Reducing endoplasmic reticulum stress does not improve steatohepatitis in mice fed a methionine- and choline-deficient diet

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Running head: Reducing ER stress does not improve murine steatohepatitis

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Abstract

Background: Endoplasmic reticulum (ER) stress has been implicated in the pathogenesis of nonalcoholic steatohepatitis. The ER stress response is activated in the livers of mice fed a methionine- and choline-deficient (MCD) diet yet the role of ER stress in the pathogenesis of MCD diet-induced steatohepatitis is unknown. We aim to determine the effects of reducing ER stress using chemical chaperones on hepatic steatosis and markers of inflammation and fibrosis in mice fed a MCD diet. Methods: C57BL/6J mice were fed a MCD diet with or without the ER chemical chaperones, 4-phenylbutyric acid (PBA) or tauroursodeoxycholic acid (TUDCA), for 2 weeks. Results: TUDCA and PBA effectively attenuated the ER stress response in MCD-fed mice as evidenced by reduced protein levels of phosphorylated eIF2-alpha and phosphorylated JNK and suppression of mRNA levels of CHOP, GRP78, and XBP1. However, PBA and TUDCA did not decrease MCD diet-induced hepatic steatosis. MCD diet-induced hepatic inflammation, as evidenced by increased plasma ALT and induction of hepatic tumor necrosis factor (TNF) alpha expression, was also not reduced by PBA or TUDCA. PBA and TUDCA did not attenuate MCD diet-induced upregulation of the fibrosis-associated genes, TIMP-1 or MMP-9. Conclusions: ER chemical chaperones reduce MCD diet-induced ER stress, yet do not improve MCD diet-induced hepatic steatosis, inflammation, or activation of genes associated with fibrosis. These data suggest that although the ER stress response is activated by the MCD diet, it does not have a primary role in the pathogenesis of MCD diet-induced steatohepatitis.

Keywords: unfolded protein response, steatosis, TUDCA, PBA, chemical chaperones
INTRODUCTION

The prevalence of nonalcoholic fatty liver disease (NAFLD) is reaching epidemic proportions in the United States, and is now estimated to affect up to a third of the US population (2). Cirrhosis resulting from NAFLD is expected to surpass hepatitis C as the leading indication for liver transplantation in the United States in the next 10-20 years (15). Unfortunately, there is no approved pharmacologic therapy to prevent the development of hepatic steatosis or the progression to more advanced liver disease. The lack of an effective treatment is due, in part, to the poor understanding of the pathogenesis of this common disease.

Recent data indicate that endoplasmic reticulum (ER) stress may have an important role in the development and/or progression of NAFLD (7, 13, 18, 24). The ER functions to maintain protein homeostasis by regulating protein synthesis, folding and processing. When normal ER function is disturbed, unfolded or misfolded proteins accumulate within the ER, triggering the unfolded protein response (UPR), also known as the ER stress response (29). The ER stress response is mediated through three ER transmembrane receptors, PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 alpha (IRE1α) and one master chaperone, glucose-regulated protein 78kDa (GRP78/BiP). In the unstressed state, GRP78 is bound to these transmembrane receptors. However, when unfolded proteins accumulate in the ER, GRP78 preferentially binds to unfolded proteins, thereby releasing PERK, ATF6, and IRE1α. Release of GRP78 triggers a signaling cascade which initially aims to restore homeostasis and allow the cell to adapt to the cellular stressor; however, if homeostasis is not restored, pathways leading to apoptosis are initiated (10, 23).

Hepatic lipid accumulation induces ER stress and, in turn, the ER stress response promotes hepatic lipogenesis thus creating a positive feedback loop, which may contribute to the development of hepatic steatosis (9, 11, 24). ER stress has been implicated not only in the development of hepatic steatosis but
also in the development of hepatocellular injury and fibrosis, which herald the progression of simple
steatosis to NASH. One of the commonly cited lines of evidence supporting the role of ER stress in the
pathogenesis of NASH is the observation that mice fed a methionine- and choline-deficient (MCD) diet, a
well-established murine model of steatohepatitis, demonstrate activation of the hepatic ER stress
response. MCD diet-induced steatosis is thought to be due, in large part, to impaired hepatic triglyceride
secretion (16, 28). However, the mechanisms underlying the development of steatohepatitis in MCD-fed
mice remain incompletely understood. Whether ER stress promotes steatohepatitis in mice fed a MCD
diet is unknown.

We aim to establish the role of ER stress in the pathogenesis of MCD diet-induced steatohepatitis in mice.
Tauroursodeoxycholic acid (TUDCA) and 4-phenylbutyric acid (PBA) are chemical chaperones that have
been shown to reduce ER stress by facilitating proper protein folding and trafficking (22, 26). Inhibiting
ER stress using these chemical chaperones in a murine model of hepatic steatosis has been shown to
reduce hepatic steatosis, suggesting that modulating the ER stress response may serve as a therapeutic
strategy for NAFLD (12). In the present study we will determine the effects of ER chemical chaperones
on the development of hepatic steatosis, inflammation, and fibrosis in mice fed a MCD diet.

MATERIALS AND METHODS

Animals and Diets

Male C57BL/6J mice (8-10 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME).
Mice were fed a MCD or methionine- and choline-sufficient (MCS) diet (MP Biomedical, Solon, OH) for
14 days. The MCS diet is identical to the MCD diet except for the content of methionine and choline.
MCD and MCS-fed mice were treated with either 4-phenylbutyric acid (PBA, Sigma-Aldrich, St. Louis,
MO, 200mg/kg/day I.P.), tauroursodeoxycholic acid (TUDCA, Calbiochem, LaJolla, CA, 500 mg/kg/day
I.P.) or vehicle (sterile saline I.P.) daily during the feeding protocol. Mice underwent 14/10-hour
light/dark cycling and were given free access to food and water. Mice were fasted for 4 hours prior to
sacrifice and were euthanized by CO₂ inhalation. Blood was collected by cardiac puncture and
centrifuged to collect the plasma. The livers were rapidly excised, weighed, and flushed with ice-cold
saline. An aliquot was fixed in 10% formalin for histologic analysis which was performed at the
Northwestern University Pathology Core (Chicago, IL). The remainder of the liver was sectioned and
snap-frozen in liquid nitrogen. All animal protocols were approved by the Northwestern University
Animal Care and Use Committee (ACUC).

Liver and Plasma Chemistries
Liver samples were homogenized in Dulbecco’s phosphate buffered saline for hepatic lipid analysis
(100mg liver tissue/1mL). Triglyceride and cholesterol levels were measured in liver homogenate and
fresh plasma using an Infinity spectrophotometric assay per the manufacturer’s protocol (Thermo
Electron Corporation, Melbourne, Australia). Plasma ALT was measured using a spectrophotometric
assay per the manufacturer’s protocol (Teco Diagnostics, Anaheim, CA).

Analysis of Gene Expression by Real-time Quantitative PCR
Total RNA from frozen liver samples was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Two
micrograms of total RNA was used for reverse transcription PCR using a qScript cDNA Synthesis Kit
(Quanta BioSciences, Gaithersburg, MD). Real-time quantitative PCR was performed using 2 µL of
cDNA from each sample in a 25 µL reaction mixture containing Quantitect SYBR Green PCR Mastermix
(Qiagen, Valencia, CA) along with primers specific for the gene of interest. GAPDH was employed as a
housekeeping gene. Amplification was performed on an ABI 7300 sequence detector (Applied
Biosystems, Foster City, CA). Gene expression was calculated relative to respective age and gender
matched controls using the comparative threshold cycle method as described in the Applied Biosystems
Sequence Detection Systems instruction guide.
**Analysis of Protein Expression by Western Blot**

Liver samples were homogenized in T-Per (Thermo Scientific, Rockford, IL) containing Halt phosphatase inhibitor (Thermo Scientific) and protease cocktail inhibitor (Calbiochem). Protein concentrations of homogenates were determined by the Bradford assay using Coomassie Blue reagent (Thermo Scientific) and subsequently diluted with Laemmli buffer (BioRad, Hercules, CA) containing beta-mercaptoethanol to a standard concentration of 2 µg/µL and heated at 95°C for 5 minutes. Samples containing 50 µg of protein were separated on a 12% SDS polyacrylamide gel by electrophoresis. Protein was then transferred to a nitrocellulose membrane by electrophoresis. Protein detection was performed using polyclonal rabbit antibodies to total and phosphorylated eIF-2α and JNK (Cell Signaling Technology, Danvers, MA). Bound antibody was detected using goat anti-rabbit polyclonal HRP antibody (Santa Cruz Biotechnology) and developed using ECL Western Blotting Substrate (Thermo Scientific). Representative Western blots of pooled samples are shown.

**Statistical Analysis**

Data are presented as mean ± standard deviation (SD). Comparisons between groups were performed using Student’s t-test analysis.

**RESULTS**

**Chemical chaperones inhibit the ER stress response in mice fed a MCD diet**

The livers of mice fed a MCD diet for 14 days showed significant activation of the UPR. Specifically we found robust activation of the PERK targets, eukaryotic translation initiation factor (eIF) 2α (Figure 1A) and C/EBP homologous protein (CHOP) (Figure 1C). Additionally, MCD feeding significantly activated
the IRE1α target, JNK (Figure 1B). There was a trend toward increased expression of the IRE1α target, spliced X-box binding protein 1 (XBP1s) and the UPR master regulator, GRP78 with MCD feeding (Figure 1D, E). Consistent with their established role as inhibitors of the ER stress response, TUDCA and PBA suppressed the expression of p-eIF2α, p-JNK, CHOP, XBP1s, and GRP78 in mice fed a MCD diet (Figure 1). Among MCS-fed mice, the chemical chaperones did not alter the expression of UPR markers with the exception of modest activation of eIF2α in response to PBA.

Effect of chemical chaperones on phenotypic sequelae of the MCD diet

We next assessed whether reducing ER stress in MCD-fed mice prevents the classic phenotypic sequelae of the MCD diet. Weight loss is a well-established consequence of MCD-feeding (19). Consistent with prior studies, MCD-feeding resulted in a 25% reduction in body weight during the feeding protocol (Table 1). Treatment with TUDCA or PBA did not prevent MCD diet-induced weight loss. Consistent with the induction of steatohepatitis, the MCD diet results in significant elevation in plasma ALT level. We determined that MCD-fed mice demonstrated a >20-fold increase in plasma ALT level compared to MCS-fed controls (259 ± 119 vs 11 ± 6 U/L, p<0.001) (Table 1). TUDCA and PBA did not attenuate the MCD diet-induced elevation in plasma ALT (240 ± 54 and 303 ± 163 in MCD+TUDCA and MCD+PBA vs 259 ± 119 U/L in MCD, NS). Treatment with PBA and TUDCA had no effect on plasma ALT in MCS-fed mice. Hypoglycemia and a reduction in plasma cholesterol level are additional known sequelae of MCD feeding (7, 17). The MCD, MCD+TUDCA, and MCD+PBA cohorts all showed reductions in blood glucose levels relative to their respective MCS controls (Table 1). The MCD diet caused a reduction in plasma total cholesterol, which was not attenuated by TUDCA or PBA.

Chemical chaperones do not reduce MCD diet-induced hepatic steatosis

We next sought to determine whether inhibition of ER stress translates to a reduction in MCD diet-induced hepatic steatosis. Mice fed a MCD diet showed macrovesicular steatosis on hematoxylin and
eosin (H&E) staining of liver samples (Figure 2A). There was no significant reduction in the degree of steatosis in mice treated with MCD+TUDCA or MCD+PBA compared to mice fed the MCD diet alone. Accordingly, quantification of the hepatic triglyceride and cholesterol level showed that MCD diet-induced hepatic lipid accumulation was not attenuated by TUDCA or PBA treatment (Figure 2 B,C). In fact, the MCD+TUDCA cohort showed a greater increase in hepatic triglyceride content compared to the MCD cohort. Given the increase in hepatic triglyceride content, we examined whether TUDCA regulates genes involved in hepatic triglyceride synthesis and/or degradation. Consistent with previous reports, the MCD diet caused suppression in sterol regulatory element binding protein-1c (SREBP-1c) and stearoyl-coenzyme A desaturase-1 (SCD-1), genes involved in triglyceride synthesis (4, 19) (Table 2). MCD-fed mice treated with TUDCA also demonstrated suppression of these genes indicating that the observed increase in hepatic triglyceride content in this cohort is not due to increased triglyceride synthesis. The MCD+TUDCA cohort showed an increase in expression the fatty acid oxidation genes, fatty acyl-CoA oxidase (AOX) and carnitine palmitoyltransferase-1 (CPT-1), which may be a compensatory response to the increased hepatic triglyceride content.

Effect of chemical chaperones on markers of hepatic inflammation and fibrosis

Hepatic inflammation and fibrosis are features that distinguish simple steatosis from steatohepatitis. TNFα is a proinflammatory cytokine that is critically important in the pathogenesis of numerous inflammatory liver diseases including NASH (25, 27). We found that the expression of TNFα mRNA was increased nearly 8-fold by MCD feeding. Chemical chaperones did not attenuate the expression of TNFα in MCD-fed mice (Figure 3A).

The MCD diet induces significant hepatic fibrosis by four to eight weeks of feeding depending on the murine strain (8, 21). As expected we did not find significant fibrosis present histologically at 2 weeks, however, even by 2 weeks, we found that the MCD diet induced expression of genes associated with the
development of fibrosis including TIMP-1 and MMP-9. TUDCA and PBA did not attenuate the MCD diet-induced upregulation of these fibrosis-associated genes (Figure 3B, C).

**DISCUSSION**

ER stress may have an essential role in the pathogenesis of NAFLD. One of the frequently cited lines of evidence supporting this assertion is the observation that MCD feeding to rodents is associated with activation of the ER stress response (6, 7, 14). However, it has never been proven that ER stress promotes MCD diet-induced steatohepatitis. We now show that reducing ER stress in mice fed a MCD diet does not result in a reduction in hepatic steatosis, inflammation, or fibrosis. The present work indicates that although components of the UPR are upregulated by MCD-feeding, ER stress may not play a primary role in the pathogenesis of MCD diet-induced steatohepatitis.

It must be considered, however, that chemical chaperones ameliorate, but do not completely prevent, ER stress. Therefore, we cannot exclude the possibility that low levels of ER stress, as observed in MCD-fed mice treated with chemical chaperones, are sufficient to induce steatohepatitis. Arguing against this hypothesis, however, is the observation that some of the negative sequelae of the MCD diet were actually exacerbated by the introduction of chemical chaperones. Most notably, MCD diet-induced hepatic triglyceride accumulation was worsened by the administration of TUDCA, which could not be attributed to enhanced hepatic lipogenesis or suppressed fatty acid oxidation. This may suggest that the ER stress response has a protective role in the setting of methionine and choline deficiency. Alternatively, TUDCA and PBA do not function exclusively as inhibitors of the ER stress response, and it is possible that modulation of other physiologic processes may be causing these untoward effects.

The accumulation of saturated fat and cholesterol in the liver is an established trigger of ER stress (3, 5, 24). It is, therefore, plausible that MCD diet-induced ER stress may be a consequence rather than a cause
of the hepatic lipid accumulation observed in this dietary model. Furthermore, it has been shown that the
ER stress response induced by saturated fatty acids is characterized by a preferential induction of PERK
signaling (3). We found that the MCD diet most robustly activated eIF-2α and CHOP, which are
components of the PERK signaling pathway. As such, if induction of hepatic ER stress by a MCD diet is
due to lipid accumulation in the liver, this may explain the disproportionate induction of PERK signaling.

It is noted, however, that eIF-2α is activated not only by the PERK arm of the UPR but also by general
control nonderepressible 2 (GCN2), a component of an “integrated stress response” (1, 20). It has
previously been shown that MCD feeding activates both PERK and GCN2 (14, 20) and the relative
importance of ER stress versus an integrated stress response in MCD diet-related eIF-2α signaling is
unclear. The fact that ER chemical chaperones attenuate MCD diet-induced activation of eIF-2α, as
shown in this study, highlights the importance of ER perturbation in MCD diet-induced eIF-2α
phosphorylation.

The effect of a MCD diet on the IRE1α pathway of the UPR is controversial. In the present study we
found an insignificant increase in the spliced transcript of XBP1 with MCD feeding indicating minimal
activation of the IRE1α pathway. Others have shown transient activation of hepatic XBP1 early in the
course of MCD feeding followed by sustained suppression (20). In our prior work, we have shown a 2-
fold elevation in hepatic XBP1s in female FVB mice fed a MCD diet (7). However, MCD feeding to
female FVB mice also induced a more severe degree of steatohepatitis than in the current study using
male C57BL/6J mice. Rinella et al recently showed that after 4 weeks of MCD feeding db/db mice
showed no induction of hepatic XBP1s, whereas db/m controls showed a >2-fold induction of XBP1s
mRNA (18). As such, we can conclude that induction of the IRE1α pathway in the MCD model is highly
dependent on the length of feeding and mouse strain.
The present work challenges the notion that ER stress promotes the development of MCD diet-induced steatohepatitis. However, our observations do not challenge the assertion that ER stress plays a role in the pathogenesis of human NAFLD. Although the MCD diet produces histologic findings similar to human NASH, this model is not associated with the classic systemic manifestations associated with human NAFLD, most notably obesity and insulin resistance. Instead, MCD-fed mice lose significant amounts of weight and become more insulin sensitive. As such it would not be surprising if the pathogenesis of MCD diet-induced steatohepatitis does not exactly parallel the mechanisms governing the progression of human NAFLD. It has been previously shown that human subjects with NAFLD demonstrate activation of the UPR in the liver, and the significance of this finding warrants further investigation to establish whether there is a causal relationship (13).

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Disclosures: The authors have no conflicts of interest to disclose
References:


15. **Rinella ME.** Will the increased prevalence of nonalcoholic steatohepatitis (NASH) in the age of better hepatitis C virus therapy make NASH the deadlier disease? *Hepatology* 54: 1118-1120.


Figure legends:

Figure 1: The effect of chemical chaperones on markers of ER stress in mice fed a MCD diet. Hepatic protein expression of A) total and phosphorylated eIF2α and B) total and phosphorylated JNK, and hepatic gene expression of C) CHOP, D) XBP1s, and E) GRP78 in C57BL/6J mice fed a MCS or MCD diet ± vehicle, PBA, or TUDCA for 14 days. Representative Western blots of pooled samples (n=4). Gene expression is relative to MCS-fed mice treated with vehicle, mean (n=5) ± SD. *p<0.05 vs vehicle-injected MCS-fed mice, ^p<0.05 vs vehicle-injected MCD-fed mice.

Figure 2: The effect of chemical chaperones on hepatic lipid accumulation in mice fed a MCD diet. A) Hematoxylin and eosin staining of liver samples, B) hepatic triglyceride levels, and C) hepatic cholesterol levels in C57BL/6J mice fed a MCS or MCD diet ± TUDCA or PBA for 14 days. Mean (n=5) ± SD. *p<0.05 relative to MCS-fed mice treated with the same I.P injection, ^p<0.05 relative to vehicle-injected MCD-fed mice.

Figure 3: Effect of chemical chaperones on the hepatic expression of genes associated with inflammation and fibrosis in mice fed a MCD diet. Quantitative PCR analysis of A) TNFα, B) TIMP-1, C) MMP-9 in C57BL/6J mice fed a MCS or MCD diet ± TUDCA or PBA for 14 days. Expression is relative to vehicle-injected MCS-fed mice, mean (n=5) ± SD. *p<0.05 relative to MCS-fed mice treated with the same I.P injection.
Table 1: Body weight change, blood glucose, and plasma total cholesterol in C57BL/6J mice fed a MCD or MCS diet ± PBA or TUDCA for 14 days

<table>
<thead>
<tr>
<th>Diet</th>
<th>Treatment</th>
<th>Body weight change (%)</th>
<th>Blood glucose (mg/dL)</th>
<th>Plasma total cholesterol (mg/dL)</th>
<th>Plasma ALT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCS</td>
<td>Vehicle</td>
<td>-0.5 ± 5.5</td>
<td>208 ± 8</td>
<td>159 ± 27</td>
<td>11 ± 6</td>
</tr>
<tr>
<td>MCS</td>
<td>TUDCA</td>
<td>-6.6 ± 7.7</td>
<td>203 ± 19</td>
<td>139 ± 16</td>
<td>13 ± 10</td>
</tr>
<tr>
<td>MCS</td>
<td>PBA</td>
<td>-12.7 ± 4.7 (^a)</td>
<td>173 ± 43</td>
<td>98 ± 44</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>MCD</td>
<td>Vehicle</td>
<td>-25.3 ± 1.6 (^a)</td>
<td>110 ± 31 (^a)</td>
<td>73 ± 30 (^a)</td>
<td>259 ± 119 (^a)</td>
</tr>
<tr>
<td>MCD</td>
<td>TUDCA</td>
<td>-30.9 ± 1.8 (^a,b,c)</td>
<td>72 ± 14 (^a,b,c)</td>
<td>73 ± 8 (^a,b,c)</td>
<td>239 ± 54 (^a,b,c)</td>
</tr>
<tr>
<td>MCD</td>
<td>PBA</td>
<td>-27.9 ± 0.2 (^a,b,c)</td>
<td>128 ± 12 (^a,b,c)</td>
<td>59 ± 13 (^a,b,c)</td>
<td>303 ± 63 (^a,b,c)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD (n=5)

\(^a\) *p*<0.05 versus MCS + vehicle

\(^b\) *p*<0.05 versus MCD + vehicle

\(^c\) *p*<0.05 versus MCS-fed mice of the same treatment (TUDCA or PBA)
Table 2: Expression of genes associated with triglyceride synthesis and oxidation in C57BL/6J mice fed a MCD diet +/- TUDCA for 14 days

<table>
<thead>
<tr>
<th></th>
<th>MCS (control) diet</th>
<th>MCD diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>TUDCA</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>1.13 ± 0.63</td>
<td>0.55 ± 0.08</td>
</tr>
<tr>
<td>SCD-1</td>
<td>1.04 ± 0.34</td>
<td>0.73 ± 0.31</td>
</tr>
<tr>
<td>AOX</td>
<td>1.01 ± 0.20</td>
<td>1.69 ± 0.45</td>
</tr>
<tr>
<td>CPT-1</td>
<td>1.09 ± 0.51</td>
<td>1.36 ± 0.26</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD (n=5)

\(^a p<0.05\) versus vehicle-treated mice on the same diet (MCS or MCD)

\(^b p<0.05\) versus MCS-fed mice of the same treatment (TUDCA or PBA)
Figure 1

A) Relative CHOP expression

B) Relative GRP78 expression

C) Relative XBP1s expression

D) vehicle TUDCA PBA

E) Relative GRP78 expression
Figure 2

A) Vehicle | TUDCA | PBA

MCS

MCD

B) Hepatic triglyceride content (mg trig/g protein)

C) Hepatic cholesterol content (mg chol/g protein)
Figure 3

A) Relative TNFα expression

B) Relative TIMP-1 expression

C) Relative MMP9 expression