutrients except L-methionine (2 g/kg) and choline chloride (2 g/kg), which were present in the MCS formula only. Both diets provided 17% kcal as protein, 62% kcal as carbohydrate (70:30 sucrose:starch), and 19% kcal as fat. All animals had free access to diet and drinking water for the duration of study. In some experiments, mice were fed MCS and MCD formulas containing 0.5% (wt/wt) clofibrate (Sigma-Aldrich, St. Louis, MO). In other experiments, MCD formulas were fed to CHOP-deficient mice (B6.129S-*Ddit3*^{tm1Dron}/J) and wild-type controls (C57Bl/6J) (The Jackson Laboratory, Bar Harbor, ME). In all dietary studies, mice were fasted for 4 hours before killing.

All animals received humane care according to the guidelines of the US Public Health Service. All experimental procedures performed on live animals were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Serum Chemistries

Alanine aminotransferase (ALT), glucose, cholesterol, and triglyceride levels were measured in mouse serum using an ADVIA 1800 autoanalyzer (Siemens Healthcare Diagnostics, Deerfield, IL) in the clinical chemistry laboratory at San Francisco General Hospital.

Liver Histology

Paraffin sections of liver tissue were stained with H&E. Slides were evaluated blindly and scored for steatosis (0–3), ballooning (0–2), and inflammation (0–3).[21] Cell death was evaluated in tissue sections by terminal deoxynucleotide transferase–mediated deoxyuridine triphosphate nick end-labeling (TUNEL) (ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit; Millipore, Billerica, MA). Sections were counterstained with hematoxylin for viewing and photography.

Measurement of Hepatic Triglycerides

Lipids were extracted from fresh liver tissue. Triglycerides were quantitated as described previously.[10] Results were reported as milligrams of triglycerides per grams of liver.

Evaluation of Stress Responses in Liver Homogenates

The expression or activation of proteins involved in the unfolded protein response was assessed in mouse liver homogenates by immunoblotting. Antibodies against β-actin, B-cell lymphoma extra-large (Bcl-xL), C/EBPα, C/EBPβ, immunoglobulin-binding protein (BiP), caspase-12, cJun/P-cJun, eIF2α/P-eIF2α, IRE1, JNK/P-JNK, PERK, and P-GCN2 were from Cell Signaling Technology (Danvers, MA). Antibodies against CHOP and X-box protein-1 (XBP-1) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–IRE1-P was from Novus Biologicals (Littleton, CO). Proteins of interest were identified by chemiluminescence (Super Signal West Dura; Thermo Fisher Scientific, Rockford, IL).