Hepatocyte CD73 Mediates Alcohol-Induced Liver Injury in a Sex-Dependent Manner

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ABSTRACT

BACKGROUND Alcoholic liver disease (ALD) caused by excessive drinking is one of the leading causes of chronic liver disease globally. Despite its significance, there is no effective treatment other than abstinence from alcohol. Combined with this clinical challenge, the fact that women have increased susceptibility to liver damage by alcohol compared to men has warranted the need to identify potential regulators in ALD that can be targeted for sex-specific diagnosis, prognosis, and treatment. One promising candidate is ecto-5'-nucleotidase (commonly known as CD73), which generates adenosine from adenosine monophosphate (AMP) in the extracellular space. We have previously shown that the loss of hepatocyte-specific CD73 (CD73-LKO) and the secondary loss of extracellular adenosine led to spontaneous sex-dependent liver injury in mice, which was exhibited as fatty liver, cell death, and inflammation. These changes are generally observed in patients diagnosed with alcoholic steatohepatitis (ASH), the most severe form of ALD with a high mortality rate. Given our observations in mice, we hypothesize that CD73 exerts sex-dependent hepatoprotective functions in alcohol-induced liver injury. **METHODS** Primary hepatocytes were isolated from mice and treated with ethanol. CD73 mRNA and protein levels were analyzed by quantitative polymerase chain reaction and immunoblot, respectively. Male and female WT and CD73-LKO mice were fed the acute-on-chronic ethanol diet, and were compared by serological, histological, and biochemical methods. Untreated hepatocytes and standard chow-fed mice were used as controls. **RESULTS** CD73 is upregulated at the mRNA and protein levels in response to ethanol in primary mouse hepatocytes. Similarly, male and female WT mice, but not CD73-LKO mice, exhibited induction of the CD73-encoding gene *Nt5e* in the liver upon alcohol consumption. In contrast to *Nt5e* expression, CD73 protein levels were altered in a sex-dependent manner, wherein male WT mice had increased levels but female WT mice had decreased levels. Mice fed the ethanol diet had increased levels of serum ALT, compared to their control-fed counterparts. In contrast to WT mice, CD73-LKO had elevated ALT levels, and was associated with increased steatosis, cell death, and inflammation, with greater severity seen in the male. **CONCLUSION** CD73 expressed on hepatocytes is regulated transcriptionally and post-transcriptionally upon alcohol exposure. Moreover, the loss of hepatocyte-CD73 exacerbated alcohol-induced liver injury in mice, which was more prominent in male than female mice. Additional studies will reveal potential sex-dependent factors that may compensate for the loss of CD73 in female mice.

BACKGROUND

- Alcoholic liver disease (ALD) is a spectrum disorder that includes fatty liver or steatosis, hepatitis, and cirrhosis (O'Shea, Dasarathy and McCullough *Hepatology* 2009)
- Global deletion of CD73 in mice prevented the development of ethanol-induced steatosis (Peng et al. J Clin Invest 2009)
- Cell-specific mechanisms by which CD73 mediates ALD are not known
- We generated a hepatocyte-specific CD73KO mouse model and characterized the phenotype under basal conditions and in the setting of ALD

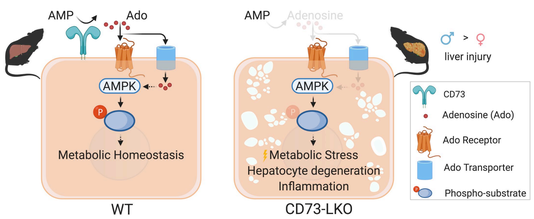


Figure 1. Loss of Hepatocyte CD73 Promotes Spontaneous Liver Injury in Mice in a Sex-Dependent Manner. Our previous studies showed that loss of hepatocyte CD73 and secondary loss of extracellular adenosine lead to steatosis, inflammation, and cell death under basal conditions. Male CD73-LKO mice were more severely affected than the female.

Hypothesis:
CD73 exerts sex-dependent hepatoprotection in alcohol-induced liver injury.

METHOD

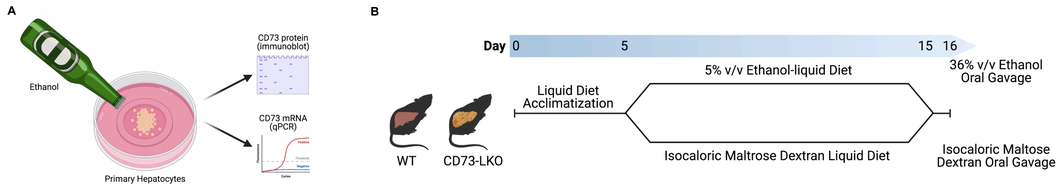


Figure 2. *In vitro* and *in vivo* Analysis of the Role of CD73 upon Alcohol Exposure. (A) Primary hepatocytes were isolated from wild-type (WT) mice expressing CD73, and treated with ethanol in a time- and dose-dependent manner. CD73 protein and mRNA expression were analyzed by immunoblot and quantitative polymerase chain reaction (qPCR), respectively. (B) WT and CD73-LKO mice were fed an isocaloric maltose dextran diet or 5% volume/volume (v/v) liquid diet for 10 days, followed by an oral gavage of 36% v/v ethanol or isocaloric maltose dextran. The alcohol diet promotes the development of steatosis (fatty liver) and inflammation in mice, which mimics acute-on-chronic alcoholic liver injury in patients. (Bertola, et al. *Nat Protoc.* 2013)

RESULTS (*IN VITRO*)

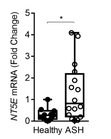


Figure 3. CD73 is Induced in Alcoholic Steatohepatitis (ASH) Patients. Liver biopsies from ASH patients (n=16) and non-ASH, healthy individuals (n=11) were obtained in collaboration with Dr. Ramon Bataller. Messenger RNA (mRNA) was extracted and analyzed by qPCR for the CD73-encoding gene *NT5E*. *p<0.05; unpaired *t*-test.

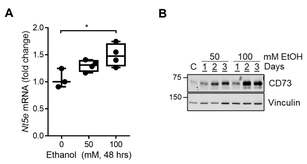


Figure 4. CD73 Expression is Upregulated Upon Alcohol Exposure in Primary Mice Hepatocytes. (A) qPCR analysis of primary hepatocytes showing a dose-dependent induction of *Nt5e* upon alcohol exposure for 48 hours. (B) Representative immunoblot analysis showing a dose- and time-dependent increase in CD73 protein. Vinculin is the loading control. n=3-4 *p<0.05; unpaired *t*-test.

RESULTS (*IN VIVO*)

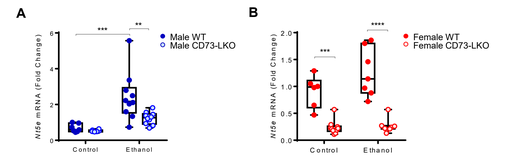


Figure 5. Hepatocyte *Nt5e* is Upregulated in Response to Alcohol *In Vivo*. Consistent with the *in vitro* data on primary hepatocytes, (A-B) *Nt5e* is upregulated in ethanol-fed WT (filled circles) male (blue) and female (red) mice compared to the control-fed group. Notably there is no significant change in CD73 mRNA expression between diet groups in CD73-LKO mice (open circles), suggesting that induction of CD73 is primarily from hepatocytes in the liver. **p<0.01, ***<0.001; two-way ANOVA. Male mice: n=7-8 WT, n=7-11 CD73-LKO; Female mice: n=6-7 WT control, n=8-10 CD73-LKO.

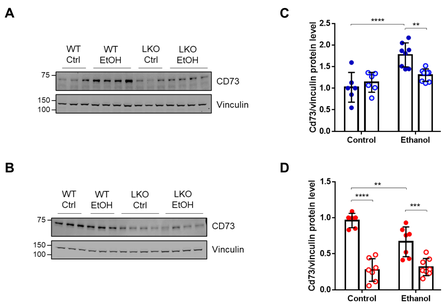


Figure 6. CD73 Protein Expression is Altered in a Sex-Dependent Manner. Representative immunoblot analysis of the total liver shows increased CD73 protein levels in ethanol-fed male WT mice (A) but a decrease in female WT mice (B) compared to the control-fed group. There is no significant change in CD73 protein expression in the total livers of CD73-LKO mice. (C-D) Quantification using densitometry analysis of the immunoblots in A and B. **p<0.01 ***p<0.001, ****p<0.0001. Two-way ANOVA. Male mice: n=6-8 WT, n=6-8 CD73-LKO; Female mice: n=6-8 WT, n=7-8 CD73-LKO.

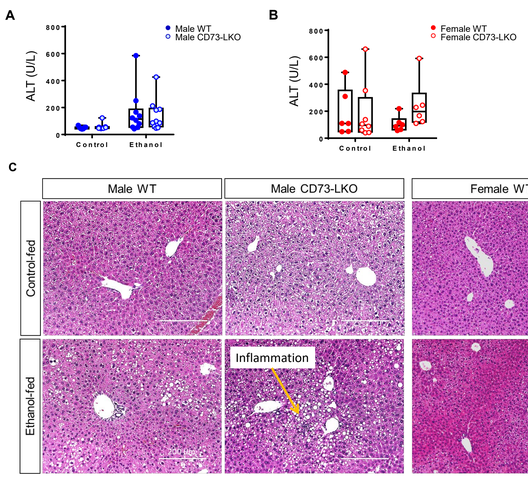


Figure 7. Loss of Hepatocyte CD73 Predisposes Mice to Alcohol-Induced Liver Injury with Male Predominance. (A-B) Serum ALT levels are increased in ethanol-fed male (A) and female (B) mice compared to the control-fed group. CD73-LKO (open circles) mice exhibit elevated ALT levels than WT (filled circles) mice. (C) Representative H&E images of liver tissues display variable signs of steatosis in ethanol-fed WT and CD73-LKO male and female mice, with more prominence in male CD73-LKO mice. Additionally, CD73-LKO male mice exhibit liver inflammation. No significance in ALT values; two-way ANOVA. Male mice: n=7-8 WT, n=7-11 CD73-LKO; Female mice: n=6-7 WT control, n=8-10 CD73-LKO.

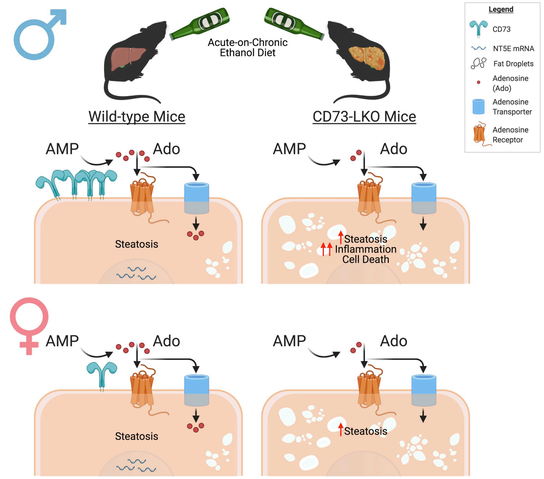
CONCLUSIONS

CD73 is regulated in alcohol-induced liver injury based on the following observations:

1. *NT5E* is induced in humans and mice (transcriptional regulation)
2. Hepatocyte CD73 protein is upregulated in male mice but not female mice (post-transcriptional regulation)

CD73 expressed on hepatocytes is protective against alcohol-induced liver injury based on the following observations in mice:

1. Elevated serum ALT levels (hepatocyte injury marker) in ethanol-fed CD73-LKO mice compared to WT mice
2. Hepatocyte-specific loss of CD73 promotes alcohol-induced liver injury characterized by steatosis and inflammation



FUTURE DIRECTION

- Delineate the hepatoprotective mechanisms of CD73 during alcohol-induced hepatocyte stress
- Determine how the enzymatic function of CD73 mediates hepatocyte response to alcohol
- **More severe alcoholic liver injury in male CD73-LKO mice suggests that sex-dependent factors may compensate for the loss of CD73 in female mice**
  - Identify sex-dependent factors that promote liver injury susceptibility to alcohol
- Examine the significance of these findings in alcoholic liver disease patients
  - CD73 post-transcriptional regulation in ASH and ALD patients
  - CD73 function in ASH and ALD patients

ACKNOWLEDGMENT

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