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Abstract

Disease progression in alcoholic and non-alcoholic fatty liver disease shows sex-specific differences and is influenced by mechanisms linked to oxidative stress. Acetaldehyde plays a critical pathogenic role but its effects are mitigated by the activity of aldehyde dehydrogenases. Aldehyde dehydrogenase 1b1 (Aldh1b1) is the aldehyde dehydrogenase isoform with the second highest affinity for acetaldehyde after Aldh2, and is highly expressed in the intestine and liver. We examined sex differences and the effect of Aldh1b1 depletion in a murine model of chronic alcohol-induced liver disease. Male and female wild-type and Aldh1b1-depleted mice received either ethanol (10-20% v/v) in drinking water or water alone for one year, and livers were examined histopathologically, histochemically and by immunohistochemistry. A significant increase in hepatic steatosis was observed in female mice after one year of ethanol consumption, and expression of ethanol-metabolising enzymes and up-regulation by ethanol was also sex-dependent. Ethanol-induced hyperproliferation of hepatocytes was observed in female and male wild-type mice, and Aldh1b1 depletion enhanced this effect in males. Further, one ethanol-treated, Aldh1b1-depleted male developed a steatohepatitic hepatocellular carcinoma. These sex-specific differences in susceptibility to hepatic steatosis and disease progression may be related to differences in expression of ethanol-metabolising enzymes, informing the clinically significant differences. Aldh1b1 plays a role in protection from ethanol-induced hepatocellular hyperproliferation and may protect from tumour development.

Keywords

Hepatic steatosis, non-alcoholic fatty lover disease, alcoholic liver disease, Aldh, acetaldehyde, EUCOMM

Introduction

Hepatic steatosis has a high prevalence in the population. Steatosis accompanied by characteristic histological inflammation and hepatocellular ballooning represents steatohepatitis, with associated fibrosis that can progress to cirrhosis carrying an increased risk of hepatocellular carcinoma (HCC)(Bellentani et al., 2004). Both alcohol consumption and obesity are recognised as major risk factors for steatotic liver disease(Bellentani et al., 1994, 2004; Völzke, 2012), alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD), respectively. Disease mechanisms and risk factor interactions for ALD and NAFLD have not been fully clarified, but their influence on disease progression and HCC development is synergistic(Hart et al., 2010; Loomba et al., 2013). Females are believed to be more susceptible to alcohol-induced liver disease, although the reasons are not completely understood. Putative mechanisms include oestrogen effects, differential expression or activity of alcohol-metabolising enzymes, and increased sensitivity to gut derived endotoxins(Eagon, 2010; Sato et al., 2001).

The pathogenesis of both NAFLD and ALD is a ‘two hit’ process. Hepatic steatosis constitutes the first cellular injury but a second event is required to induce steatohepatitis and trigger disease progression(Day, 2002; Schaffert et al., 2009). The second insult critically leads to oxidative stress-induced lipid peroxidation, and cytokine-mediated injury and inflammation. In ALD, increased plasma endotoxin levels correlate with disease progression and so represent part of the ‘second hit’(Mandrekar and Szabo, 2009).

Acetaldehyde is a highly reactive compound produced during hepatocellular oxidation of ethanol by alcohol dehydrogenases(Seitz and Stickel, 2010; Setshedi et al., 2010)). It has a central role in ALD, with direct effects on the liver as well as

indirect effects through increased intestinal permeability(Szabo and Bala, 2010), and there is increasing recognition that alcohol produced by the gut microbiome plays a role in NAFLD(Liu et al., 2016). Acetaldehyde is removed by oxidation to acetate by aldehyde dehydrogenases (ALDH). Excessive alcohol consumption leads to accumulation of NADH and acetyl-CoA, disrupting the metabolic balance and causing inhibition of fatty acid beta-oxidation and increased fatty acid synthesis(Liu, 2014).

ALDH2 is the Aldh isoform with the highest affinity for acetaldehyde(Marchitti et al., 2008). Patients with an inactivating *ALDH2* polymorphism(Seitz and Stickel, 2010) show severe responses to ethanol consumption. *Aldh2* deletion in mice led to increased plasma acetaldehyde levels after ethanol consumption, and increased risk of ethanol-toxicity(Kiyoshi et al., 2009; Yu et al., 2009). These animals showed reduced hepatic steatosis but an enhanced inflammatory response after 4 weeks of ethanol treatment(Kwon et al., 2014).

ALDH1B1 has the second highest affinity for acetaldehyde and is highly expressed by the intestinal epithelium and hepatocytes(Marchitti et al., 2008; Stagos et al., 2010). We have demonstrated that *Aldh1b1* depletion enhances ethanol-induced intestinal carcinogenesis(Müller et al., 2017) but the effect on liver damage in this clinically-relevant model has not been examined. We investigated the hepatic sequelae of Aldh1b1 loss in a mouse model of long-term ethanol consumption, using male and female mice to identify often overlooked sex differences. We took advantage of the development of late-onset obesity in C57Bl/6 mice fed a standard rodent diet(Becskei et al., 2009) to also model NAFLD alone or combined with ethanol-induced disease(Lutz and Woods, 2012). We define the sex-specific effects of long-term ethanol consumption and Aldh1b1-depletion in the murine liver,

demonstrating hepatic steatosis and mild inflammation, as well as the development of one steatohepatitic hepatocellular carcinoma.

Materials and Methods

Mouse experiments

Aldh1b1$^{tm2a(EUCOMM)Wtsi}$ homozygous mice (abbreviated *Aldh1b1*^{tm2a}) with very low level residual Aldh1b1 expression in occasional intestinal crypts were analysed in a long-term ethanol consumption experiment as described previously(Müller et al., 2017). Cohort sizes were chosen based on those used in previous similar work using. Briefly, homozygous *Aldh1b1*^{tm2a} mice and wild-type littermates were randomised (by alternating allocation as animals of each genotype were available) to receive either normal drinking water or ethanol supplemented water in escalating doses ad libitum for one year, starting with one month of 10% (v/v) ethanol, one month of 15% (v/v) ethanol and 20% (v/v) ethanol for the remaining ten months. Ethanol supplementation started at 6-7 weeks of age, and each group consisted of 15 animals (7-8 males and 7-8 females in each). Mice were fed a standard rodent maintenance diet ad libitum (RM1, Special Diets Services). All experiments were performed under a project licence issued under the United Kingdom Home Office Animals (Scientific Procedures) Act 1986.

Immunohistochemistry and histochemistry

Immunohistochemistry was performed as described previously(Müller et al., 2017) using the following primary antibodies: rabbit anti-Aldh1b1 (15560-1-AP, Proteintech), rabbit anti-Aldh2 (AB PB9472, Picoband), rabbit anti-Aldh1a1 (ab52492, abcam), rabbit anti-Ki67 (ab16667, abcam), rabbit anti-Cytochrome P450 2E1 (Cyp2e1) (ADI-100-MFO, Enzo Life Sciences) and rabbit anti-4-hydroxynonenal

(4-HNE) (ab46545, abcam). For picrosirius red (PSR) staining, sections were incubated with 0.1% Sirius red F3B in saturated picric acid solution after pre-treatment in 0.2% phosphomolybdic acid (w/v in distilled water), and counterstained in 0.1% fast green FCF (w/v in acidified water). PSR stained sections were used for automated collagen proportionate area quantification by image analysis(Calvaruso et al., 2009), modified to use the application of a machine-learning based classifier(Arganda-Carreras et al., 2017) to whole slide images after training in the FIJI implementation(Schindelin et al., 2012) of ImageJ(Rueden et al., 2017).

Immunostainings were scored based on staining intensity on blinded samples. Steatosis was scored on blinded samples by two pathologists using two different scoring systems. Pathologist 1 used the steatosis scale used in both the Non-alcoholic Steatohepatitis Clinical Research Network (NASH CRN)(Kleiner et al., 2005) and SAF(Bedossa et al., 2012) scoring systems for NAFLD from 0 to 3 to assess quantities of large or medium sized lipid droplets (score 0:<5%, score 1: 5%-33% [mild], score 2: 34%-66% [moderate], score 3: >67% [marked]). Pathologist 2 used a score from 0 (no fatty change) to 4 (severe fatty change).

Statistics

Data were analysed with GraphPad Prism® 6 software (version 6.04), using unpaired, two-tailed Student's t-test or linear regression analysis as detailed in the figure legends. Comparison of ordinal data generated by semi-quantitative scoring between treatment groups was undertaken using Mann-Whitney U-testing. Differences between groups were considered significant if $p \leq 0.05$, as further documented in the figure legends.

Results

Sex-specific differences in hepatic Aldh isoform expression in normal ageing and in response to chronic ethanol consumption

Protein expression of the three Aldh isoforms with highest affinity for acetaldehyde, Aldh2, Aldh1b1 and Aldh1a1(Marchitti et al., 2008), in the liver was quantified by immunohistochemistry (Figure 1). Aldh2 levels were identical in male and female animals. There was a significant increase in Aldh2 immunostaining intensity after one year of ethanol consumption in male and female *Aldh1b1*^{tm2a} mice, but not in wild-types (Figure 1A, B).

Aldh1b1 was only expressed in livers of wild-type mice, indicating that *Aldh1b1*^{tm2a} mice are true knockouts in the liver. Both control and ethanol-treated female mice showed significantly greater hepatocellular Aldh1b1 immunostaining than male counterparts. Aldh1b1 levels in females were not influenced by ethanol, whereas significantly increased Aldh1b1 immunostaining intensity after ethanol consumption was observed in males (Figure 1C, D).

In contrast, Aldh1a1 immunostaining was stronger in wild-type males than in females in the water-treated groups, and a significant increase after ethanol consumption was observed in wild-type and *Aldh1b1*^{tm2a} females, but not in males (Figure 1E, F).

There was no compensatory up-regulation of Aldh1a1 or Aldh2 in control *Aldh1b1*^{tm2a} mice (Figure 1A, B, E, F).

Hepatic steatosis develops spontaneously in ageing mice and is exacerbated by chronic ethanol consumption

A considerable increase in body weight was observed over the 1-year treatment period. At the end of Week 50, the mean body weight of females in the different

groups ranged from 30.7-33.5 g, with a maximum individual weight of 40.1 g, and the mean body weight of males in the different groups ranged from 32.0-40.6 g, with a maximum individual weight of 47.1 g (Figure S1).

Ethanol-treated male wild-type and *Aldh1b1*^{tm2a} animals had significantly lower body weights compared with their water-treated littermates, with a mean body weight in Week 50 of 32.0 g and 34.8 g in ethanol-treated wild-type and *Aldh1b1*^{tm2a} males and a mean body weight of 38.9 g and 40.6 g in water-treated wild-type and *Aldh1b1*^{tm2a} males, respectively. The body weights of female wild-type and *Aldh1b1*^{tm2a} animals were unaffected by ethanol treatment (Figure S1).

In accordance with the previously reported higher susceptibility of females to ethanol-induced liver damage(Iimuro et al., 1997; Wagnerberger et al., 2013), ethanol-treated wild-type and *Aldh1b1*^{tm2a} females, but not males, displayed a significant increase in hepatic steatosis compared with the respective water-treated groups. Absolute levels of steatosis were higher in wild-type females in both ethanol treatment and control groups, compared with wild-type males (Figure 2, Figure S2).

Spontaneous hepatic steatosis in the water-treated groups correlated with body weight, especially in males, indicating the development of NAFLD with age. A significant correlation of hepatic steatosis and body weight was observed in water-treated wild-type females and water-treated *Aldh1b1*^{tm2a} males (Figure 2B, H).

Steatosis was independently scored by two experienced Pathologists using two different scoring systems and a statistically significant, strong correlation between these two sets of scores was obtained (R^2=0.6457, p<0.0001) (Figure S3).

No significant evidence that normal aging and chronic ethanol consumption induce steatohepatitis or fibrosis

There was minimal necroinflammation evident in the livers of all but one animal, meriting their classification as simple steatosis but not steatohepatitis. In keeping with this, no broad scars were evident. Collagen proportionate area, derived from PSR-stained sections, similarly showed no significant difference between control and ethanol-treated animals, nor between wild-type and *Aldh1b1*^{tm2a} animals, with the exception of a slight but significant decrease in ethanol-treated *Aldh1b1*^{tm2a} males compared with water-treated *Aldh1b1*^{tm2a} males (Figure S4).

Ethanol treatment increases Cyp2e1 expression with evidence of lipid peroxidation

Chronic ethanol treatment is known to increase Cyp2e1 expression(Lu and Cederbaum, 2008). Cyp2e1 metabolises ethanol to generate acetaldehyde, and reactive oxygen and nitrogen species, leading to greater lipid peroxidation. This mechanism has been shown to drive damage in steatohepatitis(Leclercq et al., 2000).

Cyp2e1 expression in water- and ethanol-treated animals showed no sex-specific differences although there was a trend (p=0.0598) towards greater Cyp2e1expression in ethanol-treated *Aldh1b1*^{tm2a} males compared with respective females. A significant up-regulation of Cyp2e1 expression was observed in wild-type males and in *Aldh1b1*^{tm2a} males and females (Figure 3A-E) after chronic ethanol exposure.

4-HNE immunostaining was quantified as a marker of lipid peroxidation. Livers of water-treated females had more 4-HNE expression than water-treated males. There was a highly significant increase in 4-HNE immunostaining intensity in ethanol-treated wild-type and *Aldh1b1*^{tm2a} males, but not in females. After the ethanol-induced increase in male 4-HNE expression, absolute 4-HNE expression was higher

than in ethanol-treated females. 4-HNE expression was not affected by Aldh1b1-depletion (Figure 3F-G).

Increased hepatocellular proliferation induced by ethanol treatment is exacerbated by loss of Aldh1b1

Untreated female animals had significantly more nuclear Ki67-immunopositive hepatocytes than untreated male animals. Ethanol treatment significantly increased the number of Ki67-immunopositive hepatocytes in wild-type females and males, and in *Aldh1b1*^{tm2a} males (Figure 4).

Aldh1b1^{tm2a} males had significantly more Ki67-immunopositive hepatocytes than wild-type males, both in the water- and ethanol-treated groups. This is consistent with an exacerbation of ethanol-induced hyper-proliferation previously observed in the intestines of ethanol-treated *Aldh1b1*^{tm2a} mice(Müller et al., 2017).

Hepatocellular carcinoma development in an ethanol-treated Aldh1b1^{tm2a} *male*

Three large nodules of hepatocellular carcinoma were present in the liver of a single ethanol-treated *Aldh1b1*^{tm2a} male animal (Figure 5A). Microscopically, nodules lacked portal tracts but contained unaccompanied arterial branches, and the normal sinusoidal structure was absent (Figure 5B). Lesional cells demonstrated nuclear enlargement and pleomorphism, with variable cellular ballooning, small Mallory-Denk bodies and steatosis (Figure 5C), meriting classification as steatohepatitic HCC(Salomao et al., 2012). Intralesional fibrosis was prominent (Figure 5D).

Discussion

Fatty liver disease represents a significant health burden; alcoholic liver disease is the largest single primary aetiology requiring liver transplantation in the UK(NHS Blood and Transplant, 2016), whilst in four European countries (Germany, France,

Italy, and UK) there are an estimated 52 million people with NAFLD at an annual cost of €35 billion(Younossi et al., 2016).

Women have a greater risk of chronic damage in alcoholic liver disease. In accordance with this, hepatic steatosis after long-term ethanol-treatment was only observed in females in this study, and ethanol induced more hepatic hyperproliferation in wild-type females than in males. The putative mechanisms behind this are unclear but may include the effects of oestrogen, differential expression or activity of alcohol-metabolising enzymes, and increased sensitivity to gut derived endotoxins(Eagon, 2010; Sato et al., 2001). The incidence of non-alcoholic fatty liver disease in lean individuals is also higher in females(Younossi et al., 2012), and there is also twice the rate of non-alcoholic steatohepatitis, predicting fibrosis progression, in female NAFLD patients(Neuschwander-Tetri et al., 2010).

We have shown complex sex-specific differences in the Aldh enzymes responsible for metabolising acetaldehyde in response to both ageing and alcohol. In a recent mass spectrometry-based proteomics approach, the two Aldh isoforms with the highest affinity for acetaldehyde, Aldh2 and Aldh1b1, were up-regulated after ethanol-consumption in male, but not in female mouse livers(Wang et al., 2014). In agreement with this, we detected increased Aldh1b1 immunostaining intensity after one year of ethanol consumption in male but not in female wild-type and *Aldh1b1*^{tm2a} mice in this study. Interestingly, our data show that Aldh1b1 immunostaining intensity in females was not responsive to ethanol consumption, but was significantly stronger than in males in both water- and ethanol-treated wild-type females.

Animals were examined at an advanced age more in keeping with the development of NAFLD in humans than is often used in rodent modelling of chronic liver disease. Steatosis was present in the livers of some male and female control animals; in

female wild-type animals there was a correlation between extent of steatosis and body weight. This indicates the spontaneous development of NAFLD in ageing C57/Bl6 mice, and indicates that disease in ethanol-treated animals represents the additive effect of both aetiologies.

We have previously observed a nearly twofold increase in plasma acetaldehyde levels in ethanol-treated *Aldh1b1*^{tm2a} mice compared with ethanol-treated wild-type littermates(Müller et al., 2017). Acetaldehyde is critically involved in the development of ALD, and ALDHs have a crucial role in protecting from ethanol-induced liver damage. Ethanol consumption led to increased plasma and liver acetaldehyde levels in a mouse model, along with steatosis, inflammation and an increase in liver 4-HNE levels, which was alleviated by co-administration of an ALDH activator(Zhong et al., 2015).

In *Aldh1b1*^{tm2a} mice lacking hepatic Aldh1b1, the only compensatory change was increased Aldh2 expression after chronic ethanol exposure, which was not observed in wild-type littermates. Increased 4-HNE levels, reflecting lipid peroxidation, after ethanol treatment were demonstrated, as expected(Zhong et al., 2015), in male wild-type and *Aldh1b1*^{tm2a} mice. Increased Cyp2e1 immunostaining, an important contributor to hepatic oxidative stress after ethanol-consumption(Lu and Cederbaum, 2008), was confirmed in ethanol-treated wild-type and *Aldh1b1*^{tm2a} males as well as in *Aldh1b1*^{tm2a} females. However, there was no significant difference in 4-HNE or Cyp2e1 between genotypes, nor differences in the steatotic response to chronic ethanol exposure.

These data demonstrate that compensatory hepatic over-expression of Aldh2 is sufficient to reduce potential increases in plasma acetaldehyde and prevent increased liver damage and is an example of inherent, protective, biological

redundancy. This also suggests profound functional differences between Aldh2 and Aldh1b1. In contrast to Aldh1b1 loss, *Aldh2* deletion in mice aggravated alcohol-induced hepatic oxidative stress and inflammation, but ameliorated steatosis and hepatocellular injury after 4 weeks of ethanol consumption(Kinoshita et al., 2008; Stagos et al., 2010). Steatosis after ethanol consumption is caused by accumulation of NADH and acetyl-CoA that results in inhibition of fatty acid beta-oxidation and increased fatty acid synthesis. Depletion of Aldh2 causes a more than tenfold increase of plasma acetaldehyde in ethanol-treated *Aldh2* knockout mice compared with wild-types(Kiyoshi et al., 2009), compared with a twofold increase in *Aldh1b1*-depleted mice(Müller et al., 2017; Singh et al., 2015).

Increased hepatic proliferation after ethanol consumption has been previously reported(Baroni et al., 1994; Chung et al., 2001). In our study, this response was greater in wild-type females than in males. Aldh1b1-depletion exacerbated ethanol-induced hyperproliferation in males, although absolute proliferation rates remained higher in female animals for each condition. Development of steatohepatitic HCC is associated with ALD and NAFLD(Salomao et al., 2012), including in non-cirrhotic liver(Baffy et al., 2012). Steatohepatitic HCC developed in the only animal showing background steatohepatitis, in an ethanol-treated, Aldh1b1-depleted male. Together, these data suggest that Aldh1b1, particularly in males, plays a protective role from ethanol-induced hepatocellular proliferation. As only a single ethanol-treated, Aldh1b1 depleted male developed HCC, definitive conclusions are precluded, but the male sex and genotype are intriguing and a longer ethanol treatment study of Aldh1b1 knockout mice is warranted to further study the protective role of Aldh1b1 in the liver.

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