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

PUNEET PURI,* FARIDODDIN MIRSHAHI,* ONPAN CHEUNG,* RAMESH NATARAJAN,5 JAMES W. MAHER,2 JOHN M. KELLUM,1 and ARUN J. SANYAL*4

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 Hyperhomocysteinemia, 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.6,18

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; MAFK, 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, α1-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 (Microvasive, 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, cytoligic 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

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 co-amplifying 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
(Bio-Rad). The cycling parameters for qPCR reaction included 45 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 74°C for 60 seconds. After amplification, a final melting curve was recorded by heating to 95°C.

The specificity of qPCR was established by incorporating no template and no reverse transcript controls. The amplification of specific transcripts was confirmed by melting curve profiles generated at the end of the PCR program. Total RNA extracted from a histologically normal liver of a lean normal individual obtained at the time of living donor liver transplantation was used as an internal calibrator across all experiments. Cycle threshold (Ct) values were normalized to β-actin, and comparative quantification of target mRNA was done by the ΔΔCt method using integrated software with the Stratagene Mx3000P QPCR system.

**Western Blot Analysis**

Liver biopsy tissues were lysed using lysis buffer (catalogue No. C2978; Sigma Chemical Co, St. Louis, MO). Samples (10 μg protein/lane) were separated by 4–12% Bis-Tris Nu-PAGE gel (Invitrogen Life Technologies) (phos-eIF-2α, ATF4, and XBP-1) or 10% SDS-PAGE (JNK and p38 MAPK). Western blots were performed with appropriate antibodies (ATF4 and XBP-1; Santa Cruz Biotechnology, Santa Cruz, CA) and (JNK and p38 MAPK; Signalchem, Richmond, BC, Canada). β-Actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal controls. Immunoblots were developed with horse-radish peroxidase (HRP)-linked secondary antibodies (Amersham catalogue No. RPN210891; Cell Signaling catalogue No. 7074) and a chemiluminescence kit (ECL) (Amersham catalogue No. RPN210891; Cell Signaling) and a chemiluminescence kit (ECL) (Amersham catalogue No. RPN210891; Cell Signaling) and a chemiluminescence kit (ECL) (Amersham catalogue No. RPN210891; Cell Signaling).

**Evaluation of Apoptosis**

Apoptotic activity was evaluated by the TUNEL assay, which was performed using the in situ death detection kit (catalog no. 11684817910; Roche Diagnostics, Penzberg, Germany) according to the manufacturer’s instruction manual. Methyl green (5%) was used as a counterstain. For each subject, a total of 10 high-power fields (×40) from each of 2 sections were studied, and the number of brown-stained TUNEL-positive cells counted. Thus, approximately 20,000 cells from each group were evaluated. The mean number of TUNEL-positive cells per high-power field was compared across study groups.

**Statistical Analysis**

The RNA levels for a given gene were compared across groups using Kruskal–Wallis analysis of variance (ANOVA), a distribution-free test. A Dunn posttest was used for multiple comparisons as indicated. Linear regression was used to analyze the relationship between the levels of expression of multiple genes along a given pathway. ANOVA was used to compare the slopes of 2 or more linear regressions. Ordinal logistic regression was used to evaluate the relationship between expression of genes in specific UPR pathways and the severity of histologic abnormalities. Significance was set at a P value of .05.

**Results**

A total of 89 subjects were screened: 59 subjects were enrolled (controls [normal histology + normal ALT] = 17, NAFL = 21, and NASH = 21). The reasons for exclusion included refusal to consent (n = 17), insufficient amount of tissue for experimental studies (n = 9), and alanine aminotransferase (ALT) elevation in those with normal histology (n = 4). The baseline characteristics of the study population are shown in Table 2. The 3 groups were comparable with respect to gender, ethnicity, and features of the metabolic syndrome. However, subjects with NASH were older and, surprisingly, less obese. As expected, subjects with NASH had higher aspartate aminotransferase (AST), ALT, and alkaline phosphatase levels. The liver biopsy specimen length used for histologic studies varied from 1.7 to 3.5 cm (mean, 2.3 cm). Subjects with NAFL had a mean steatosis grade of 2.1 ± 0.6 and had no other features of NAFLD. Subjects with NASH all had steatosis, inflammation, cytologic ballooning, and pericellular fibrosis. The mean NAFLD activity score was significantly higher in those with NASH.

**Activation and Dysregulation of PERK Pathway in NAFLD**

The phosphorylated eIF-2α levels were significantly increased in both NAFL and NASH subjects compared with controls (Figure 1A). Despite the increase in phosphorylated eIF-2α, the majority of subjects with NAFL or NASH did not have increased levels of the downstream effectors ATF4 mRNA or protein, CHOP, and GADD34 mRNA (Figures 1B and 2A–C). A few subjects with NAFL or NASH had elevation of ATF4, CHOP, and GADD34 mRNA. CHOP mRNA was proportional to
the upstream ATF4 mRNA and downstream GADD34 mRNA. Regardless of the group, GADD34 mRNA levels were also directly proportional to the ATF4 mRNA ($r = 0.45$, $P = .001$). Thus, although the upstream element of the PERK pathway, ie, eIF-2α was activated in both NAFL and NASH subjects, the downstream elements (ATF4, CHOP, and GADD34) required for recovery from ER stress were not activated in most subjects with either condition.

**Activation and Dysregulation of ATF6 Pathway in NAFLD**

BiP mRNA levels in subjects with NAFL were comparable with normal histology controls. However, NASH was associated with a significant increase in BiP mRNA compared with NAFL ($P = .01$) (Figure 3A). Although there was a trend for the median uXBP-1 mRNA to increase in NASH subjects, this was not significant (Figure 3B). However, there was increased uXBP-1 protein in NASH subjects (Figure 3C). In contrast, despite a trend for increased uXBP-1 mRNA in NASH subjects, the uXBP-1 protein did not rise above levels seen in those with normal histology (Figure 3C).

**Activation and Dysregulation of IRE-1 Pathway in NAFLD**

Although the median sXBP-1 mRNA levels were not significantly different across the groups, 3 of 21 subjects with NAFL and NASH each had greater than 4.5-fold increase in sXBP-1 mRNA (Figure 4A). There was a significant decrease in sXBP-1 protein in NASH subjects (Figure 3C). Although there was a progressive increase in the median mRNA levels of EDEM from subjects with normal histology to NAFL to NASH, these were not statistically significant (Figure 4B). However, although EDEM mRNA levels were directly and linearly related to sXBP-1 mRNA in those with normal liver histology, the slope of this relationship flattened out in NAFL subjects, and the relationship reversed in subjects with NASH (Figure 4C). The slope of this regression in NASH subjects was significantly different from that for the other groups ($P = .04$ by ANOVA). Specifically, 3 of 21 subjects with NASH had the lowest quartile EDEM mRNA while having the highest quartile sXBP-1 mRNA. These subjects had the lowest sXBP-1 protein. Interestingly, some subjects with NASH had high EDEM mRNA despite low sXBP-1 protein levels.

**Status of Alarm Pathway Activation**

The amount of phosphorylated JNK in those with normal histology was similar to those with NAFL (Figure 5). However, there was a significant increase in phosphorylated JNK levels in those with NASH. In contrast, both subjects with NAFL and NASH had a significant increase in phosphorylated p38 MAPK activity. This was accompanied by a progressive increase in apoptotic activity, measured by TUNEL assay, in NAFL and NASH subjects (Figure 6).

**Relationship of Histologic Changes to Parameters of UPR**

The sXBP-1 mRNA, a reflection of IRE-1 activity, was directly related to the severity of histologic changes as measured by the NAFLD activity score ($r = 0.34$, $P = .03$). JNK phosphorylation was specifically associated with the presence of NASH. However, none of the other

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**Table 2. Baseline Characteristics of the Study Groups**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal (n = 17)</th>
<th>NAFL (n = 21)</th>
<th>NASH (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>41.5 ± 12.7</td>
<td>44.1 ± 10.1</td>
<td>52 ± 11.3</td>
</tr>
<tr>
<td>Sex (female)</td>
<td>14 (82.4)</td>
<td>16 (76.2)</td>
<td>16 (76.2)</td>
</tr>
<tr>
<td>Ethnicity (Caucasian)</td>
<td>13 (76.5)</td>
<td>16 (76.2)</td>
<td>18 (85.7)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>45 ± 6.3</td>
<td>43.9 ± 10.4</td>
<td>36.5 ± 9.6</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>128.1 ± 6.5</td>
<td>126.4 ± 6.8</td>
<td>122.9 ± 7.4</td>
</tr>
<tr>
<td>Overweight/obese</td>
<td>1/16</td>
<td>2/19</td>
<td>5/16</td>
</tr>
<tr>
<td>Hypertension</td>
<td>10 (58.8)</td>
<td>13 (61.9)</td>
<td>13 (61.9)</td>
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<tr>
<td>Diabetes</td>
<td>5 (28.4)</td>
<td>8 (38.1)</td>
<td>6 (28.6)</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>6 (35.3)</td>
<td>6 (28.6)</td>
<td>7 (33.3)</td>
</tr>
<tr>
<td>Features of the metabolic syndrome (≥3)</td>
<td>17 (100)</td>
<td>21 (100)</td>
<td>21 (100)</td>
</tr>
<tr>
<td>AST (0–65 IU/L)</td>
<td>25.6 ± 4.9</td>
<td>32.8 ± 18.2</td>
<td>84.2 ± 58.6</td>
</tr>
<tr>
<td>ALT (0–65 IU/L)</td>
<td>32.4 ± 13.5</td>
<td>53.6 ± 37.9</td>
<td>102.8 ± 59.2</td>
</tr>
<tr>
<td>Alkaline phosphatase (0–110 IU/L)</td>
<td>77.3 ± 20.1</td>
<td>80.7 ± 18.6</td>
<td>102.9 ± 25.7</td>
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<tr>
<td>Total bilirubin (0.3–0.8 mg/dl)</td>
<td>0.5 ± 0.2</td>
<td>0.6 ± 0.4</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Albumin (3.5–4.5 g/dl)</td>
<td>4.4 ± 0.3</td>
<td>4.3 ± 0.7</td>
<td>4.4 ± 0.3</td>
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<tr>
<td>Platelets (1000/mm³)</td>
<td>295.5 ± 65.8</td>
<td>311.8 ± 96.6</td>
<td>252.9 ± 70.7</td>
</tr>
<tr>
<td>Steatosis grade</td>
<td>0.0 ± 0.0</td>
<td>2.1 ± 0.6</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>NAFLD activity score</td>
<td>0.0 ± 0.0</td>
<td>2.19 ± 0.6²</td>
<td>4.33 ± 0.57²</td>
</tr>
</tbody>
</table>

**NOTE.** Data are expressed as mean ± SD or absolute number of subjects (n) and percentage in parentheses.

BMI, body mass index; NAFL, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

Across group comparisons by ANOVA: aP < .05 vs normal, bP < .05 vs NAFL, cP < .05 NAFL vs control.
parameters of the UPR measured correlated significantly with the NAFLD activity score or individual histologic parameters.

**Discussion**

The delicate balance between a cell’s synthetic needs and the ability of the ER to meet these demands is an important determinant of whether a given cell lives or dies. Perturbation of this balance triggers the UPR, which can either correct the imbalance and help the cell adapt or condemn the cell to death.\(^6\) In this study, we demonstrate

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**Figure 1.** Western blot analysis of proteins related to the PERK pathway in subjects with the metabolic syndrome and normal liver histology, NAFL, and NASH. Data for phosphorylated and total eIF-2\(\alpha\) (P-eIF-2\(\alpha\) and T-eIF2\(\alpha\), respectively) are shown on the top (panel A) and ATF4 on the bottom (panel B). \(\beta\)-actin was used as an internal control for both. There was a significant increase in P-eIF-2\(\alpha\):T-eIF2\(\alpha\) in NAFL and NASH compared with those with normal histology. The ATF4 protein levels in all 3 groups were comparable. The graphs represent the mean (±SD) of data from the first 6 subjects in each group, \(^*P < .05\) vs normal.

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**Figure 2.** Activation status of downstream effectors of the PERK pathway in subjects with the metabolic syndrome and normal liver histology, NAFL, and NASH. ATF4, CHOP, and GADD34 mRNA were measured by quantitative real-time PCR using RNA from the liver of a lean normal individual without the metabolic syndrome as an internal calibrator set at 1 across all studies. The horizontal bars in each study group represent median mRNA fold change. (A) The ATF4 mRNA levels were generally low in the majority of subjects and comparable among the 3 study groups. (B) The data for CHOP were qualitatively similar, although a few subjects with NASH had greater than 2-fold increase. (C) The levels of GADD34 mRNA were also low across all 3 groups.
that there is activation of the UPR in both NAFL and NASH. However, NASH is distinguished from NAFL subjects and controls with the metabolic syndrome and normal histology by low sXBP-1 protein levels and JNK activation.

Figure 3. Activation status of downstream effectors of the ATF6 pathway in subjects with the metabolic syndrome and normal liver histology, NAFL, and NASH. BIP and unspliced XBP-1 (uXBP-1) mRNA were measured by quantitative real-time PCR using RNA from the liver of a lean normal individual without metabolic syndrome as an internal calibrator set at 1 across all experiments. The horizontal bars in each study group represent median mRNA fold change. (A) NASH was associated with a significant increase in BIP mRNA compared with NAFL (P = .01). (B) There was a trend for the mean uXBP-1 mRNA to increase in NASH, but this did not reach statistical significance. (C) Western blot analysis of uXBP-1 and spliced XBP-1 (sXBP-1) proteins using β-actin as an internal control. Whereas uXBP-1 and sXBP-1 proteins were modestly increased in NAFL, uXBP-1 protein in NASH was comparable with controls with normal histology, and sXBP-1 protein was markedly decreased.

Figure 4. The mRNA expression of sXBP-1 and EDEM and their regulation in subjects with the metabolic syndrome and normal liver histology, NAFL, and NASH was assessed. The horizontal bars in each study group represent median mRNA fold change. (A) All groups showed similar median mRNA levels of sXBP-1 with 2.5-fold increase compared to the internal calibrator set at 1 across all experiments. (B) There was a progressive increase in median EDEM mRNA levels in the NAFLD group compared with normal histology, but this did not reach statistical significance. (C) There was an inverse relationship between sXBP-1 and EDEM mRNA in NASH, and the slope was significantly different when compared with the other groups (P = .04; ANOVA).
JNK activation, which is known to increase inflammation and apoptosis, has previously been shown in animal models of steatohepatitis.15–17 The current study confirms these findings in human NASH. The findings further demonstrate that JNK activation is specifically associated with NASH but not in NAFL. Importantly, with the sample size of the current study, the power of the observed differences in JNK phosphorylation between NAFL and NASH (0.25 ± 0.07 vs 0.75 ± 0.1, respectively) (Figure 5) exceeded 90%. Also, the phosphorylated JNK levels correlated well with the increase in TUNEL activity. Moreover, IRE-1 activation, as measured by JNK and sXBP-1 mRNA, correlated with histologic activity. It is thus likely that IRE-1 activation, which causes JNK phosphorylation, plays an important role in the genesis of cell injury in NASH.

Although UPR can activate JNK, there are, however, other mechanisms that are also known to activate JNK. Several such mechanisms, eg, free fatty acid toxicity, increased tumor necrosis factor α levels, and mitochondrial dysfunction, are present in NASH.4,30,31 The relative importance of these mechanisms in the development and progression of NASH requires further clarification.

Failure to generate EDEM in response to sXBP-1 mRNA is another key observation in this study. Luman/CREB3 and sXBP-1 protein are the only 2 known transcriptional factors that affect EDEM activation.32 Although decreased sXBP-1 protein is an obvious explanation for the failure to

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**Figure 5.** The total and phosphorylated levels of p38 MAPK and JNK proteins were evaluated by Western blot analysis. The density of individual protein bands was normalized to GAPDH. Whereas both NAFL and NASH were associated with increased phosphorylated p38 MAPK levels, phosphorylated JNK was increased only in NASH (\( P = .001 \) vs others). The graph shows the mean (±SD) of 6 subjects in each group. *\( P < .05 \) vs normal.

**Figure 6.** Apoptotic activity in the liver of subjects with metabolic syndrome and normal liver histology (left panel), NAFL (middle panel), or NASH (right panel) as measured by the TUNEL assay. TUNEL-positive cells show brown staining of nuclei, whereas nuclei of viable cells are stained with methyl green, which was used as the counterstain. For each subject, a total of 10 high-power fields (×40) from each of the 2 sections were studied, and brown-stained TUNEL-positive hepatocytes were counted. There was a highly significant increase in TUNEL-positive cells/high-power field in NASH compared with both those with normal histology and NAFL (\( P = .001 \) by ANOVA). The graph shows the mean (±SD) from 10 subjects in each group, *\( P < .05 \) vs normal.
generate EDEM mRNA in proportion to sXBP-1 mRNA, the high levels of EDEM mRNA in some subjects despite low sXBP-1 proteins suggest there are other mechanisms for EDEM activation that are also present in NASH. These data further indicate that a low EDEM is not critical for the development of NASH.

Does the failure to generate an appropriate EDEM response to rising sXBP-1 mRNA in NASH drive the progression to cirrhosis? A priori, one would predict that those with the lowest EDEM levels would be least able to degrade unfolded proteins and thus be at risk for ongoing ER stress and hepatocyte injury. Prospective studies are needed to confirm, in subjects with similar NAFLD activity, whether those with the lowest EDEM levels are most likely to progress over time.

Another important adaptive response to UPR involves translational arrest of protein synthesis by increasing phosphorylated eIF-2α via PERK activation. Although the increased eIF-2α phosphorylation observed in NAFL and NASH suggests that the PERK pathway is activated, it is possible that there are other alternate mechanisms responsible for eIF-2α phosphorylation. Importantly, phosphorylated eIF-2α specifically increases ATF4 mRNA and protein levels, which help the cell recover from ER stress. In NAFL as well as NASH, the ATF4 mRNA and protein as well as GADD34 mRNA levels were low in most subjects, suggesting that the recovery mechanisms had not been activated and that the cells were under continued stress. The mechanism for the failure to upregulate ATF4 despite increased phosphorylated eIF-2α remains to be defined.

The lack of lean individuals with normal liver histology in this study prevents comparisons to be drawn against such individuals. These subjects could not be studied because of ethical concerns about performing liver biopsies, especially with 2 cores, in such individuals. Moreover, many lean subjects in the general population have insulin resistance. It was therefore elected to use subjects with the metabolic syndrome and normal liver histology as a control group.

Another potential confounding factor is the problem of sampling variability in histologic assessment of NAFL vs NASH. This was minimized by ensuring an adequate liver biopsy specimen length (mean, 2.3 cm) and including the histologic extremes of the NAFLD, ie, those with steatosis alone, ie, NAFL or full blown NASH with steatosis, inflammation, cytologic ballooning, and pericellular fibrosis. The potential confounding effects of advanced fibrosis and cirrhosis were further avoided by excluding those with bridging fibrosis or cirrhosis. It has recently been shown that different causes of ER stress induce UPR of differing quantitative and temporal profiles. As with much of the literature on UPR, these were based on acute interventions in a cell line and are not easily translatable to the chronic human disease state. Given the relative paucity of data on the mechanisms of long-term adaptation to ER stressors in vivo, the current study provides impetus to future studies on both the triggers and mechanisms of regulation of the UPR and their role in the pathogenesis of chronic diseases, specifically NAFLD.

In summary, the current study demonstrates that the UPR is activated to varying degrees in the liver of subjects with NAFLD. Whereas the upstream effector of the PERK pathway eIF-2α is activated in NAFL and NASH, the downstream effectors ATF4, CHOP, and GADD34 remain inactive in most subjects. The development of steatohepatitis is associated with a failure to generate sXBP-1 protein. NASH is also associated with activation of JNK, which is downstream of IRE-1. IRE-1 activation is directly associated with histologic activity of NASH.

References

16. Rahman SM, Schroeder-Gloeckler JM, Jansen RC, et al. CCAAT/enhancing binding protein β deletion in mice attenuates inflam-


