Insulin Resistance, Ceramide Accumulation and Endoplasmic Reticulum Stress in Experimental Chronic Alcohol-Induced Steatohepatitis

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INTRODUCTION

On a global scale, chronic alcohol abuse is a leading cause of liver-associated morbidity and mortality (Paula et al., 2010; McCullough et al., 2011; Miller et al., 2011a). Alcohol-related liver injury leads to steatohepatitis, which can progress to chronic alcoholic liver disease (ALD) (O’Shea et al., 2010). In chronic ALD, liver function is compromised due to insulin resistance (de la Monte et al., 2008; Pang et al., 2009; Longato et al., 2012a). Ethanol impairs insulin and insulin-like growth factor type 1 (IGF-1) signaling in the liver (Sasaki and Wands, 1994; Onishi et al., 2003; He et al., 2007; Ronis et al., 2007; de la Monte et al., 2008; Pang et al., 2009; Denucci et al., 2010; Setshedi et al., 2011) by inhibiting tyrosine phosphorylation of the insulin and IGF-1 receptors (Patel et al., 1991; de la Monte et al., 2008). These effects are mediated by reduced ligand-receptor binding, decreased activation of receptor tyrosine kinases (Patel et al., 1991; de la Monte et al., 2008; Denucci et al., 2010), and increased activation of phosphatases that negatively regulate receptor function (Xu et al., 2003; Yeon et al., 2003; Gao et al., 2010). In addition, ethanol inhibits insulin/IGF-1 downstream signaling by impairing tyrosine phosphorylation of insulin receptor (InR) substrate (IRS) proteins (Sasaki and Wands, 1994; de la Monte, 2012). Consequently, IRSs interactions with adaptor molecules are reduced, thereby inhibiting the activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) and Akt (Sasaki et al., 1994; Mohr et al., 1998; de la Monte et al., 2008) networks. Insulin resistance may be an early pathophysiologic effect of ethanol-induced hepatotoxicity because it develops after short-term exposures (He et al., 2010). Furthermore, since ethanol compromises insulin/IGF regulated hepatocyte growth, regeneration, survival, glucose utilization, energy metabolism and protein synthesis (Mohr et al., 1998; Lonardo et al., 2005; Ronis et al., 2007; de la Monte, 2012), hepatic insulin/IGF resistance most likely contributes to ALD progression.

Despite the pivotal role of insulin resistance in the pathogenesis of ALD, growing evidence supports the concept that other pathophysiological processes including cytotoxic and lipotoxic injury (McVicker et al., 2006; de la Monte et al., 2009a; Cohen and Nagy, 2011; Derdak et al., 2011), inflammation (Ronis et al., 2008; Cohen and Nagy, 2011), oxidative and endoplasmic reticulum (ER) stress (Kaplowitz and Ji, 2006; Pandol et al., 2010; Feldstein and Bailey, 2011; Malhi and Kaufman, 2011), metabolic and mitochondrial dysfunction (Purowit et al., 2009; Ding et al., 2011), decreased DNA synthesis (Sasaki et al., 1994; Pang et al., 2009), and increased cell death (Derdak et al., 2011) not only cause injury, but also reinforce the adverse effects of insulin resistance and thereby drive ALD progression. Progression of chronic ALD is marked by activation of pro-fibrogenic pathways (Cohen and Nagy, 2011), setting the stage for eventual development of cirrhosis and finally liver failure (Vidali et al., 2008; Purowit et al., 2009). Improved understanding of the mechanisms of liver dysfunction caused by chronic alcohol abuse could lead to new treatments for reducing morbidity and mortality from ALD.
Insulin resistance perturbs lipid homeostasis and promotes lipolysis (Kao et al., 1999), which increases the generation of toxic lipids, i.e. ceramides, that further impair insulin signaling (Holland and Summers, 2008; Kraegen and Cooney, 2008; Langeveld and Aerts, 2009). Ceramides also activate pro-inflammatory cytokines and inhibit PI3K-Akt (Hajducz et al., 2001; Bourbon et al., 2002; Powell et al., 2003; Nogueira et al., 2008). We suspect that ceramide accumulation plays a key role in ALD pathogenesis and progression because: (a) mice deficient in acidic sphingomyelinase are resistant to ethanol-induced hepatic steatosis (Garcia-Ruiz et al., 2003); (b) ceramides inhibit S’ adenosine monophosphate-activated protein kinase (AMPK) (Liangpunsakul et al., 2010) and promote local hepatocellular injury (Anderson and Borlak, 2008), possibly because AMPK reduces ER stress and apoptosis (Kuznetsov et al., 2011) and (c) preliminary studies in ethanol exposure models showed that concurrent treatment with inhibitors of ceramide biosynthesis reduces hepatic insulin resistance (Setsshedhi et al., 2010). Together with the recent finding that in advanced human ALD, hepatic ceramide profiles are substantially altered (de la Monte, 2012; Longato et al., 2012a), it is likely that ceramide accumulation and altered profiles contribute to ALD pathogenesis and progression.

Insulin resistance drives ER stress because vital ER functions such as protein synthesis, modification and folding, calcium signaling, and lipid biosynthesis (Hotamisligil, 2010) all utilize glucose as the main source of energy, and insulin resistance impairs glucose uptake and metabolism. Ethanol-impaired insulin signaling promotes hepatocellular injury and death via all three major ER stress sensor cascades: PERK, IRE-1α, and activating transcription factor 6 (ATF6) (Kaplowitz et al., 2007; Sundar-Rajan et al., 2007; Malhi and Gores, 2008; Sharma et al., 2008). In addition, ethanol increases ER resident sterol regulatory-binding proteins (SREBP)-1c and 2, resulting in up-regulation of fatty acid/ triglyceride synthesis, beta oxidation (SREBP-1a) and cholesterol synthesis (SREBP2) (Kaplowitz and Ji, 2006). On the one hand, ER stress may potentiate insulin resistance and lipolysis leading to increased ceramide production (Kaplowitz et al., 2007; Sundar-Rajan et al., 2007; Anderson and Borlak, 2008; Malhi and Gores, 2008) and worsening of inflammation and insulin resistance (Kaplowitz and Ji, 2006; Ronis et al., 2008). On the other hand, disease-associated lipolysis can be initiated by ER stress and mitochondrial dysfunction (Kaplowitz et al., 2007; Sundar-Rajan et al., 2007; Anderson and Borlak, 2008; Malhi and Gores, 2008). Therefore, it is not surprising that ER stress in ALD is marked by lipid dyshomeostasis and activation of pro-ceramide and pro-inflammatory pathways that increase toxic lipid generation (Ozcan et al., 2004; Kaplowitz et al., 2007; Sundar-Rajan et al., 2007; Malhi and Gores, 2008; Ronis et al., 2008).

The present study was designed to better characterize structural and functional correlates of chronic ALD using a robust experimental model, and further examine the roles of ceramide accumulation and ER stress in relation to hepatic insulin resistance. In addition to providing evidence that ALD must be approached using multi-pronged therapeutic measures, the findings suggest that biomarker panels for detecting disease and monitoring responses to treatment could be greatly expanded beyond the current standard of care measures.

MATERIALS AND METHODS

Materials

The Akt Total and Phospho 7-Plex Panels, Taqman Gene expression master mix, Amplex Red Cholesterol Assay Kit and Amplex UltraRed soluble fluorophore were purchased from Invitrogen (Carlsbad, CA). The bead-based 24-plex magnetic immunoassay for cytokine quantification was from Biorad (Hercules, CA). QIAzol Lysis Reagent for RNA extraction was obtained from Qiagen, Inc (Valencia, CA). The AMV First-Strand cDNA Synthesis Kit, the Universal Probe Library probes and reference gene assays were purchased from Roche Applied Science (Indianapolis, IN). The Nile Red fluorescence-based assay for lipids was obtained from Molecular Probes (Eugene, OR). The OxyBlot assay for measuring protein carbonylation was purchased from Millipore Corp (Billerica, MA). MaxiSorb 96-well plates used for enzyme-linked immunosorbent assays (ELISAs) were from Nunc (Thermo Fisher Scientific; Rochester, NY). The MDA-586 kit to measure malondialdehyde (MDA) was purchased from Oxis International, Inc. (Foster City, CA). The monoclonal anti-ceramide, Triglyceride Determination kit and synthetic oligodeoxynucleotides were purchased from Sigma-Aldrich Co (St. Louis, MO).

Experimental model

Adult male (~200–250 g) Long Evans rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) were pair-fed with isocaloric liquid diets (BioServ, Frenchtown, NJ) containing 0% (n = 12) or 37% (n = 12) ethanol (caloric content) for 8 weeks (Cohen et al., 2007; Pang et al., 2009; Denucci et al., 2010). Two weeks prior to initiating the experiment, rats were adapted to the liquid diets by incrementing the ethanol content from 0 to 11.8%, 23.6% and then 37% of the caloric content. Controls were adapted to ethanol-free liquid diets over the same period. Rats were monitored daily to ensure adequate nutritional intake and maintenance of body weight. Blood alcohol levels were measured at 8 a.m. using the Analox GM7 apparatus (Analox Instruments USA, Lunenburg, MA) according to the manufacturer’s protocol. Using this approach, blood alcohol concentrations ranged from 110 to 130 mg/dl as previously reported (Denucci et al., 2010). Throughout the experiment, rats were housed under humane conditions and kept on a 12-h light/dark cycle with free access to food. All experiments were performed in accordance with protocols approved by Institutional Animal Care and Use Committee at the Lifespan-Rhode Island Hospital, and they conform to guidelines established by the National Institutes of Health.

Lipid and protein adduct assays

To assess the role of adducts as mediators of oxidative stress and insulin resistance, we measured MDA using the MDA-586 kit, and protein carbonylation using the OxyBlot assay. Carbonyl dot blot assay results were normalized to total protein spotted onto nitrocellulose membranes. Proteins were detected with SYPRO Ruby and quantified by digital imaging (Kodak Digital Science Image Station, Rochester, NY). Lipids were chloroform-methanol (2:1) extracted from fresh frozen tissue (Lyn-Cook et al., 2009). Total neutral
lipid content was measured using the Nile Red microplate assay (McMillian et al., 2001), and fluorescence (Ex 485/Em 572) was measured in a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA). Hepatic triglyceride and cholesterol levels were measured using commercially available kits, and results were normalized to liver sample weight.

Liver tissue processing
At the end of the experiment, the rats were sacrificed by iso- fluorane inhalation. Portions of liver were snap-frozen in a dry ice/methanol bath and stored at −80°C for protein, lipid, and RNA studies. Portions of liver were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Adjacent histological sections (5-µm thick) were stained with hematoxylin and eosin (H&E) and Sirius Red (collagen stain) dyes. Fresh frozen liver tissue was cryostat sectioned (5-µm thick) and stained with Oil Red-O to assess hepatic steatosis. Liver sections were examined using a Morgagni 268 transmission electron microscope (EM) and immunoreactivity to: InR, IGF-1R, IRS-1, Akt, pYpY1162/1163-InR, pYpY1135/1136-IGF-1R, pSer1139-GSK3β, pThr451/458-IRS-1, pS312-Akt, pSer473-Akt, pSer406-Akt, pThr246-PRAS40, pSer241/242-p70S6K and pSer9-GSK3β. In addition, a 24-plex bead-based immunoassay was used to measure pro-inflammatory cytokines and chemokines. Samples containing 100 µg protein for the 7-plex, and 200 µg protein for the 24-plex assays were incubated with the beads according to the manufacturer’s protocol. Captured antigens were detected with secondary antibodies and phycoerythrin-conjugated anti-rabbit immunoglobulin G. Plates were read in a Bio-Plex 200 system (Bio-Rad, Hercules, CA).

Quantitative reverse transcriptase polymerase chain reaction assays
RNA extracted from fresh frozen liver tissue using the RNeasy Mini Kit was reverse transcribed using random oligonucleotide primers and the AMV First-Strand cDNA Synthesis Kit. Gene expression was measured using a hydrolysis probe-based duplex quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay with β-actin as a reference gene as previously described (de la Mont e et al., 2012). Gene-specific primers and matched probes were determined with the ProbeFinder Software (Roche, Indianapolis, IN) (Supplementary data, Table S1). PCR amplifications were performed in a LightCycler® 480 PCR machine (Roche, Indianapolis, IN) and results were analyzed using LightCycler® Software 4.0.

Bead-based multiplex ELISA
We used bead-based multiplex ELISAs to examine signaling through insulin and IGF-1 receptors (IGF-1R), IRS-1 and downstream through Akt-related pathways using Akt Total and Phospho 7-Plex Panels. The Akt Total 7-Plex panel measured immunoreactivity to: InR, IGF-1R, IRS-1, Akt, proline-rich Akt substrate of 40 kDa (PRAS40), ribosomal protein S6 kinase (p70S6K) and glycogen synthase kinase 3β (GSK-3β). The Akt Phospho 7-Plex panel measured immunoreactivity to: pSer1162/1163-InR, pThr315/316-IGF-1R, pS312-IRS-1, pS473-Akt, pT451/458-PRAS40, pS421/422-p70S6K and pS9-GSK3β. In addition, a 24-plex bead-based immunoassay was used to measure pro-inflammatory cytokines and chemokines. Samples containing 100 µg protein for the 7-plex, and 200 µg protein for the 24-plex assays were incubated with the beads according to the manufacturer’s protocol. Captured antigens were detected with secondary antibodies and phycoerythrin-conjugated anti-rabbit immunoglobulin G. Plates were read in a Bio-Plex 200 system (Bio-Rad, Hercules, CA).

Liquid chromatography, tandem mass spectrometry for quantification of ceramides in liver tissue
Ceramide profiling was performed with lipids extracted from 60 to 70 mg samples of fresh frozen liver tissue as previously described (Bielawski et al., 2006; Bielawski et al., 2009; Longato et al., 2012a). Dried extracts were re-dissolved in methanol. Lipids were subjected to chromatographic separation through a Thermo-Hypersil GOLD PFP (50 × 2.1 mm, 3.0 µm) analytical column. The system consisted of a binary pump and auto-sampler (Shimadzu, Kyoto, Japan) coupled to an API 3200 triple quadruple mass spectrometric detector (AB Sciex, Toronto, Ontario, Canada), equipped with a Turbo V source electrospray ionization probe. Chromatographic data were collected and analyzed using the Analyst® package (version 1.4.1., AB Sciex). Multiple reaction monitoring scanning was used for mass spectral detection and quantification of C14-Ceramide (N-miristoyl-o-erythro-sphingosine), C16-Ceramide (N-palmitoyl-o-erythro-sphingosine), C18-Ceramide (N-stearoyl-o-erythro-sphingosine), C20-Ceramide (N-arachidonyl-o-erythro-sphingosine) and C24-Ceramide (N-lignoceroyl-o-erythro-sphingosine). C17-Ceramide (heptadecanoyl-o-erythro-sphingosine) was used as an internal standard.

Specific pairs of precursor/product ions are as follows: the ion transitions were C14-Ceramide (510.5→492.4), C16-Ceramide (538.8→520.5), C17-Ceramide (552.6→534.4), C18-Ceramide (566.4→548.4), C20-Ceramide (594.7→576.2) and C24-Ceramide (650.6→632.5). The retention times for C14-Ceramide, C16-Ceramide, C17-Ceramide, C18-Ceramide, C20-Ceramide and C24-Ceramide were 9.4, 10.0, 10.4, 10.7, 11.1 and 12.1 min, respectively. The ionization was set at an electrospray positive ion mode and at a temperature of 150°C utilizing nitrogen for the drying and collision gas (cabinet gas, 10 psi; collision gas, 3 psi; ionspray voltage, 5500 V; ion source gas 1, 25 psi; ion source gas 2, 25 psi). Separate stock solutions of all compounds each containing 1.0 mM were prepared in methanol. Working standard solutions of combined analytes were prepared by serial dilution in methanol. Calibration standards were prepared in concentrations ranging from 0.5 to 500 µM (0.1, 0.5, 1, 5, 10, 50, 100 and 500 µM). Internal standard was used at a final concentration of 20 nM. In addition, total ceramide immunoreactivity was measured by ELISA as described (Longato et al., 2012a,b).

Statistical analysis
Data depicted in box plots reflect group medians (horizontal bar), 95% confidence interval limits (upper and lower box limits) and range (whiskers). Tabulated data reflect means ± SEM for each group. Data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA), and inter-group comparisons were made using Student’s t-tests. Computer software generated P-values are shown over the graph panels or within the table.
RESULTS

Pathology of experimental alcohol-induced steatohepatitis

Control livers exhibited the expected chord-like architecture and were free of inflammation, steatosis, fibrosis and cholestasis (Fig. 1A and B). Livers from chronic ethanol-fed rats had steatohepatitis characterized by diffuse hepatocellular micro-vesicular steatosis, dilatation of sinusoids, loss of the chord-like architecture, multifocal areas of inflammation with hepatocellular apoptosis or necrosis and increased cell turnover with easily detected nuclear mitoses (Fig. 1C and D), as previously reported (Denucci et al., 2010). The histopathological abnormalities seen in the ethanol-exposed livers were not observed in control livers. Ethanol-induced steatosis was confirmed by the prominent Oil red O staining of hepatocytes (Fig. 2A and B) and increased hepatic lipid content (see below). In addition, chronic ethanol feeding resulted in increased intra-lobular and peri-hepatocyte ‘chicken-wire’ fibrosis, as demonstrated with the Sirius Red stain (Fig. 2C and D). Transmission EM demonstrated regular parallel organized ER in close association with regularly distributed mitochondria that had well-organized cristae. In contrast, chronic ethanol-exposed livers had increased densities of lipid vacuoles, marked variability in shape, size and physical relationships among the organelles, e.g. ER and mitochondria were displaced by lipid droplets, enlargement of mitochondria, irregularity of mitochondrial cristae and abnormalities in the ER ranging from dilation with irregular spacing and density of ribosomes to pronounced architectural disruption (Fig. 3).

Biochemical correlates of chronic alcohol-induced steatohepatitis

Despite ongoing chronic inflammation and focal necrosis/apoptosis, serum alanine aminotransferase (ALT) levels were only modestly elevated in the ethanol-fed group (Fig. 4A), consistent with our previous observations (Denucci et al., 2010). In contrast, ethanol-induced steatohepatitis was associated with significantly higher levels of Nile Red fluorescence, triglyceride and cholesterol content in liver (Fig. 4B and D), corresponding with the Oil red staining results. Moreover, ethanol-induced steatohepatitis was associated increased oxidative stress and DNA damage, as demonstrated by the significantly higher levels of 4-hydroxy-nonenal, MDA, nitro-tyrosine and DNA nicking (increased labeling for single-stranded DNA) (Fig. 4E, F, H and I). Increased DNA damage and oxidative stress indices are recognized features of chronic ALD in this model (de la Monte et al., 2008; Pang et al., 2009; Denucci et al., 2010). Protein carbonyl levels were also higher in the ethanol group (Fig. 4G), but the difference from control did not reach statistical significance due to the large variance in the ethanol group.

Effects of chronic ethanol feeding on cytokine levels in liver

We used a 24-plex bead-based assay to measure pro-inflammatory cytokine and chemokine levels in relation to chronic ethanol feeding (Table 1; Supplementary data, Table S2 for cytokine and chemokine functions). Among the 24 cytokines and chemokines tested, livers of ethanol-exposed rats had significantly higher levels of interleukin (IL)-7, IL-10,
Fig. 2. Increased hepatic steatosis and early fibrosis in experimental chronic ethanol-feeding model. Cryostat sections of liver from (A) control and (B) ethanol-fed rats were stained with Oil Red O to detect cytoplasmic lipid accumulation (punctate for oil red O labeling). Formalin-fixed paraffin embedded sections of liver were stained with Sirius red to detect collagen. Note minimal labeling in the (C) control liver compared with (D) the delicate peri-hepatocyte labeling in the ethanol-exposed liver (collagen fibrils have dark linear profiles). (Original magnifications, ×650).

Fig. 3. Ultrastructural features of experimental chronic alcohol-induced liver injury. Transmission EM studies demonstrating (A and C) regular parallel organized ER (er) in close association with mitochondria (m) in control livers (n, nucleus) and (B and D) and irregularly arranged and disrupted ER, striking variability in mitochondrial size and shape with enlargement and disruption of the normal cristae in ethanol-exposed livers (L = lipid droplet). (Original magnifications: A and B, 44,000x; C and D, 86,000x).
GRO/KC (growth-related oncogene-alpha CXCL1), interferon-γ and macrophage inflammatory protein-1α (MIP-1α) relative to control (all \( P < 0.05 \)). In addition, significant trends toward higher levels of IL-2, IL-13, MIP-3α and erythropoietin were measured in the chronic ethanol-fed group (0.05 < \( P < 0.10 \)). In contrast, no significant inter-group differences or trends were observed with respect to IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-12 p70, IL-17, Eotaxin, G-colony-stimulating factor (CSF), granulocyte-macrophage (GM)-CSF, macrophage-CSF, RANTES, tumor necrosis factor alpha (TNF-α) or vascular endothelial growth factor (VEGF).

Chronic ethanol exposure impairs hepatic insulin signaling

Multiplex ELISAs demonstrated significantly reduced levels of InR and IRS-1 proteins in ethanol-exposed livers, but similar levels of hepatic IGF-1R in the two groups (Fig. 5A, D and G). In addition, immunoreactivity corresponding to \( \text{pTy}^{1162/1163}\)-InR, \( \text{pTy}^{1135/1136}\)-IGF-1R, \( \text{pS}312\)-IRS-1 were lower in ethanol-exposed relative to control livers, but the inter-group differences were only statistically significant for \( \text{pTy}^{1162/1163}\)-InR and \( \text{pS}312\)-IRS-1 (Fig. 5B, E and H). The calculated \( \text{pTy}^{1162/1163}\)-InR/InR and \( \text{pS}312\)-IRS-1/IRS-1 ratios, reflecting relative phosphorylation of InR and IRS-1 were significantly lower in the ethanol-exposed group, whereas the mean \( \text{pTy}^{1135/1136}\)-IGF-1R/IGF-1R ratios were similar for the two groups (Fig. 5C, F and I). In previous

Table 1. Effects of chronic ethanol exposure on hepatic cytokine levels

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control</th>
<th>Ethanol</th>
<th>( P )-value</th>
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<tbody>
<tr>
<td>IL-1α</td>
<td>422.0 ± 107.1</td>
<td>431.1 ± 72.9</td>
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<tr>
<td>IL-1β</td>
<td>345.2 ± 64.4</td>
<td>380.3 ± 82.7</td>
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<tr>
<td>IL-2</td>
<td>485.9 ± 265.4</td>
<td>946.8 ± 442.6</td>
<td>0.062</td>
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<tr>
<td>IL-4</td>
<td>133.4 ± 81.7</td>
<td>152.2 ± 67.9</td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>85.7 ± 28.1</td>
<td>80.1 ± 39.4</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>187.3 ± 110.9</td>
<td>260.7 ± 106.5</td>
<td></td>
</tr>
<tr>
<td>IL-7</td>
<td>1774.4 ± 1087.4</td>
<td>3237.9 ± 783.0</td>
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</tr>
<tr>
<td>IL-10</td>
<td>2384.2 ± 1169.7</td>
<td>4001.8 ± 591.1</td>
<td>0.0243</td>
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<tr>
<td>IL-12 p70</td>
<td>229.1 ± 130.3</td>
<td>255.7 ± 94.2</td>
<td></td>
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<tr>
<td>IL-13</td>
<td>288.9 ± 96.6</td>
<td>423.2 ± 147.7</td>
<td>0.089</td>
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<tr>
<td>IL-17</td>
<td>181.5 ± 76.9</td>
<td>199.1 ± 34.5</td>
<td></td>
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<tr>
<td>Eotaxin</td>
<td>99.0 ± 22.6</td>
<td>103.7 ± 26.4</td>
<td></td>
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<tr>
<td>G-CSF</td>
<td>28.6 ± 19.7</td>
<td>31.7 ± 16.4</td>
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<tr>
<td>GM-CSF</td>
<td>148.6 ± 75.5</td>
<td>142.8 ± 31.4</td>
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<tr>
<td>GRO/KC</td>
<td>752.5 ± 265.3</td>
<td>1293.1 ± 168.9</td>
<td>0.046</td>
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<td>Interferon-γ</td>
<td>322.3 ± 161.5</td>
<td>635.9 ± 231.4</td>
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<td>M-CSF</td>
<td>90.3 ± 30.2</td>
<td>102.3 ± 27.7</td>
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<tr>
<td>MIP-1α</td>
<td>1362.6 ± 762.8</td>
<td>2506.7 ± 870.1</td>
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<tr>
<td>MIP-3α</td>
<td>354.5 ± 210.11</td>
<td>614.6 ± 221.7</td>
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<tr>
<td>RANTES</td>
<td>3670.2 ± 2874.1</td>
<td>2180.3 ± 777.4</td>
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<tr>
<td>TNF-α</td>
<td>82.3 ± 68.4</td>
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<tr>
<td>VEGF</td>
<td>979.7 ± 656.4</td>
<td>749.9 ± 2009.9</td>
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<tr>
<td>EPO</td>
<td>1514.7 ± 827.7</td>
<td>2474.5 ± 831.1</td>
<td>0.076</td>
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</table>

Liver protein homogenates were used to measure immunoreactivity of the indicated cytokines by multiplex ELISA. Immunoreactivity was normalized to protein concentration and data are expressed as mean ± SD of fluorescence light units (arbitrary). Inter-group comparisons were made using \( t \)-test and significant \( P \)-values are indicated in the right column.
studies, we demonstrated that chronic ethanol feeding causes hepatic insulin resistance manifested by reduced levels of ligand-receptor binding and mRNA expression of the ligands, receptors and/or IRS molecules (de la Monte et al., 2008, 2011; Pang et al., 2009; Denucci et al., 2010). The present findings corroborate the conclusions of those studies, and also extend our knowledge about how ethanol impairs hepatic insulin signaling. In this regard, we provide new information showing that chronic ethanol feeding impairs phosphorylation and activation of the insulin and IGF-1 receptors and IRS-1.

Additional studies characterized the effects of chronic ethanol feeding on downstream signaling through Akt pathways. Chronic ethanol feeding significantly reduced hepatic total, phosphorylated and relative phosphorylated levels of Akt (Fig. 6A, E and I), the PRAS40 (Fig. 6C and G), p70S6K (Fig. 6D, H and L), although the mean levels of relative PRAS40 phosphorylation were similar in control and ethanol-exposed livers. At the same time, chronic ethanol feeding significantly increased hepatic levels of GSK-3β, and reduced pSer9-GSK-3β and pSer9-GSK-3β/GSK-3β (Fig. 6B, F and J), which together reflect increased GSK-3β activity.

**Chronic ethanol feeding increases hepatic ceramide-related gene expression**

To examine the effects of chronic ethanol feeding on ceramide-related gene expression in liver, we used qRT-PCR analysis to measure mRNA levels of genes corresponding to de novo ceramide biosynthesis mechanisms (Ceramide synthases (CERS) 1, 2, 4 and the subunits 1 (regulatory) and 2 (catalytic) of serine palmitoyl transferase, long-chain subunit (SPTLC 1 and 2)), catabolic enzymes [sphingomyelin phosphodiesterases (SMPD) 1 and 3 and ceramidases (CERD) and glycosphingolipid synthesis (ganglioside GM3 synthase and UDP-glucose ceramide glucosyltransferase (UGCG)]. See Supplementary data, Table S2 for ceramide gene functions. Chronic ethanol feeding significantly increased hepatic expression of CERS4 (Fig. 7B), CERD3 (Fig. 7D), SMPD1 (Fig. 7E) and SPTLC2 (Fig. 7H), and significantly reduced expression of SMPD3 (Fig. 7F) and GM3 synthase (Fig. 7J). In contrast, no significant inter-group differences were observed with respect to CERS2, CERD2, SPTLC1 or UGCG (Fig. 7A, C, G and I).

**Chronic ethanol feeding increases ceramide levels and alters ceramide profiles in liver**

We measured ceramide immunoreactivity in liver by ELISA and quantified the levels of ceramides C14:0, C16:0, C18:0, C20:0 and C24:0 by liquid chromatography, tandem mass spectrometry (LC-MS)/MS (Fig. 8). Livers from ethanol-fed rats had significantly higher mean levels of ceramide immunoreactivity relative to controls (Fig. 8A). The LC-MS/MS studies demonstrated that the most abundant ceramide species in rat livers were C16:0, followed by C18:0 and C24:0, while the least abundant species was C14:0 (Fig. 8C). Chronic ethanol feeding significantly increased hepatic levels of C14:0 (Fig. 8B) and C18 (Fig. 8D), and reduced the levels of C16:0 (Fig. 8C) relative to control, C20:0 was also marginally lower in ethanol-exposed relative to control livers (Fig. 8E), whereas C24:0 levels were similar in control and ethanol-exposed livers (Fig. 8F).
ER stress in experimental chronic ethanol-induced steatohepatitis

ER stress mechanisms are activated in steatohepatitis, and in association with hepatic ceramide accumulation and insulin resistance. ER stress contributes to progression of ALD by promoting oxidative injury and inflammation. To determine if chronic ethanol feeding in our rat model of alcohol-induced steatohepatitis was associated with increased ER stress, we measured mRNA levels of genes that mediate ER stress at various levels in the cascade (Jager et al., 2012).

Chronic ethanol feeding significantly up-regulated the homocysteine-responsive ER-resident ubiquitin-like domain member 1 (HERPUD) (Fig. 9A), protein disulphur isomerase (PDI) (Fig. 9F) and the transcription factor ATF-4 (Fig. 9G), down-regulated the chaperone Bip/GRP78 (Fig. 9C), ER degradation-enhancing α-mannosidase-like 1 (EDEM) (Fig. 9I), and had no significant effect on the expression of the protein kinase inhibitor p58 (P58IPK) (Fig. 9B) and tryptophanyl-tRNA synthetase (WARS) (Fig. 9E). In addition, we detected significantly higher levels of C/EBP homologous protein (CHOP) (Fig. 9D) and BAX (Fig. 9H), corresponding to unfolded protein response (UPR)-driven pathological responses that promote apoptosis. Also see Supplementary Table data, S3 for ER stress gene functions.

DISCUSSION

This study was designed to gain a better understanding of the molecular pathogenesis of chronic alcohol-induced liver disease using a robust experimental animal model. Herein, we examined several interrelated aspects of liver injury including: (a) histopathology and ultrastructural pathology; (b) integrity of insulin/IGF signaling through Akt pathways; (c) activation of pro-inflammatory mediators; (d) ceramide production, accumulation and profiles and (e) ER stress pathway activation. Although we have previously described this model and shown evidence of hepatic insulin resistance following chronic ethanol feeding (de la Monte et al., 2008; Pang et al., 2009; Denucci et al., 2010; Derdak et al., 2011), we have not previously characterized signaling impairments downstream of Akt, altered hepatic ceramide profiles or activation of ER stress networks in experimental ALD. We now discuss how these aspects of chronic alcohol-induced steatohepatitis are interrelated.
Pathologic correlates in experimental chronic alcohol-induced steatohepatitis

Histopathologic studies revealed that chronic ethanol feeding caused steatohepatitis with loss of the normal chord architecture, multifocal areas of intra-lobular lymphomononuclear cell inflammation, apoptotic bodies as previously described (de la Monte et al., 2008; Denucci et al., 2010). In addition, we demonstrated ethanol-induced early chicken-wire (peri-hepatoocyte) fibrosis. Ultrastructural studies demonstrated abundant membrane-bound lipid vacuoles, enlargement and irregularity of mitochondria and variable degrees of ER disruption. Ethanol-induced hepatic steatosis corresponded with the significantly higher levels of Nile Red fluorescence, triglyceride and cholesterol measured in liver tissue homogenates.

Steatohepatitis was also associated with increased levels of chemo-attractant cytokines and chemokines (GROK/CXCL1 and MIP-1α/CCL3), and pro-inflammatory activators of T cells, B cells, NK cells or macrophages (IL-7, IL-2 and interferon-γ). The persistent activation of pro-inflammatory cytokines and chemokines most likely contributed in a direct way to the ongoing alcohol-related liver injury, but also could have promoted injury by worsening hepatic insulin resistance and ER stress (Hotamisligil, 2010). The contribution of pro-inflammatory cytokines and chemokines most likely contributed in a direct way to the ongoing alcohol-related liver injury, but also could have promoted injury by worsening hepatic insulin resistance and ER stress (Hotamisligil, 2010). The contribution of pro-inflammatory cytokines and chemokines most likely contributed in a direct way to the ongoing alcohol-related liver injury, but also could have promoted injury by worsening hepatic insulin resistance and ER stress (Hotamisligil, 2010). The contribution of pro-inflammatory cytokines and chemokines most likely contributed in a direct way to the ongoing alcohol-related liver injury, but also could have promoted injury by worsening hepatic insulin resistance and ER stress (Hotamisligil, 2010). The contribution of pro-inflammatory cytokines and chemokines most likely contributed in a direct way to the ongoing alcohol-related liver injury, but also could have promoted injury by worsening hepatic insulin resistance and ER stress (Hotamisligil, 2010). The contribution of pro-inflammatory cytokines and chemokines most likely contributed in a direct way to the ongoing alcohol-related liver injury, but also could have promoted injury by worsening hepatic insulin resistance and ER stress (Hotamisligil, 2010). The contribution of pro-inflammatory cytokines and chemokines most likely contributed in a direct way to the ongoing alcohol-related liver injury, but also could have promoted injury by worsening hepatic insulin resistance and ER stress (Hotamisligil, 2010). The contribution of pro-inflammatory cytokines and chemokines most likely contributed in a direct way to the ongoing alcohol-related liver injury, but also could have promoted injury by worsening hepatic insulin resistance and ER stress (Hotamisligil, 2010). The contribution of pro-inflammatory cytokines and chemokines most likely contributed in a direct way to the ongoing alcohol-related liver injury, but also could have promoted injury by worsening hepatic insulin resistance and ER stress (Hotamisligil, 2010). The contribution of pro-inflammatory cytokines and chemokines most likely contributed in a direct way to the ongoing alcohol-related liver injury, but also could have promoted injury by worsening hepatic insulin resistance and ER stress (Hotamisligil, 2010).
Finally, the presence of patchy peri-hepatocyte and sinusoidal (chicken-wire) fibrosis indicates that after 8 weeks of ethanol feeding, the liver injury had begun to progress toward a more chronic disease state. These results corroborate previous reports on the effects of chronic ethanol feeding on the liver (Denucci et al., 2010; Setshedhi et al., 2011), but also provide new information that integrates histopathology and ultrastructural pathology with molecular and biochemical indices of liver injury.

**Role of impaired insulin/IGF-1/IRS-1 signaling**

Although there is evidence that ethanol exposure can lead to body insulin resistance (Yao et al., 2006), our analyses were focused on the liver to gain a better understanding of ALD. The significantly reduced levels of InR and IRS-1 in ethanol-exposed livers indicate that chronic ethanol feeding impairs insulin/IRS-1 signaling in part by reducing the expression of proteins needed to transduce the signals. In addition, we detected lower levels of InR (significant) and IRS-1 receptor (trend) tyrosine phosphorylation in ethanol-exposed livers, indicating that signaling through the available receptors was also constitutively impaired. This phenomenon most likely reflects a state of chronic insulin/IGF-1 resistance in the liver, corresponding with our previous analysis of ethanol-exposed brains (de la Monte et al., 2001; de la Monte, 2012). There is now ample evidence that chronic ethanol exposure impairs insulin and IGF-1 signaling by inhibiting tyrosine phosphorylation of the insulin and IGF-1 receptors, and IRS-1 (de la Monte, 2012). These effects are mediated by reduced receptor binding, and inhibition of downstream signaling through Ras/Raf/Erk MAPK, which impairs DNA synthesis and liver regeneration, and through PI3K-Akt, which sustains cell viability, energy metabolism and mitochondrial function (de la Monte, 2012). Inhibition of PI3K-Akt promotes apoptosis, DNA damage, mitochondrial dysfunction and oxidative stress (Yeon et al., 2003; Ronis et al., 2007; de la Monte et al., 2008; Pang et al., 2009). Correspondingly, the chronic ethanol-exposed livers had profoundly impaired signaling through Akt, as was manifested by the significantly reduced levels of total and phosphorylatedAkt, increased levels of total GSK-3β and decreased levels pSer9-GSK-3β and pSer9-GSK-3β/GSK-3β (Pang et al., 2009). Similarly, activation of mTOR networks via p70S6K and PRAS40 were impaired. The net effect was constitutive inhibition of Akt and activation of GSK-3β, which would have contributed to the higher hepatic indices of oxidative stress, lipid peroxidation, DNA damage and cell death, as well as metabolic impairments mediating to lipid dyshomeostasis and ultimately ER stress in chronic ethanol-fed rats.

**Role of ceramides in experimental chronic alcohol-induced steatohepatitis**

Two factors that likely mediate progression of ALD include: (a) aberrant shifts in membrane lipid composition leading to disrupted intracellular signaling (Ikonen and Vainio, 2005;
Experimental alcohol-related liver injury

Anderson and Borlak, 2008; Inokuchi, 2011) and (b) accumulation of toxic lipids that promote cellular pathology via increased oxidative stress, reactive oxygen species (ROS) generation, adduct formation and ER stress (de la Monte, 2012). Although diacylglycerol, free fatty acids, free cholesterol and ceramides can all have lipotoxic effects in hepatocytes (Malhi and Gores, 2008; Alkhouri et al, 2009), we focused on the potential contributions of ceramides because they: (a) contribute to the pathogenesis of steatohepatitis, irrespective of cause (Lyn-Cook et al, 2009; Tong et al, 2009; Setschedi et al, 2011); (b) promote insulin resistance, inflammation and oxidative stress in various insulin resistance states (Turinsky et al, 1990; Holland et al, 2007; Yang et al, 2009) and (c) impair Akt/PKB signaling through activation of protein phosphatase 2A, which dephosphorylates Akt (Teruel et al, 2001; Ruvolo, 2003) and via inhibition of protein kinase C-ζ-dependent translocation of Akt to the plasma membrane, which is required for its function (Powell et al, 2003). Since the Akt pathway was strikingly inhibited in our model, it is possible that abnormal ceramide accumulation in liver contributed to this effect.

Livers of chronic ethanol-fed rats had increased expression of multiple ceramide-related genes, and higher levels of ceramide immunoreactivity. The significance of these responses is underscored by the constitutive up-regulation of ceramide genes in several pathways. The increased levels of CERS4 and SPTLC2 expression would have contributed to hepatic ceramide accumulation via biosynthetic mechanisms, whereas the increased expression of SMPD1 would have promoted hepatic ceramide accumulation via catabolic mechanisms. It is noteworthy that similar alterations in gene expression have been observed in other models of chronic steatohepatitis with hepatic insulin resistance (de la Monte et al, 2009b; Lyn-Cook et al, 2009; Setschedi et al, 2011). One notable discrepancy was that, in contrast to previous reports (Setschedi et al, 2011) and evidence that glycosphingolipids/gangliosides such as GM3 synthase can mediate insulin resistance (Yamashita et al, 2003; Holland et al, 2007; Langeveld and Aerts, 2009), we did not detect increased GM3 synthase expression in our ethanol-exposed model. However, the differences could be accounted for as follows: (a) up-regulation of GM3 synthase occurs in response to TNF-α (Tagami et al, 2002; Kabayama et al, 2005), which was not elevated in our model and (b) GM3 synthase’s role in insulin resistance may be more related to non-alcohol-induced steatohepatitis, leptin deficiency/resistance, effects in adipocytes or chronic feeding with high-fat diets (Kabayama et al, 2007; Setschedi et al, 2011). Since the regulation of ceramide-related genes is still under investigation, it is not possible to know which component of the liver pathology mediated the alterations in mRNA levels observed herein. Nonetheless, the aggregate findings from both the published literature and the present work suggest that in the context of steatohepatitis with insulin resistance, hepatic ceramide accumulation is mediated by the combined effects of increased enzymatic activity and up-regulated gene expression.

We extended our analyses by characterizing ceramide profiles in liver tissue using LC-MS/MS. The rationale was that: (a) ceramides associated with specific fatty acids can produce diverse biological functions and may be generated in response to diverse stimuli (Hamun and Obeid, 2008, 2011; Levy and Futerman, 2010) and (b) intracellular partitioning of fatty acids and their metabolites plays a role in mediating insulin resistance and lipotoxicity (Li et al, 2010). Coupling LC with tandem MS enabled us to identify the different ceramide species in liver based on retention time and specific mass transition using synthetic reference standards (Bielawski et al, 2009, 2010).

The LC-MS/MS studies demonstrated that the hepatic ceramide profiles in chronic ethanol-fed rats differed significantly from control due to higher levels of C14:0 and C18:0, and relatively lower levels of C16 (significant) and C20 (trend). Previously, it was shown that increased levels of C16:0 ceramides could promote cell death (Oswa et al, 2005; Seumois et al, 2007), while C18:0 ceramide inhibits cell growth (Koybasi et al, 2004). Mechanistically, CERS1 has a high specificity for C18:0-CoA generating C18:0-ceramide, and CERS2 and CERS4 mainly synthesize C20:0-, C22:0-, C24:1-, C24:0-, C26:1- and C26:0-ceramide. In contrast, shorter chain ceramides like C14:0, C16:0 are primarily generated by CerS5 and CerS6 (Levy and Futerman, 2010). As the field of lipidomics grows, our ability to interpret these results in relation to disease pathogenesis, including insulin resistance, lipotoxicity and inflammation will improve. In addition, ongoing research may lead to strategies for targeting-specific pro-ceramide mediators and reduce the severity and progression of alcohol-related liver injury.

ER stress mediates chronic ethanol-induced steatohepatitis

Insulin resistance, inflammation and ceramide accumulation can promote ER stress, and ER stress exacerbates insulin resistance, inflammation, oxidative stress and ceramide accumulation (Urano et al, 2000; McCullough et al, 2001; Orcan et al, 2004; Carracedo et al, 2006; Sauerne et al, 2010; Senkal et al, 2010; Boslem et al, 2011). Our results demonstrate that steatohepatitis caused by chronic ethanol feeding activates ER stress signaling, as was manifested by the up-regulation of genes at multiple levels in the pathway including, HERPUD, PDI, ATF-4 CHOP and BAX. PDI helps to mediate early stages of the UPR by functioning as a chaperone that escorts mal-folded proteins to the ubiquitin proteasome pathway, and it also directly oxidizes disulfide bonds in folding proteins. HERPUD is an ER stress target gene that mediates ER-associated protein degradation (ERAD) by facilitating protein ubiquitination. Increased ATF-4 expression in ethanol-exposed livers corresponds with the significantly higher levels of chaperones, components of the folding and ERAD machinery, i.e. PDI and HERPUD, and pro-apoptosis mechanisms, i.e. CHOP. Consequences of constitutive activation of the UPR in chronic ethanol-exposed livers include: (a) increased apoptosis via CHOP (Oyadomari and Mori, 2004); (b) disruption of calcium and redox homeostasis (Malhi and Kaufman, 2011) and (c) further deregulation of lipid homeostasis (Werstuck et al, 2001) leading to enhanced production of cholesterol and triglycerides via activation of the transcription factor SREBP. CHOP activates caspases and inhibits Bcl2 pro-survival function by activating BAX. The constitutively up-regulated expression of CHOP and Bax in chronic ethanol-fed rats corresponds with the histopathologic findings of increased necrosis and apoptosis in the livers. Our findings corroborate recent studies showing that ethanol exposure in experimental animals leads
to increased activation of ER stress responses in liver (Ji and Kaplowitz, 2003; Esfandiari et al., 2005; Dara et al., 2011), but also extend the concept by having demonstrated simultaneous disruption of insulin signaling networks and activation of pro-ceramide and pro-inflammatory mechanisms.

**SUMMARY AND CONCLUSIONS**

Experimental chronic alcohol-induced steatohepatitis is associated with impaired insulin and IGF signaling through insulin/IGF receptors, IRS-1, Akt and Akt6 downstream pathways that mediate cell survival and energy metabolism. The molecular and biochemical indices of liver injury, inflammation and stress correlated with the histopathologic and ultrastructural findings, particularly with regard to disruption of the ER structure and increased ER stress. The prominent activation of pro-ceramide mechanisms with ceramide accumulation and altered ceramide profiles, together with increased activation of the UPR via several pathways and at multiple levels of the network underscore the concept that chronic progressive alcohol-related liver disease is mediated by concurrent mal-signaling through multiple inter-connected cascades. We hypothesize that ethanol-induced hepatic insulin resistance activates mal-signaling cascades whereby lipolysis promotes ceramide generation and accumulation in cell membranes including the ER. Ceramide accumulation in the ER promotes ER stress, and ceramide incorporation into lipid rafts exacerbates insulin resistance, inflammation, oxidative stress and pro-apoptosis signaling. Increased oxidative stress leads to ROS formation, lipid peroxidation, DNA damage and cell death. This hypothesis suggests that the treatment of chronic progressive alcohol-related liver disease requires multi-pronged strategies to reverse or stabilize consequences of hepatic insulin resistance and dysregulated lipid metabolism.

**SUPPLEMENTARY MATERIAL**

Supplementary material is available at Alcohol and Alcoholism online.

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