Research Article

JOURNAL OF HEPATOLOGY

Aging aggravates alcoholic liver injury and fibrosis in mice by downregulating sirtuin 1 expression

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Background & Aims: Aging is known to exacerbate the progression of alcoholic liver disease (ALD), but the underlying mechanisms remain obscure. The aim of this study was to use a chronic plus binge ethanol feeding model in mice to evaluate the effects of aging on alcohol-induced liver injury.

Methods: C57BL/6 mice were subjected to short-term (10 days) ethanol plus one binge or long-term (8 weeks) ethanol plus multiple binges of ethanol. Liver injury and fibrosis were determined. Hepatic stellate cells (HSCs) were isolated and used in *in vitro* studies.

Results: Middle-aged (12–14 months) and old-aged (>16 months) mice were more susceptible to liver injury, inflammation, and oxidative stress induced by short-term plus one binge or long-term plus multiple binges of ethanol feeding when compared to young (8–12 weeks) mice. Long-term plus multiple binges of ethanol feeding induced greater liver fibrosis in middle-aged mice than that in young mice. Hepatic expression of sirtuin 1 (SIRT1) protein was downregulated in the middle-aged mice compared to young mice. Restoration of SIRT1 expression via the administration of adenovirus-SIRT1 vector ameliorated short-term plus binge ethanol-induced liver injury and fibrosis in middle-aged mice. HSCs isolated from middle-aged mice expressed lower levels of SIRT1 protein and were more susceptible to spontaneous activation in *in vitro* culture than those from young mice. Overexpression of SIRT1 reduced activation of HSCs from middle-aged mice *in vitro* with downregulation of PDGFR-α and c-Myc, while deletion of SIRT1 activated HSCs isolated from young mice *in vitro*. Finally, HSC-specific *SIRT1* knockout mice were more susceptible to long-term chronic-plus-multiple binges of ethanol-induced liver fibrosis with upregulation of PDGFR-α expression.

Conclusions: Aging exacerbates ALD in mice through the downregulation of SIRT1 in hepatocytes and HSCs. Activation of SIRT1 may serve as a novel target for the treatment of ALD.

Lay summary: Aged mice are more susceptible to alcohol-induced liver injury and fibrosis, which is, at least in part, due to lower levels of sirtuin 1 protein in hepatocytes and hepatic stellate cells. Our findings suggest that sirtuin 1 activators may have beneficial effects for the treatment of alcoholic liver disease in aged patients.

Introduction

Alcoholic liver disease (ALD) is considered to be a major cause of morbidity and mortality nationwide [1–5]. It has an array of liver pathology that ranges from simple fatty liver to more severe forms of liver injury such as steatohepatitis, cirrhosis, and hepatocellular carcinoma. Many factors have been shown to affect the development and progression of ALD, including age, gender, ethnicity, genetic factors, drinking pattern, type of alcohol consumed, dose, duration, obesity, viral hepatitis infection, and environment [1–5]. Aging has been demonstrated to be associated with a progressive and widespread impairment of cellular functions. Therefore, resulting in an increasing risk of diseases in humans including diabetes, inflammatory diseases, and cancers [6]. In addition, liver function declines during aging, which involves alterations to the hepatic structure, hepatic sinusoid and function, thereby leading to the development of age-related liver diseases [7–9]. It has been reported that aged mice are more susceptible to hepatocellular injury, inflammation, and liver fibrosis after high-fat diet feeding. This, is partly due to the sensitization to the Fas death pathway, increased M1 macrophage polarization, and increased innate immune responses [10,11]. Moreover, accumulating evidence suggests that the aged liver is more susceptible to injury due to alcohol abuse [12], which may be due to many mechanisms such as alterations to ethanol metabolism, microsomal ethanol oxidation, CYP2E1, and microsomal activity, and a reduction in hepatic mitochondrial function in the aged liver [13–16].

Keywords: Fatty liver; Steatohepatitis; Ethanol; PDGFR-α; Middle-age; Hepatic stellate cells (HSCs).

Received 10 June 2016; received in revised form 14 October 2016; accepted 8 November 2016; available online 18 November 2016

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Although several mechanisms have been identified to be responsible for the increased susceptibility of aged livers to ALD, the exact underlying mechanisms are still unclear. Recent studies suggest that sirtuins (SIRT) play critical roles in regulating many biological and cellular processes during aging [17]. SIRTs are classified as class III histone deacetylases, in which seven mammalian sirtuin (SIRT1-7) isoforms have been identified [17]. They are part of a family of nicotinamide adenine dinucleotide (NAD^+) dependent protein deacetylases and ADP-ribosyltransferases. SIRTs are able to catalyze at specific lysine substrate deacetylation/deacylation, playing important roles in the pathogenesis of metabolic stress and control, caloric restriction, and cancer [17]. They are also associated with aging and are found at diverse subcellular locations like in the nucleus, cytosol and mitochondria [17]. For example, SIRT1, named after the *Saccharomyces cerevisiae* Sir2 (silent information regulator 2) protein, has been proposed to serve as an anti-aging protein [18]. SIRT1 is predominately expressed in the nucleus and cytoplasm, whereas SIRT-2, 3 and 4 are expressed in the mitochondria [17]. Previous studies have reported that hepatic SIRT1 plays a major role in ameliorating steatosis and inhibiting inflammation in alcoholic and non-alcoholic fatty liver diseases (NAFLD) by modifying the acetylation status of the different target molecules [19–21]. In addition, it has also been reported that a variant of histone H2A, macroH2A1.1 is highly involved in lipid metabolism in NAFLD and binds SIRT1, which helps protect hepatocytes from lipid accumulation [22]. In the present study, we used the chronic plus binge ethanol feeding model to evaluate the effects of aging on alcohol-induced liver injury. Chronic plus binge ethanol feeding mimics the drinking pattern of alcoholic hepatitis patients who have had a history of heavy drinking for many years (chronic) and with recent excessive alcohol consumption (binge). Chronic plus binge ethanol feeding may also induce steatosis, liver injury and inflammation in mice [23]. Our results revealed that aged and middle-aged mice are more susceptible to chronic plus binge ethanol feeding-induced liver injury and fibrosis by downregulating SIRT1 in both hepatocytes and HSCs.

Materials and methods

Chronic plus binge ethanol feeding model

Wild-type (WT) and C57BL/6N mice were used. Female C57BL/6N mice were predominantly used experimentally, however, male C57BL/6N mice were also used in some instances. HSC-specific SIRT1 knockout ($SIRT1^{HSC-/-}$) mice were obtained via the several steps of crossing with $SIRT1^{flox/flox}$ mice [24] and LratCre$^+$ mice (a kind gift from Dr. Robert Schwabe, Columbia University, NY) [25]. Since Cre in LratCre$^+$ $SIRT1^{flox/flox}$ mice do not efficiently delete the floxed gene in male mice (a personal communication with Dr. Schwabe), only female $SIRT1^{HSC-/-}$ and their WT littermate controls were used in our study.

For the short-term feeding, mice were subjected to short-term (10 days) plus one binge ethanol feeding as previously published [23]. For the long-term feeding, the same protocol was followed with the exception that mice were fed for 8 weeks with multiple binges (twice a week) of ethanol (5 g/kg [b.w.]) feeding as described previously [26]. Animal studies were approved by the NIAAA Animal Care and Use Committee.

Statistical analysis

For statistical analysis, which are expressed as the means ± SEM for each group, GraphPad Prism software (version 5.0a; GraphPad Software, La Jolla, CA) was used. To compare values obtained from three or more groups, a one-way ANOVA was used, followed by a Tukey post-hoc test. To compare values obtained from two groups, the Student's *t* test was performed. *p* values of <0.05 were considered significant.

Further detailed methods are reported in the Supplementary material.

Results

Middle-aged and aged mice are more susceptible to short-term (10 days) plus binge ethanol-induced liver injury and inflammation

Previous studies have reported that chronic ethanol feeding-induced greater liver damage and steatosis in aged rats compared to young rats [14]. In order to test whether aging also exacerbates the chronic plus binge ethanol feeding-induced liver injury in mice, we first tested the short-term 10-day ethanol feeding plus one binge (E10d + 1B) model. After the E10d + 1B ethanol feeding, female middle-aged mice (12–14 months) and aged mice (>16 months) showed significantly higher levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), liver to body weight ratio, and hepatic triglyceride contents when compared to young mice (8–12 weeks) (Fig. 1A, Supplementary Fig. 1A). Both middle-aged and aged mice exhibited greater liver damage when compared to young mice. Due to the cost limitations of housing aged mice, only middle-aged mice were used in the further parts of the study, and compared to young mice.

Hematoxylin and Eosin (H&E) and Oil Red O staining analyses revealed that E10d + 1B treated middle-aged mice exhibited a higher degree of steatosis when compared to young mice that underwent the same ethanol treatment (Fig. 1B). TUNEL analyses demonstrated that the number of TUNEL positive apoptotic hepatocytes were higher in middle-aged mice post E10d + 1B treatment (Fig. 1C). Furthermore, real-time PCR analyses of neutrophil marker (*Ly6g*) demonstrated that E10d + 1B treated middle-aged mice had higher levels of hepatic neutrophil infiltration when compared with young mice, while hepatic expression of *Adgre1*, which encodes the macrophage marker F4/80, was comparable between these two groups (Fig. 1D).

Aging exacerbates long-term (8 weeks) plus multiple binges-induced liver injury and inflammation but attenuates liver regeneration

The above data shows that short-term plus binge ethanol feeding induces liver injury and inflammation. Next, we utilized a long-term 8-week ethanol feeding model with multiples binges (E8w + nB) that caused severe liver injury than that seen in the E10d + 1B ethanol feeding [26]. As illustrated in Fig. 2A–C, E8w + nB feeding-induced increased levels of serum ALT, AST, hepatic triglycerides, and TUNEL positive apoptotic hepatocytes in middle-aged mice when compared to young mice. E8w + nB feeding also induced greater levels of liver inflammation in middle-aged mice than in WT mice (Supplementary Fig. 2). Moreover, liver regeneration was measured by using bromodeoxyuridine (BrdU) immunohistochemistry. As illustrated in Fig. 2D, the number of $BrdU^+$ hepatocytes were lower in E8w + nB-treated middle-aged mice compared to young mice.

Middle-aged mice exhibited stronger oxidative stress post chronic plus binge ethanol feeding when compared to young mice

Oxidative stress was determined by measuring 4-hydroxy-nonenal (4-HNE) adducts and protein nitration levels in livers