Precision-cut liver slices from diet-induced obese rats exposed to ethanol are susceptible to oxidative stress and increased fatty acid synthesis

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Duryee MJ, Willis MS, Schaffert CS, Reidelberger RD, Dusad A, Anderson DR, Klassen LW, Thiele GM. Precision-cut liver slices from diet-induced obese rats exposed to ethanol are susceptible to oxidative stress and increased fatty acid synthesis. Am J Physiol Gastrointest Liver Physiol 306: G208–G217, 2014. First published December 5, 2013; doi:10.1152/ajpgi.00124.2013.—Oxidative stress from fat accumulation in the liver has many deleterious effects. Many believe that there is a second hit that causes relatively benign fat accumulation to transform into liver failure. Therefore, we evaluated the effects of ethanol on ex vivo precision-cut liver slice cultures (PCLS) from rats fed a high-fat diet resulting in fatty liver. Age-matched male Sprague-Dawley rats were fed either high-fat (obese) (45% calories from fat, 4.73 kcal/g) or control diet for 13 mo. PCLS were prepared, incubated with 25 mM ethanol for 24, 48, and 72 h, harvested, and evaluated for ethanol metabolism, triglyceride production, oxidative stress, and cytokine expression. Ethanol metabolism and acetaldehyde production decreased in PCLS from obese rats compared with age-matched controls (AMC). Increased triglyceride and smooth muscle actin production was observed in PCLS from obese rats compared with AMC, which further increased following ethanol incubation. Lipid peroxidation, measured by thiobarbituric acid reactive substances assay, increased in response to ethanol, whereas GSH and heme oxygenase I levels were decreased. TNF-α and IL-6 levels were increased in the PCLS from obese rats and increased further with ethanol incubation. Diet-induced fatty liver increases the susceptibility of the liver to toxins such as ethanol, possibly by the increased oxidative stress and cytokine production. These findings support the concept that the development of fatty liver sensitizes the liver to the effects of ethanol and leads to the start of liver failure, necrosis, and eventually cirrhosis.

alcohol liver disease; fatty liver disease; obesity; oxidative stress; precision-cut liver slices

MACROVESICULAR STEATOSIS, due to long-chain fatty acid accumulation in liver cells, is the result of inadequate oxidation and transport of lipids out of the liver, most commonly seen in patients with obesity, alcoholic liver disease, insulin resistance, and inherited metabolic disorders (2, 35, 39). Although it is initially benign, additional insults can incite liver necrosis, leading to fibrosis, scarring, and hepatocellular carcinoma. This underlying insult has been hypothesized to be the second hit needed to push the liver to cirrhosis (15). Insults include, but are not limited to, increases in fatty acid oxidation, oxidative stress, free radical generation, gut-derived endotoxin increasing inflammation, and higher cytochrome P-450 2E1 (CYP2E1) expression in the liver (12, 17, 23, 36, 46).

Alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD) have many of the same features, although they are histologically distinct and NAFLD is not associated with alcohol abuse (4, 40, 42). Gut-derived bacteria have been hypothesized to initiate a “second hit” in both ALD and NAFLD, inciting cytokine release and the production of reactive oxygen species (ROS) in the liver (6, 16, 47). High-fat meals alone alter gut microbiota composition and result in the subsequent increase in endotoxin levels, likely by increasing gut permeability, allowing endotoxin to gain access to the vasculature (30). Similarly, increased gut permeability has been proposed as a source of endotoxin in the pathogenesis of ALD (12, 26). Treating animals fed a high-fat diet with antibiotics alone can attenuate the resulting steatosis in the liver (7).

A common theme of both ALD and NAFLD is an increase in ROS and the induction of CYP2E1 (32, 36). Increased ROS results in enhanced lipid peroxidation and damage to cellular membranes and mitochondria (36), eventually leading to cell death. Damage to cells in this magnitude has been hypothesized to occur secondarily to the oxidative posttranslational modification of “self” proteins that could trigger an autoimmune response against liver proteins (36, 45).

A diversity of animal feeding models for ALD and NAFLD have been developed [recently reviewed by Takahashi et al. (44) and Arteel (1), respectively]. Although these models have provided insight into the complex interactions of oxidative stress and endotoxin on the liver, none develop end-stage liver disease mimicking human pathology. In contrast, recent studies have reported an ex vivo model of alcoholic liver fibrosis using precision-cut liver slices (PCLS) that develop fibrosis comparable to that seen in human disease (28, 41).

Whereas multiple in vivo animal models of NAFLD have been described (24, 31, 43, 44), only a few in vitro studies using cell lines or isolated liver cells have been reported (10, 21, 27). Most investigators agree that heavy alcohol consumption on an already fatty liver would be detrimental. This is the case in both patients and animals that already have fatty liver.
then binge drink or consume high amounts of ethanol (8, 11, 33). In these studies oxidative stress and inflammation is increased providing greater stress on the liver. However, animal models sometimes are limited by how much alcohol they consume, the actual amount of liver damage present, or the contributions of other cells and biological intermediates. Therefore, the purpose of this study was to evaluate ex vivo PCLS cultures from rats with diet-induced fatty liver incubated with ethanol. We hypothesize that incubating ethanol with PCLS from high-fat liver will be the second hit needed to increase oxidative stress and induce damage to the liver.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (225–350 g) were purchased from Sasco Charles River (Portage, MI) and housed in a temperature controlled room (19–21°C) with a 12:12-h light-dark cycle (lights off at 1700). Rats were provided pelleted rat chow (Labdiet, 5001 Rodent Diet; PMI Nutrition International) and water ad libitum. All procedures were approved by the Animal Subcommittee of the Omaha VA Medical Center and are in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals.

Induction of obesity. Animals were provided over a 13-mo period both a high-fat pelleted food (45% calories from fat, 4.73 kcal/g, D12451; Research Diets, New Brunswick, NJ) and vanilla Ensure Plus liquid food (29% calories from fat, 1.5 kcal/ml; Ross Nutrition, Abbott Laboratories, Columbus, OH). These rats are defined as obese rats, and the subsequent age-matched controls are defined as the AMC group in this study. There appears to be no consensus in defining obesity in rats, although percent body fat above 20% body weight is most likely considered obese (5). Therefore, an EchoMRI-700 quantitive nuclear magnetic resonance (QMR) analyzer (Echo Medical Systems, Houston, TX) was used to measure total fat mass and lean mass (not including skeletal mass) in the rats at monthly intervals.

Liver pathology. Livers extracted from AMC and obese rats were placed in 10% formalin PBS for 48 h and embedded in paraffin. Sections were stained with hematoxylin and eosin and blindly scored by a pathologist.

Serum endotoxin levels. Serum from obese and AMC rats was collected to assay for the presence of endotoxin by using a Chromogenic Limulus amebocyte lysate (LAL) Endotoxin Assay Kit (GenScript, Piscataway, NJ). Assays were run according to manufacturers’ directions and analyzed by use of an MRX II plate reader with Revelation Software (Dynex Technologies, Chantilly, VA).

Precision-cut liver slices. PCLS were prepared as previously described (28) from obese rats weighing 700–800 g and AMC rats 550–650 g. Briefly, rats were anesthetized with isoflurane, and the liver was excised and then placed into oxygenated V-7 cold preservation buffer (Vitron, Tucson, AZ). Cylindrical tissue cores (8 mm) were cut with a hand-held coring tool, loaded into the Vitron Tissue Slicer (Vitron). Slices (250 μm thickness) were cut with a 45-mm rotary blade and floated into ice-cold oxygenated V-7 preservation buffer. Slices were equilibrated by gentle shaking in serum-free Williams E Medium (Sigma Chemical, St. Louis, MO) containing d-glucose and gentamicin (WEGG) under 95% O2-5% CO2 (carboxen) at 37°C for 30 min. Some slices were processed at this point and were designated as time 0. The remaining slices were floated onto titanium screen-containing rollers from Vitron. These rollers were inserted into sterile 20-ml glass vials containing 1.7 ml of serum-free WEGG medium or WEGG medium containing 25 mM ethanol. The vials were capped with lids containing a 1-mm hole for oxygen transfer. Vials were placed into the dynamic organ culture incubator from Vitron and were incubated at 37°C with carbogen (1.5 l/min). The medium was replenished every 24 h up to 72 h.

Ethanol metabolism and viability measurements. Ethanol metabolism and acetaldehyde (AA) production were measured in media at each time point by headspace gas chromatography as described previously (18). Viability was determined by measuring lactate dehydrogenase (LDH) activity in the slices and corresponding media from each time point using the Cytotoxicity Detection Kit (LDH) (Roche Applied Science, Indianapolis, IN) as per manufacturer’s directions.

ADH/ALDH activity. Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) enzyme levels were determined by lysing slices at appropriate time points, subjecting them to 10 mM ethanol/3 mM NAD+/0.5 M Tris-HCl (pH 7.4), and detecting at 340 nm wavelength as previously described (13).

TG analysis. At indicated times, PCLS were placed in PBS containing 0.5% Triton X-100, sonicated, and assayed for triglycerides (TGs; 100–200 μg) by using a serum TG kit according to the manufacturer instructions (Sigma Chemical).

GSH assay. At indicated times, PCLS were rinsed with PBS, pH 7.2 and assayed for total cellular glutathione using a total glutathione detection system from Assay Designs (Ann Arbor, MI). Briefly, PCLS were sonicated on ice in PBS, pH 7.2 (150 μl PBS/slice), precipitated with metaphosphoric acid, and centrifuged at 14,000 g 10 min at 4°C. The clarified supernatant was used in the GSH assay using an MRXII plate reader with Revelation Software (Dynex Technologies). Cellular GSH concentrations were calculated against a GSH standard and were normalized to the total protein detected in the samples.

Lipid peroxidation. PCLS were analyzed for lipid peroxidation by using the thiobarbituric acid reactive substances assay (TBARS) system from Cayman Chemical (Ann Arbor, MI). Briefly, PCLS were lysed with PBS-RIPA buffer [PBS, pH 7.4, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM Na-EDTA, and 5 μl/mle protease inhibitor cocktail (Sigma Chemical)]. The supernatant was assayed for protein concentration and TBARS. Samples (30 μl) and malondialdehyde (MDA) standards were incubated in a thiobarbituric acid solution for 1 h in a boiling water bath according to the manufacturer’s instructions. Samples and standards were placed in a microtiter plate and the absorbance at 540 nm was measured. The amount of MDA was calculated against the standard curve and was normalized to the amount of protein in each sample.

CYP2E1 and HO-1. Twenty-five micrograms of PCLS were resolved under reducing conditions on a 10% SDS-PAGE to detect CYP2E1 and hem oxygenase-1 (HO-1). Proteins were transferred to Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA), blocked 30 min in Odyssey blocking buffer (LI-COR) at 37°C, and incubated with anti-CYP2E1 rabbit polyclonal antibody (1:5,000, EMD Biosciences, San Diego, CA) or anti-HO-1 rabbit polyclonal antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), followed by IRDye-conjugated anti-rabbit secondary antibodies (1:20,000, LI-COR). Blots were scanned with an Odyssey IR Scanner (LI-COR). Anti-tubulin (1:4,000, Sigma Chemical) was used as loading control.

Cytokine detection. Detection of IL-6, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) in media collected at 24, 48, 72 and 96 h of incubation was performed by using OpTEIA ELISA kits (Pharmingen, Palo Alto, CA) according to the manufacturer’s instructions. Assays were evaluated for reactivity at 450 nm (570 nm correction) on a MRXII plate reader with Revelation Software (Dynex Technologies).

Real-time PCR. RNA was extracted from PCLS by use of the RNeasy Mini Kit Qiagen (Valencia, CA). Sample concentration was determined by using a Nanodrop 2000c (Thermo Scientific, Waltham, MA) and adjusted to equal concentrations. cDNA was made using a High Capacity cDNA Archive system from Applied Biosystems...
RT-PCR was performed with TaqMan primers (Collagen Iα1, Timp-1, IL-1β, TNF-α, and IL-6) and amplified with a 7500 Real-Time PCR System from Applied Biosystems. Message levels were analyzed with Applied Biosystems software. All samples were normalized to their corresponding preincubated PCLS.

**Statistical analysis.** Results are expressed as means ± SE. Statistical significance was achieved if *P* values were less than 0.05. All statistical analysis was performed by using the Sigma Plot 10.0 with SigmaStat (Jandel Scientific, 2006) program and one-way or multiple analysis of variance (ANOVA) as indicated.

**RESULTS**

To determine how both metabolic syndrome and alcohol consumption affect the liver, rats were fed a high-fat diet similar to the Western diet, resulting in obesity (33% body fat vs. 14% in AMC, Fig. 1A, *P* < 0.001) determined by QMR. Previous studies have demonstrated that patients with metabolic syndrome have increased levels of serum LPS (6, 7, 26, 30). To identify whether our obese rat model similarly had serum LPS, presumably from increased gut permeability, we assayed for LPS and identified a significant increase in the serum from obese rats (1.28 EU/ml) compared with AMC (0.46 EU/ml, *P* < 0.002) (Fig. 1B). Blinded histological analysis of liver sections from AMC and obese rats revealed that AMC livers had no evidence of pathology (*N* = 4; Fig. 2A), whereas livers from obese rats illustrated a diffuse periportal (zone 1) microvesicular steatosis (*N* = 4; Fig. 2B). No evidence of inflammation beyond the rare chronic leukocytes was seen.

To determine how livers from either obese or AMC rats metabolized ethanol, PCLS from these rats were incubated with ethanol. Ethanol metabolism by PCLS from AMC and obese rats decreased significantly from baseline levels over the 72 h assayed (Table 1, *P* < 0.001). Small yet significant differences in ethanol metabolism between PCLS from obese and AMC rats were detected at 48 h (*P* < 0.04). As expected, parallel increases in acetaldehyde (AA) were detected in PCLS from both obese and AMC rats over time (Table 1, *P* < 0.001), with PCLS from obese rats producing significantly less AA at 48 h (*P* < 0.04). To determine whether differences in ethanol and AA detected were due to changes in viability over time, parallel LDH release was investigated (Table 1). Changes in viability were observed at 48 and 72 h, as previously reported, and at no time were significant differences in viability between PCLS from AMC or obese rats observed. ADH and ALDH were looked at to determine their involvement in the decrease in ethanol metabolism seen in the obese rat PCLS. There was no significant difference in ADH when comparing the obese and AMC ethanol-fed PCLS. However, there was a significant decrease in the ALDH at 24 and 48 h when comparing the AMC control and ethanol-fed PCLS to the

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**Fig. 1.** Percent body fat and serum endotoxin levels in rats fed a high-fat diet for 13 mo. Rats were examined for percent body fat by MRI (A) or serum endotoxin levels using an endotoxin assay kit (B). Data expressed are ± SE. Percent body fat significantly increased in obese rats compared with age-matched controls (AMC) (*P* < 0.001) (*N* = 6). Endotoxin significantly increased in obese rats compared with AMC (*P* = 0.002) (*N* = 6).

**Fig. 2.** Livers from obese or AMC rats were excised and subjected to hematoxylin and eosin staining and analyzed for presence of fat by a pathologist. A: AMC; B: obese rats. There appears to be damage in the hepatic portal vein. Images are a representation of all the animals in the study; ×10 magnification.
Table 1. Viability and biochemical metabolism data in response to incubation with 25 mM ethanol in AMC or obese rat
PCLS

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Cytoxicity</th>
<th>Ethanol Metabolized, mmol·24 h⁻¹·mg of protein⁻¹</th>
<th>Acetaldehyde Production/24 h, μmol</th>
<th>ADH Activity, mmol·min⁻¹·mg protein⁻¹</th>
<th>Total ALDH Activity, mmol·min⁻¹·mg protein⁻¹</th>
<th>CYP2E1 Expression, counts/μg protein</th>
</tr>
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<tbody>
<tr>
<td>T-0 AMC</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T-0 Obese</td>
<td>0</td>
<td></td>
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<tr>
<td>AMC control 24 h</td>
<td>19.69 ± 2.60</td>
<td>9.76 ± 2.30</td>
<td>6.84 ± 0.43</td>
<td>1008.10 ± 38.31</td>
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<tr>
<td>AMC control 48 h</td>
<td>31.70 ± 4.06</td>
<td>7.79 ± 1.80</td>
<td>4.17 ± 0.17</td>
<td>613.60 ± 31.71</td>
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<tr>
<td>AMC control 72 h</td>
<td>49.0 ± 5.46</td>
<td>7.04 ± 2.01</td>
<td>2.54 ± 0.19</td>
<td>546.0 ± 66.78</td>
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<tr>
<td>AMC control 84 h</td>
<td>59.95</td>
<td>7.76 ± 2.09</td>
<td>3.64 ± 0.24</td>
<td>990.50 ± 59.24</td>
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<tr>
<td>AMC ethanol 24 h</td>
<td>33.39 ± 3.20</td>
<td>30.20 ± 0.90</td>
<td>86.0 ± 4.12</td>
<td>40.8 ± 1.53</td>
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<tr>
<td>AMC ethanol 48 h</td>
<td>37.19 ± 3.07</td>
<td>9.20 ± 5.01</td>
<td>102.0 ± 6.63</td>
<td>3.25 ± 1.33</td>
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<tr>
<td>AMC ethanol 72 h</td>
<td>49.19 ± 2.83</td>
<td>14.22 ± 0.79</td>
<td>66.78 ± 2.13</td>
<td>1.61 ± 0.33</td>
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<tr>
<td>Obese control 24 h</td>
<td>15.11 ± 0.75</td>
<td>8.42 ± 2.49</td>
<td>2.10 ± 0.49</td>
<td>401.10 ± 50.36</td>
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<tr>
<td>Obese control 48 h</td>
<td>25.70 ± 3.76</td>
<td>8.44 ± 3.33</td>
<td>1.42 ± 0.67</td>
<td>388.30 ± 32.95</td>
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<tr>
<td>Obese control 72 h</td>
<td>44.75 ± 5.26</td>
<td>6.48 ± 3.74</td>
<td>1.62 ± 0.51</td>
<td>320.50 ± 59.95</td>
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<tr>
<td>Obese ethanol 24 h</td>
<td>11.44 ± 0.47</td>
<td>9.76 ± 2.36</td>
<td>18.0 ± 3.74</td>
<td>3.64 ± 0.24</td>
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<tr>
<td>Obese ethanol 48 h</td>
<td>37.19 ± 3.07</td>
<td>54.0 ± 8.12</td>
<td>0.62 ± 0.22</td>
<td>781.10 ± 170.62</td>
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<tr>
<td>Obese ethanol 72 h</td>
<td>48.73 ± 6.49</td>
<td>11.96 ± 0.99</td>
<td>92.0 ± 8.01</td>
<td>1.46 ± 0.47</td>
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Data expressed are calculated from an $N$ = 5 individual rats with an $N$ = 2 slices/rat average for each test. *$P < 0.01$ significantly increased from 24 h. **$P < 0.001$ significantly decreased from 24 h. ***$P < 0.05$ significantly decreased compared with age-matched control (AMC), ****$P < 0.01$ significantly increased from 24 h. ^*$P = 0.008 significantly decreased in the obese group at 48 h. $\dagger$ $P = 0.36$ significantly decreased compared with 24 h of obese ethanol administration. *$P < 0.01$ significantly decreased compared with AMC control and ethanol fed PCLS at 24 h. ^$P < 0.05$ significantly decreased compared with AMC control and ethanol fed PCLS at 48 h. %$P < 0.01$ significantly decreased compared with age-matched control (AMC). **$P < 0.01$ significantly increased from 24 h. ^$P = 0.008$ significantly decreased compared with 24 h of age-matched control at 24 h. $\dagger$ $P = 0.36$ significantly decreased compared with 24 h of obese ethanol administration. *$P < 0.01$ significantly decreased compared with AMC control and ethanol fed PCLS at 24 h. 

Obese group (Table 1, $P < 0.01$). Since the CYP2E1 enzyme is critical to ethanol metabolism, we next determined protein expression of CYP2E1. At baseline, PCLS from obese rats had a nearly 50% increase in CYP2E1 levels (Table 1, $P < 0.04$) compared with PCLS from AMC rats. After 24 h in control medium, this difference changed such that obese rat PCLS had significantly less CYP2E1 than AMC (Table 1, $P = 0.012$). However, their changes in baseline CYP2E1 protein expression were only significant in PCLS exposed to ethanol at the 24-h time point (Table 1, $P < 0.01$), although CYP2E1 expression also trended to decrease in PCLS exposed to ethanol at 48 and 72 h (Table 1).

With histological evidence for increased fat storage in the liver (Fig. 2), we next assayed PCLS TG levels to determine the effect of ex vivo ethanol exposure on fat storage. Initially, PCLS from obese rats had significantly higher TG levels compared with AMC (Fig. 3, $P < 0.001$). This significant increase in TG found in PCLS from obese rats continued during the 72 h that were assayed (Fig. 3). With ex vivo exposure to ethanol, PCLS from obese rats further stored TGs, with significant increases from their baseline levels at 48 and 72 h of ex vivo exposure to ethanol (Fig. 3, $P < 0.05$). These findings demonstrate that PCLS from obese rats with prestored TG continue to further store TGs upon ethanol exposure, whereas PCLS from AMC do not have the ability to store further TGs.

To begin looking at the fibrotic response in this model system, SMA expression in PCLS from AMC and obese rat PCLS was examined. SMA was increased at baseline and continued throughout the experiment in the obese PCLS (Fig. 4). When ethanol was introduced levels increased at 24 and 48 h in both the AMC and obese PCLS compared with controls at the corresponding time point ($P < 0.05$). Differences were present between ethanol-fed AMC and obese slices at 24 and 72 h ($P < 0.05$).

We next determined the amount of ROS present in PCLS by quantifying the lipid peroxidation products, mainly MDA, using the TBARS assay. In parallel, we measured the amount of GSH and HO-1 activity in PCLS exposed to ethanol over time to determine how antioxidant systems were responding to the oxidative stress of the ethanol challenge. TBARS reactivity did not change over the first 24 h in PCLS from AMC and obese rats (Fig. 5A). After 24-h ex vivo exposure to ethanol, PCLS from AMC and obese rats demonstrated that TBARS reactivity increased significantly in both (Fig. 5A, $P < 0.01$). However, at this 24-h point, TBARS reactivity is less in PCLS from obese rats compared with AMC (Fig. 5A, $P < 0.05$). At 48 and 72 h, only PCLS from obese rats exposed to ex vivo ethanol demonstrated increases in TBARS reactivity compared with baseline and with their AMCcs (Fig. 5A, $P < 0.01$). Both GSH and HO-1 activity were decreased in both PCLS from AMC and obese rats exposed to ex vivo ethanol for 24 and 48 h, consistent with their utilization in ethanol metabolism (Fig. 5B, $P < 0.05$). Although the levels of the antioxidant HO-1 were not initially different between PCLS from AMC and obese rats, PCLS from obese rats had significantly decreased HO-1 levels compared with those from AMC at every time whether exposed to ethanol or control medium after the initial time 0 (Fig. 5C, $P < 0.05$), suggesting a defect in the obese rat PCLS ability to produce HO-1.

With significant increases in LPS circulating in the obese rats compared with AMC, such as that found in obese patients, we next identified the inflammatory cytokine profile of the PCLS over time and when further challenged with ethanol exposure. After 24 h in culture, supernatant TNF-α concentrations were significantly greater in PCLS from obese rats compared with AMC in the presence or absence of ethanol (Fig. 6A, $P < 0.01$). At 48 h of ethanol exposure, the TNF-α levels found in the media doubled in PCLS from obese and AMC rats, with obese PCLS being larger, yet not significant

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from the AMC PCLS (Fig. 6A, \( P < 0.05 \)). The proinflammatory cytokine IL-6 was significantly increased in PCLS from obese rats at 24, 48, and 72 h of ex vivo ethanol exposure compared with parallel PCLS from AMC (Fig. 6B, \( P < 0.001 \)). Only PCLS from obese rats had increases in IL-6 at 72 h in control medium (Fig. 6B, \( P < 0.05 \)). Like TNF-\( \alpha \), MCP-1 cytokine levels were increased in PCLS from obese rats compared with AMC in control medium at 24 h (Fig. 6C, \( P < 0.01 \)). However, following ethanol incubation these levels went down in the obese slices, suggesting that subsequent ethanol challenge to PCLS from obese rats inhibits MCP-1 release (Fig. 6C, \( P < 0.001 \)).

To further determine the extent of fibrosis and inflammatory events in this model system, RT-PCR was performed with the following primers: Collagen 1a1, Timp-1, IL-1\( \beta \), TNF-\( \alpha \), and IL-6. Collagen 1a1 was increased over time in all four of the groups (Table 2) and was significantly increased when comparing AMC control to obese control PCLS at 72 h (Table 2, \( P < 0.001 \)). However, ethanol decreased the collagen 1a1 in both the AMC and obese ethanol-fed PCLS (Table 2, \( P < 0.02 \)). Collagen 1a1 was increased in the obese control group compared with the AMC control at 72 h (Table 2, \( P < 0.01 \)). Timp-1 mRNA levels were increased at 72 h in all four groups (Table 2). In the AMC and obese group ethanol decreased the Timp-1 levels at 24 and 48 h (Table 2, \( P < 0.01 \)). However,
this level was significantly increased in the obese PCLS ethanol-exposed slices compared with the AMC ethanol slices (Table 2, \( P < 0.001 \)). IL-1\( \beta \) expression was increased in the AMC and obese ethanol group compared with control at 24 and 48 h (Table 2, \( P < 0.001 \)). There was a small insignificant increase in the obese ethanol-fed slices at 72 compared with AMC ethanol. TNF-\( \alpha \) levels were significantly increased in the AMC ethanol group compared with AMCs (Table 2, \( P < 0.04 \)). Obese PCLS exposed to ethanol were increase over obese controls; however, significance was not achieved. The obese ethanol-exposed slices were significantly decreased compared with AMC slice exposed to ethanol. Levels of IL-6 expression were increased by ethanol in the AMC group at 24 h and both AMC and obese groups at 48 and 72 h compared with control AMC and obese (Table 2, \( P < 0.04 \)). Obese ethanol-exposed slices decreased IL-6 levels compared with AMC ethanol slices at 24 and 48 h (Table 2, \( P < 0.01 \)). These levels were significantly elevated at 72 h in the obese ethanol-exposed slices compared with AMC ethanol (Table 2, \( P < 0.05 \)).

DISCUSSION

This study was undertaken to assess the usefulness of PCLS from diet-induced obese rats compared with AMC to examine a multiple-hit model of NAFLD and the effects of ethanol on liver damage. On the basis of previous studies, the obese rats exhibit characteristics of metabolic syndrome (hyperinsulinemia, hyperglycemia, increased serum LPS, increased serum TG, increased serum cytokines) (34, 37). They also exhibit many NAFLD characteristics in common with the human disease, including macro- and microvesicular steatosis, inflammation, increased lipid peroxidation, and increased cytokine expression. These parameters were met with a diet that contains 45% of its calories as fat, and the age of these rats is equivalent to middle age in humans, the age when most of the population is diagnosed with diabetes. Therefore, the purpose of this study was to evaluate the ethanol effects on obese rat PCLS and determine whether it would be a good multihit model of liver injury.

Previous studies have shown that high-fat feeding of animals increases both adipose abdominal fat and systemic endotoxic
load (6, 16, 34). Rats in this model system showed that as changes in percent body fat increased so did the amount of serum endotoxin. This increase in body fat affected the liver, which was shown by the increased concentration of lipid droplets and ballooning cells in the obese rat livers. Histological analysis of the obese rat liver was significant for periportal microsteatosis, affecting primarily zone 1 hepatocytes, specializing in fatty acid oxidation and cholesterol synthesis (38). With increases in fat coming from the gut via the portal vein, it is consistent that lipid accumulation is occurring in this model at the sites where fatty acid oxidation is occurring. This is in contrast to recent diagnostic guidelines published for nonalcoholic steatohepatitis (NASH) in humans (4). However, significant histological and biochemical changes are seen, which may parallel NASH patients earlier in the pathophysiological process. Thus this model appears to demonstrate the early stages of NAFLD. This is consistent with observations other investigators have reported when feeding rats a high-fat diet (9, 10, 16, 31). The effects of ethanol on the early stages of NAFLD may make this a unique model for studying these outcomes.

PCLS from both AMC and obese rats were incubated with ethanol for 3 days. Ethanol metabolism was significantly decreased in the PCLS from obese rats compared with the AMCs, suggesting an altered response by the enzymes responsible for ethanol degradation. This decrease was most likely due to a decrease in the ALDH enzyme in the obese animals. This finding was confirmed when acetaldehyde production was shown to be decreased in the PCLS from obese rats compared with AMC. It is well established that CYP2E1 is highly inducible in both ALD and NAFLD (32). In this model system, obese rats initially had extremely high levels of activity, which was decreased in culture over time. These levels were increased in response to ex vivo ethanol exposure. However, no differences were observed between the PCLS from obese or AMCs exposed to ethanol.

ALD and NAFLD livers both contain increased amounts of TG, accounting for a large portion of the fats accumulated in

Fig. 6. Cytokine levels in the supernatant of PCLS from AMC and obese rats following ex vivo ethanol exposure at 24, 48, and 72 h. Data are expressed in picograms per milliliter of cytokine ± SE. A: TNF-α levels were significantly increased in the PCLS from obese rats at 24 h regardless of ex vivo ethanol exposure (*P < 0.01). At 48 h PCLS from both obese and AMC rats incubated with ethanol were increased with the PCLS from obese rats being higher (yet not significant) than the AMC (#P < 0.05). B: IL-6 levels were significantly increased over time in the PCLS from obese rats following ex vivo ethanol exposure. At 72 h the obese rat PCLS exposed to control medium had significant increases in IL-6 (*P < 0.001), N = 5. At 48 h the PCLS from AMC rats exposed ex vivo to ethanol were increased compared with AMC PCLS incubated with control medium (#P < 0.05). C: monocyte chemotactic protein-1 (MCP-1) levels were significantly increased in the PCLS from obese rats exposed to control medium at 24 h compared with PCLS from AMC rats (*P < 0.01). PCLS from obese rats exposed ex vivo to ethanol at 24 and 48 h displayed decreased levels of MCP-1 compared with AMC (#P < 0.001). Data are expressed are calculated from N = 5 individual rats with N = 2 slices/rat average for each test.
Table 2. Expression of fibrotic and inflammatory markers by RT-PCR following incubation of 25 mM ethanol in AMC or obese rat PCLS

<table>
<thead>
<tr>
<th>Condition</th>
<th>Collagen 1α1</th>
<th>Timp-1</th>
<th>IL-1β</th>
<th>TNF-α</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-0 AMC</td>
<td>0.68 ± 0.12</td>
<td>1.28 ± 0.16</td>
<td>1.15 ± 0.23</td>
<td>3.14 ± 0.47</td>
<td>0.78 ± 0.59</td>
</tr>
<tr>
<td>T-0 Obese</td>
<td>0.63 ± 0.08</td>
<td>1.16 ± 0.13</td>
<td>1.09 ± 0.06</td>
<td>3.51 ± 0.67</td>
<td>1.28 ± 0.45</td>
</tr>
<tr>
<td>AMC control 24 h</td>
<td>0.12 ± 0.04</td>
<td>16.06 ± 0.91</td>
<td>1.47 ± 0.09</td>
<td>13.21 ± 1.89</td>
<td>91.47 ± 7.13</td>
</tr>
<tr>
<td>AMC control 48 h</td>
<td>1.71 ± 0.25</td>
<td>13.05 ± 1.55</td>
<td>0.75 ± 0.12</td>
<td>35.91 ± 6.93</td>
<td>56.99 ± 10.37</td>
</tr>
<tr>
<td>AMC control 72 h</td>
<td>8.80 ± 1.49</td>
<td>25.88 ± 3.11</td>
<td>1.03 ± 0.17</td>
<td>49.17 ± 5.38</td>
<td>129.91 ± 29.21</td>
</tr>
<tr>
<td>AMC ethanol 24 h</td>
<td>0.13 ± 0.02</td>
<td>7.87 ± 0.70a</td>
<td>4.54 ± 0.64d</td>
<td>32.91 ± 4.71f</td>
<td>352.77 ± 119.89p</td>
</tr>
<tr>
<td>AMC ethanol 48 h</td>
<td>0.40 ± 0.07b</td>
<td>10.73 ± 0.73</td>
<td>2.87 ± 0.35f</td>
<td>61.49 ± 7.71e</td>
<td>320.36 ± 38.39p</td>
</tr>
<tr>
<td>AMC ethanol 72 h</td>
<td>0.75 ± 0.11c</td>
<td>10.61 ± 1.72</td>
<td>1.75 ± 0.44</td>
<td>76.56 ± 15.13e</td>
<td>344.36 ± 54.99p</td>
</tr>
<tr>
<td>Obese control 24 h</td>
<td>0.23 ± 0.03</td>
<td>6.84 ± 0.58d</td>
<td>1.12 ± 0.07</td>
<td>10.01 ± 1.15</td>
<td>74.10 ± 12.20</td>
</tr>
<tr>
<td>Obese control 48 h</td>
<td>1.70 ± 0.16</td>
<td>12.13 ± 1.25</td>
<td>0.64 ± 0.05</td>
<td>9.30 ± 1.59</td>
<td>49.88 ± 6.36</td>
</tr>
<tr>
<td>Obese control 72 h</td>
<td>37.52 ± 3.20e</td>
<td>34.23 ± 2.71</td>
<td>1.30 ± 0.26</td>
<td>17.44 ± 2.53</td>
<td>294.85 ± 56.33</td>
</tr>
<tr>
<td>Obese ethanol 24 h</td>
<td>0.32 ± 0.54</td>
<td>6.21 ± 0.76d</td>
<td>3.59 ± 0.43f</td>
<td>11.46 ± 1.76b</td>
<td>104.03 ± 19.12</td>
</tr>
<tr>
<td>Obese ethanol 48 h</td>
<td>0.35 ± 0.54a</td>
<td>6.43 ± 0.89c</td>
<td>2.35 ± 0.18f</td>
<td>12.08 ± 1.25b</td>
<td>232.51 ± 20.03f</td>
</tr>
<tr>
<td>Obese ethanol 72 h</td>
<td>6.08 ± 0.73a</td>
<td>26.18 ± 3.02a</td>
<td>2.36 ± 0.71</td>
<td>22.36 ± 6.35b</td>
<td>499.83 ± 70.31a</td>
</tr>
</tbody>
</table>

Data expressed are calculated from an N = 7 individual rats with an N = 2 slices/rat average for each test. *P < 0.02 significantly decreased compared with AMC and obese controls at 48 and 72 h. **P < 0.01 significantly increased compared with AMC control at 72 h. ***P < 0.01 significantly increased compared with AMC ethanol treated slices at 72 h. &P < 0.01 significantly decreased compared with AMC and obese controls. **P < 0.01 significantly increased compared with AMC ethanol at 72 h. 0.01 Significantly increased compared with AMC and obese control at 24 and 48 h. &P < 0.04 significantly increased compared with AMC control. +P < 0.01 Significantly decreased compared AMC ethanol. 0.04 Significantly increased compared with AMC and obese controls. &P < 0.05 Significantly decreased compared with AMC ethanol. 0.05 Significantly increased compared with AMC ethanol.

HO-1 has been shown to have a role in the degradation of cytchrome P-450, resulting in decreased ethanol detoxification and fat metabolism in hepatocyte (22). HO-1 was decreased in the PCLS from obese rats over time. This decrease in HO-1 doesn’t result in the degradation of CYP2E1 indicating other mechanisms involved in this process. This may be attributed to other cell types present in the PCLS increase in CYP2E1 expression. However, the addition of ex vivo ethanol to the PCLS from obese rats had no effect, suggesting that HO-1 was already maximally altered by the high-fat diet. Overall, we can conclude that oxidative stress is exacerbated when either fat or ethanol are present in the liver.

It is well established that cytokines are involved in the pathogenesis and/or progression of both NAFLD and ALD (3, 14, 20). For example, IL-6 and TNF-α are released in response to oxidative stress. TNF-α release is thought to cause hepatocellular damage, whereas IL-6 may be involved in hepatoprotective and anti-inflammatory responses (20). PCLS from obese rats in our study had significantly higher levels of TNF-α compared with AMC. At 48 h, the ex vivo ethanol exposure caused a spike in this cytokine. IL-6 levels were only slightly elevated in the obese rat PCLS that were incubated with media only. However, following ethanol incubation the IL-6 levels increased dramatically at 24 h and remained increased over time. These data indicate that fat and ethanol may be synergistically causing the increase in these cytokines and thus a greater chance for the development of hepatotoxicity to the liver. The high increase in IL-6 may play an initial protective role in these animals by limiting the total amount of damage necessary to drive the liver to end-stage failure in the short time period. However, sustained levels of IL-6 have been shown to sensitize the liver to injury and cell death (25). Interestingly, MCP-1 was increased in the PCLS from AMC rats following ex vivo ethanol exposure compared with PCLS from obese rats. This may be due to the possibility that cells in the obese rats in vivo already released MCP-1. Increase in cytokine levels in the obese rat PCLS prior to any incubation with ethanol suggest that other stimuli in the system prior to the cultures was responsible for these increased.

the liver following either high-fat diet or chronic alcohol consumption (9, 19). Recently it has been demonstrated that feeding mice both a high-fat diet and ethanol would increase TGs and make animals more susceptible to fibrosis (19). These investigators determined that the high-fat diet had a more significant role in inducing steatosis than ethanol alone did. When the two were combined, a synergistic increase in TG was observed. In our model system, PCLS from obese rats exposed ex vivo to ethanol maintained a steady level of TGs until 48 h when the level was increased dramatically. This synergistic effect increases when the liver fat load is increased, putting a greater strain on its ability to metabolize other deleterious compounds.

As previously reported, exposure of ethanol to PCLS accelerates the fibrotic response (41). In this study, ethanol-fed slices from AMC and obese rats expressed higher levels of SMA compared with control fed slices. The obese rat PCLS had a higher level of SMA prior to exposure of ethanol. SMA expression increased dramatically when slices were treated with ethanol, indicating the start of the fibrogenic response.

Oxidative stress is a hallmark of both NASH and ALD. This stress caused by ROS affects the mitochondrial, endoplasmic reticulum, peroxisomal, and microsomal organelles (17, 36, 38, 48). Electron leakage caused by β-oxidation in the mitochondrion induces lipid peroxides to form and damage the membranes and other proteins within the cell. In this study, TBAR levels increased in PCLS from AMC rats following ex vivo ethanol incubation at 24 h. However, after 48 h these levels increased in the PCLS from obese rats exposed to ex vivo ethanol. Glutathione, a detoxifier of endogenous peroxidized lipids, was decreased in the ethanol-exposed PCLS from both obese and AMC rats early and then increased after 72 h, indicating that oxidation of lipids had occurred. In this PCLS model, obese rats showed equivalent responses to alcohol up until 72 h, whereas GSH increase in AMC rats and remained low in obese rat. These differences in GSH didn’t negatively correlate with the TBARs data, making it not the only antioxidant responsible for elimination of lipid peroxides.
levels. Increased levels of endotoxin in the serum of these animals could be responsible for this predisposed increase prior to ex vivo ethanol administration.

To further gain insight into the fibrogenic and inflammatory response in this model system, RT-PCR was performed with specific markers. Collagen 1α1 expression was increased over time in the obese PCLS compared to AMC. Addition of ethanol decreased the expression in both the AMC and obese PCLS. The obese ethanol PCLS did have higher levels compared with the AMC ethanol PCLS, indicating a greater fibrogenic response with the combination of both insults. Similar results were present when TIMP-1 expression was looked at. IL-1β was increased in both the AMC and obese ethanol PCLS compared with AMC and obese controls, indicating that a proinflammatory response due to ethanol was present. The TNF-α expression was increased in response to ethanol in the AMC and obese PCLS. Ethanol and fat did cause IL-6 expression to remain higher compared with AMC and obese control throughout the 72-h incubation period. These numbers were decreased at 24 and 48 h when comparing the effects of ethanol until 72 h when the obese ethanol became increased. These data indicate that both the fibrogenic and inflammatory response are present in this model system with the combination of ethanol and fat increasing the responses. Ethanol appears to decrease the expression of collagen and TIMP-1 message, which can’t be explained with the present data. This has been seen before in our hands with these PCLS cultures. However, with use of obese slices these numbers are increased over the AMC ethanol-fed PCLS, indicating that another insult is needed to increase the expression of this fibrotic marker. More work is needed to determine why collagen message is decreased in response to ethanol in our control slices. The inflammatory markers are interesting because they are increased with ethanol. However, when comparing AMC ethanol PCLS to obese ethanol PCLS the numbers are decreased in the obese PCLS. This would indicate that the presence of fat may be suppressing the inflammatory response.

This model system mimics several phenotypes present in patients with ALD and NAFLD. It appears that there is an increase in endotoxin most likely due to the fat increase in the gut increasing permeability and leakage into the vasculature. Histologically these livers are loaded with fat with some of the characteristics of NAFLD. However, this may be a better model of NAFLD then NASH because of the lack of scarring in the obese rat livers. Although these livers are fatty, they lack the changes seen in end-stage liver failure. It is possible another agent or “hit” is needed to drive these animals to end-stage liver disease. The introduction of ethanol to the PCLS, as presented in this study, provides this “hit” and many of the chemical changes seen in liver disease. However, this hit with ethanol failed to induce end-stage liver failure. There is a good possibility that other factors in addition to fat and ethanol are needed to produce this phenotype. It would be interesting to add different or additional agent(s) to these PCLS and evaluate the effects on the liver. With the right combination, it may be possible to help solve many of the questions into how fats and ethanol initiate liver disease. It may also be possible to use this PCLS model to evaluate the impact of disease treatment strategies on the liver prior to initiating patient clinical trials.

Data generated from this study using PCLS show that the presence of both preexisting fats and ethanol ingestion increases hepatic fat and oxidative stress and alters cytokine expression. This ex vivo culture system would provide the needed tools to further examine the effects of hepatotoxic agents (alcohol, acetaminophen, etc.) on NAFLD patients. Also, it may be useful for evaluating the effects of diet, alcohol, and therapeutics on the liver.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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OBESE RAT LIVER SLICES ARE SUSCEPTIBLE TO OXIDATIVE STRESS

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