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Activation and Dysregulation of the Unfolded Protein Response in Nonalcoholic Fatty Liver Disease

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Background & Aims: **Nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH) are associated with known triggers of the unfolded protein response (UPR). The aims were to (1) evaluate the activity of UPR in NAFL and NASH and (2) correlate expression of UPR pathways with liver histology.** ***Methods:*** **Messenger RNA (mRNA) and protein expression were measured by quantitative real-time PCR and Western blot, respectively. Apoptosis was assessed by TUNEL assay. Liver histology was scored using the NASH clinical research network criteria.** ***Results:*** **Compared with subjects with the metabolic syndrome and normal liver histology (n = 17), both NAFL (n = 21) and NASH (n = 21) were associated with increased eukaryotic initiation factor-2α (eIF-2α) phosphorylation. Activating transcription factor 4 (ATF4) mRNA and protein, C/EBP homologous protein (CHOP), and growth arrest, DNA damage-34 (GADD34) mRNA were not increased in NAFL or NASH. Whereas immunoglobulin heavy chain binding protein mRNA was significantly increased in NASH, unspliced X-box protein-1 (XBP-1) protein did not increase. Also, endoplasmic reticulum degradation-enhancing α-mannosidase-like protein mRNA levels were inversely related to spliced XBP-1 mRNA in NASH. NASH was specifically associated with low sXBP-1 protein and increased JNK phosphorylation. This correlated with increased TUNEL activity in NASH. The histologic severity correlated with sXBP-1 mRNA and JNK phosphorylation.** ***Conclusions:*** **There is a variable degree of UPR activation in NAFL and NASH. Although both NAFL and NASH are associated with eIF-2α phosphorylation, there is a failure to activate downstream recovery pathways, ie, ATF4-CHOP-GADD34. NASH is specifically associated with (1) failure to generate sXBP-1 protein and (2) activation of JNK.**

Nonalcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease in North America.[1] The clinical-histologic spectrum of NAFLD extends from a nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH).[2] NASH can progress to cirrhosis in up to 15% of subjects.[3] Although oxidative stress and cytokines have been implicated, the mechanisms of cell injury and progression of liver disease in NASH are not fully understood.[4,5]

The endoplasmic reticulum (ER) plays a central role in the synthesis, folding, and trafficking of proteins. ER dysfunction is characterized by accumulation of unfolded proteins within the ER, which triggers the unfolded protein response (UPR). The UPR is initially characterized by translational arrest of protein synthesis, increased ER-associated degradation of proteins via a proteosomal pathway, and activation of genes that allow the cell to adapt to the trigger for ER dysfunction.[6] If the cell fails to adapt, alarm pathways are activated including c-jun-N-terminal kinase (JNK), which results in apoptosis and inflammation.[7] Activation of UPR has been implicated in the pathogenesis of insulin resistance, diabetes, and alcohol-induced liver disease.[8–11] Saturated fat feeding, which is known to induce insulin resistance, activates UPR in the liver in mice.[12] Hyperhomocyteniemia, commonly present in the insulin-resistant state, induces UPR in cultured hepatocytes.[13] Also, C/EBP homologous protein (CHOP) and JNK activation has been noted in animal models of steatohepatitis.[14–17] NAFLD is strongly associated with insulin resistance and several known triggers of UPR, eg, ATP depletion.[4,18]

We hypothesized that (1) NAFLD was associated with activation of UPR and that (2) increasing disease activity

Abbreviations used in this paper: ATF4, activating transcription factor 4; BiP, immunoglobulin heavy chain binding protein; CHOP, C/EBP homologous protein; EDEM, ER degradation-enhancing α-mannosidase-like protein; eIF-2α, eukaryotic initiation factor-2α; ER, endoplasmic reticulum; GADD34, growth arrest and DNA damage-34; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IRE-1, inositol requiring enzyme-1; JNK, c-jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PERK, PKR-like ER kinase; sXBP-1, spliced X-box protein-1; TUNEL, terminal dUTP nick-end labeling; UPR, unfolded protein response; uXBP-1, unspliced X-box protein-1.

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correlated with the severity of the UPR. This was tested by (1) evaluating the expression of the UPR in subjects with the metabolic syndrome with and without NAFL or NASH and (2) correlation of expression of specific pathways with liver histology.

Materials and Methods

Study Cohort

Consecutive subjects who were referred for either obesity management or suspected NAFLD were screened for this study. All subjects underwent routine clinical assessment and radiologic, hematologic, biochemical, and serologic testing. The metabolic syndrome was diagnosed using the Adult Treatment Panel III criteria.[19] Alcohol consumption was assessed clinically and considered to be significant when >20 g/day for females and >30 g/day for males. All subjects were tested for hepatitis B and C, autoimmune hepatitis, hemochromatosis, Wilson disease, α_1-antitrypsin deficiency, and primary biliary cirrhosis. NAFLD was suspected in those with (1) either abnormal liver enzymes or radiologic evidence of a fatty liver along with negative studies for other common etiologies of liver disease and (2) absence of clinically significant alcohol consumption.

Subjects with the metabolic syndrome with or without features suggestive of NAFLD were considered for this study. Consecutive subjects who gave informed consent underwent a core liver biopsy, using a 15-gauge Microvasive gun (Microinvasive, Quincy, MA), by a percutaneous route under ultrasound or laparoscopic guidance. One liver core was fixed in formalin for assessment of histology, and another was snap frozen in liquid nitrogen and stored at −80°C for future studies. Based on the liver histology, 3 groups were identified: (1) subjects with the metabolic syndrome and normal liver histology and liver enzymes, (2) subjects with the metabolic syndrome and NAFL, and (3) subjects with the metabolic syndrome and NASH. Subjects with bridging fibrosis or cirrhosis were excluded. The study was performed according to Virginia Commonwealth University regulations for the protection of human research subjects, and the protocol was reviewed and approved by the Institutional Review Board.

Histologic Assessment

A true core biopsy of the liver was used for histologic assessment in all cases. A minimum of 1.7 cm of liver tissue was sent for histopathologic studies. Hepatic steatosis and other histologic parameters of fatty liver disease were scored separately using the NASH clinical research network criteria.[20] Only those with steatosis alone were classified as NAFL for this study. Steatohepatitis was diagnosed by the presence of steatosis, cytologic ballooning, and inflammation.[21]

Evaluation of the Adaptive Pathways of the UPR

RNA-activated protein kinase (PKR)-like ER kinase (PERK) activation was evaluated by measurement of phosphorylated eukaryotic initiation factor-2α (eIF-2α), activating transcription factor (AFT) 4 messenger RNA (mRNA) and protein, CHOP, and growth arrest and DNA damage-34 (GADD34) mRNA expression.[22] ATF6 activation was assessed from immunoglobulin heavy chain binding protein (BiP) and unspliced X-box protein-1 (uXBP-1) mRNA and uXBP-1 protein.[23] Inositol requiring enzyme-1 (IRE-1) activation splices uXBP-1 to form spliced XBP-1 (sXBP-1) mRNA; sXBP-1 protein transcriptionally activates ER degradation-enhancing α-mannosidase-like protein (EDEM), which promotes proteasomal protein degradation.[24] IRE-1 activation was assessed from the EDEM mRNA, sXBP-1 mRNA, and protein levels.[25,26]

Evaluation of the Alarm Pathways of the UPR

Alarm signaling in the UPR is mediated by JNK and p38 mitogen-activated protein kinase (MAPK).[27,28] The status of the alarm/death pathways was evaluated by measurement of total and phosphorylated JNK and p38 MAPK proteins and apoptotic activity, as measured by the terminal dUTP nick-end labeling (TUNEL) assay.[29]

RNA Preparation and Real-Time Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted from the frozen liver tissue using 1 mL TRIzol (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's protocol and quantified spectrophotometrically (Bio-Rad, Hercules, CA) from absorbance at 260 nm. Three measures for quality control were taken to ensure a high quality of the extracted RNA: 260 nm/280 nm ratio, NanoDrop ND-1000 UV-Vis Spectrophotometer to look for any additional genomic products and agarose gel electrophoresis for 18S and 28S components.

Sequence-specific primers were designed to assess the mRNA expression of specific genes (Table 1). β-Actin was used as the normalizing gene. The design ensured that the polymerase chain reaction (PCR) product spanned an intron/exon boundary to minimize the possibility of coamplifying genomic DNA. The oligonucleotides were synthesized from Sigma Genosys (Sigma-Aldrich Co, St. Louis, MO).

Real-time quantitative polymerase chain reaction (qPCR) was performed in a 2-step reaction. Complementary DNA (cDNA) was synthesized with oligo-dT from 1.25 μg total RNA in a final volume of 20 μL using ThermoScript Reverse Transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions. The qPCR was performed in duplicate using the Stratagene Mx3000P QPCR system and 2X SYBR Green Master Mix