



Impact of Dyrk1A level on alcohol metabolism



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ABSTRACT

Alcoholic liver diseases arise from complex phenotypes involving many genetic factors. It is quite common to find hyperhomocysteinemia in chronic alcoholic liver diseases, mainly due to deregulation of hepatic homocysteine metabolism. Dyrk1A, involved in homocysteine metabolism at different crossroads, is decreased in liver of hyperhomocysteinemic mice. Here, we hypothesized that Dyrk1A contributes to alcohol-induced hepatic impairment in mice. Control, hyperhomocysteinemic and mice overexpressing Dyrk1A were fed using a Lieber-DeCarli liquid diet with or without ethanol (5% v/v ethanol) for one month, and liver histological examination and liver biochemical function tests were performed. Plasma alanine aminotransferase and homocysteine levels were significantly decreased in mice overexpressing Dyrk1A compared to control mice with or without alcohol administration. On the contrary, the mean plasma alanine aminotransferase and homocysteine levels were significantly higher in hyperhomocysteinemic mice than that of control mice after alcohol administration. Paraoxonase 1 and CYP2E1, two phase I xenobiotic metabolizing enzymes, were found increased in the three groups of mice after alcohol administration. However, NQO1, a phase II enzyme, was only found increased in hyperhomocysteinemic mice after alcohol exposure, suggesting a greater effect of alcohol in liver of hyperhomocysteinemic mice. We observed positive correlations between hepatic alcohol dehydrogenase activity, Dyrk1A and ADH4 protein levels. Importantly, a deleterious effect of alcohol consumption on hepatic Dyrk1A protein level was found. Our study reveals on the one hand a role of Dyrk1A in ethanol metabolism and on the other hand a deleterious effect of alcohol administration on hepatic Dyrk1A level.

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1. Introduction

Alcohol is one of the most commonly used substances in the world and the most socially accepted drug [1]. Excessive drinking and long-term chronic abuse of alcohol represent a major public health issue

Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; ALT, alanine aminotransferase; CYP2E1, cytochrome P450 2E1; CBS, cystathionine beta synthase; CTL, control; DCPIP, 2,6-dichlorophenolindophenol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); Dyrk1A, dual specificity tyrosine (Y) phosphorylation-regulated kinase 1A; FAD, flavin adenine dinucleotide; hcy, homocysteine; hhcy, hyperhomocysteinemia; NAD, nicotinamide adenine dinucleotide, reduced; NQO1, NAD(P)H: quinone oxidoreductase; NRF2, nuclear factor-erythroid 2-related factor 2; PON1, paraoxonase 1; SAHH, S-adenosylhomocysteine hydrolase; TgDyrk1A, mBACtgDyrk1A transgenic mice; XMEs, xenobiotic metabolizing enzymes.

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since 140 million people worldwide are dependent on alcohol. However, only 20–30% of treated patients respond to anti-craving and anti-relapse compounds because of the large variability in treatment response due to complex gene and environment interactions. For this reason, biomarkers such as genetic markers and peripheral protein markers could be used to predict treatment response for those medications. Alcohol abuse significantly contributes to damage in a variety of tissues including liver, which is the primary site of alcohol metabolism, and a major target of injury [2]. Alcoholic liver disease is a significant cause of morbidity and mortality globally and refers to a spectrum of hepatic pathologies resulting from acute/ binge or chronic alcohol exposure/abuse for which disease progression develops in a dose and time dependent manner [3].

Alcohol is first mainly oxidized by alcohol dehydrogenase (ADH) to acetaldehyde, an electrophilic metabolite potentially toxic by formation of adduct with proteins and DNA. At high levels of alcohol consumption, an enzyme of the endoplasmic reticulum, the cytochrome P450 I.E. (CYP2E1), participates in the oxidation of ethanol into acetaldehyde.

Subsequently, acetaldehyde is oxidized to acetate by aldehyde dehydrogenase (ALDH) [4]. In addition, numerous studies have demonstrated deregulation of homeostasis of methionine cycle in alcoholic liver disease.

Indeed, it is quite common to find hyperhomocysteinemia (hhcy) in chronic alcoholism, mainly due to liver damage deregulating methionine metabolism [5]. Alcoholic liver disease is also associated with folate deficiency, and the addition of folate deficiency to a diet containing excessive ethanol enhances abnormal hepatic methionine metabolism and promotes the development of alcoholic liver injury [6]. Homocysteine (hcy) is a critical intermediate of methionine metabolism and has profound importance in health and diseases. Obviously, the metabolism of hcy mainly takes place in liver and growing evidence also indicates that chronic liver injury is closely associated with an impairment of hepatic sulfur-containing amino acid metabolism, which is attributed to abnormality in the critical enzymes involved in the metabolic reactions including cystathionine beta synthase (CBS), the first enzyme involved in the transsulfuration pathway [7–9]. We characterized hepatic lesions in hhcy mice due to CBS deficiency [10] and demonstrated that hhcy alters activities of phase I (paraoxonase 1, PON1) and phase II (NAD(P)H: quinone oxidoreductase, NQO1) xenobiotic metabolizing enzymes (XMEs). These results could reflect a susceptibility to alcohol in case of hhcy [10–13]. More recently, we found that overexpression of the dual specificity tyrosine (Y) phosphorylation-regulated kinase 1A (Dyrk1A) increases the nuclear factor-erythroid 2-related factor 2 (NRF2) quantity, a factor implicated in anti-oxidant and anti-inflammatory responses by controlling NQO1 gene expression [14]. A negative correlation between plasma hcy levels and hepatic expression of Dyrk1A has also been demonstrated [15,16], this kinase being involved in several steps of hcy metabolism [16,17]. Over-expression of Dyrk1A increased the hepatic NQO1 activity [16]. The increased activity of NQO1 is followed by an increase of a by-product of the enzyme reaction, NAD⁺, which is a cofactor of *S*-adenosylhomocysteine hydrolase (SAHH) activity. As hcy can revert back to *S*-adenosylhomocysteine via the SAHH mediated reverse reaction, we also analyzed this activity and found an increased SAHH activity concomitant with decreased plasma hcy level in mice overexpressing Dyrk1A [16]. The positive correlation found between liver Dyrk1A protein expression and CBS activity reinforces the role of this kinase in one carbon metabolism [17].

Lieber-DeCarli ethanol liquid diet has been widely used as a classical approach to mimic chronic alcoholic hepatic injury model in animals [18]. Therefore in the current study, we investigated the contribution of Dyrk1A to hepatic alcohol metabolism by submitting two well characterized murine models to ethanol using the Lieber-DeCarli ethanol diet: the model of hhcy due to CBS deficiency [19], which have a diminished hepatic protein Dyrk1A by 1.5-fold [15] and the BAC transgenic with one copy of the murine Dyrk1A gene, which have an increased hepatic protein Dyrk1A by 1.5-fold [16,20]. We found on the one hand that hepatic Dyrk1A protein level plays a role in ethanol metabolism, and on the other hand an impaired hepatic Dyrk1A protein level during alcohol intake.

2. Materials and methods

2.1. Mice and genotype determination

All procedures were carried out in accordance with internal guidelines of the French Agriculture Ministry for animal handling. Mice were housed in a controlled environment with unlimited access to food and water on 12-h light/dark cycle. Number of mice and suffering were minimized as possible. Mice heterozygous for targeted disruption of the *Cbs* gene (*Cbs* +/-) were generously donated by Dr. N. Maeda (Department of Pathology, University of North Carolina, Chapel Hill, NC, USA) [19]. *Cbs* +/- mice, on a C57BL/6 background were obtained by mating male *Cbs* +/- mice with female wild-type C57BL/6 (CTL) mice. DNA isolated from 4 week-aged mice tail biopsies was subjected

to genotyping of the targeted CBS allele using a polymerase chain reaction (PCR) assay [10]. Female from each genotype from the same litter, two months of age, were used. The murine bacterial artificial chromosome 189 N3 (mBACtgDyrk1A) strain has been constructed by electroporating HM-1 embryonic stem cells with the retrofitted BAC-189N3 [20]. Female mBACtgDyrk1A transgenic mice and control from the same litter, two months of age, were used.

2.2. Ethanol treatment

For alcoholization, mice were randomly divided into 2 groups and fed using a Lieber-DeCarli liquid diet with or without ethanol (5% (v/v) ethanol) ad libitum for one month (Bio Serv) [18]. To accustom the alcohol group mice to the alcohol-containing liquid diet, they were fed with the alcohol-containing liquid diet (1–4% ethanol) ad libitum for 1 week prior to the start of experiments. For the control liquid diet, 35% of energy was derived from fat, 18% from protein, and 47% from carbohydrates; the liquid ethanol diet contained 35% of energy from fat, 18% from protein, 11% from carbohydrates, and 36% from ethanol.

2.3. Preparation of serum samples, tissue collection, and plasma assays

Upon euthanization of mice, blood samples were obtained by retro-orbital sinus sampling with heparinized capillaries, collected into tubes containing a 1/10 volume of 3.8% sodium citrate, and immediately placed on ice. Plasma was isolated by centrifugation at 2500 × *g* for 15 min at 4 °C. Livers were harvested, snap-frozen, and stored at –80 °C until use. Plasma total hcy, defined as the total concentration of hcy after quantitative reductive cleavage of all disulfide bonds, was assayed using the fluorimetric high-performance liquid chromatography (HPLC) method as previously described [21]. Alanine aminotransferase (ALT) level was assayed using the Alanine Aminotransferase Activity Assay Kit (Sigma-Aldrich, France), based on the pyruvate generated.

2.4. Morphological studies

Microtome-generated 8 μm serial sections were collected from liver fixed in 10% formaldehyde on Superfrost slides, dried at 56 °C and stained with Hematoxylin and Eosin to assess liver histology.

2.5. Triglyceride content

The liver triglyceride content was measured with a method based on measurement of glycerol released from triglycerides, using the PicoProbe Triglyceride quantification Assay Kit (Abcam).

2.6. Enzyme activities

CBS activity assay was performed on 300 μg of protein extracts as described previously [22]. Proteins were incubated for 1 h at 37 °C with 1 mM DL-propargylglycine, 0.2 mM pyridoxal 5'-phosphate, 10 mM L-serine, 10 mM DL-hcy, and 0.8 mM *S*-(5'-adenosyl)-L-methionine, using a DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) based-assay. The reaction was performed at 37 °C by measuring the absorbance at 412 nm over 10 min, using a spectrophotometer (Lambda XLS, PerkinElmer). PON1 activity assay was performed on 100 μg of liver protein extracts or 5 μL of plasma. PON1 arylesterase activity toward phenyl acetate was quantified spectrophotometrically using 20 mM Tris-HCl (pH 8.3), 1 mM CaCl₂, and 10 mM phenyl acetate. The reaction was performed at room temperature by measuring the extent of phenyl acetate hydrolysis at 270 nm every 10 s for 1 min using a spectrophotometer (Lambda XLS, PerkinElmer). NQO1 activity was assayed on 150 μg of protein extracts as described [23]. Proteins were incubated for 8 min at room temperature in PBS containing 0.07% bovine serum albumin (pH 7.4) and 0.01% Tween-20. Then, a mixture containing 0.2 mM

β -nicotinamide adenine dinucleotide, reduced (NADH), 5 μ M flavin adenine dinucleotide (FAD), and 25 mM Tris-HCl (pH 7.4) was added to the protein preparations. Two conditions were prepared, with or without 10 μ M of dicoumarol (Calbiochem, MERCK) used to specifically block NQO1. The reaction was started by adding 40 μ M of 2,6-dichlorophenolindophenol (DCPIP). The reduction of DCPIP was assayed by measuring the absorbance at 600 nm every 30 s for 3 min using a spectrophotometer (Lambda XLS, PerkinElmer). NQO1 activity was determined by subtraction of the activity recorded in the presence of dicoumarol. ADH activity assay was performed using the Alcohol Dehydrogenase Activity Assay Kit (Sigma-Aldrich) via a colorimetric method, using 170 μ g protein extracts and isopropanol as the substrate. The absorbance was measured at 450 nm after 2–3 min of incubation at 37 °C and then every 5 min up to 30 min. ALDH activity assay was performed using the Aldehyde Dehydrogenase Activity Assay kit (Sigma-Aldrich) via a colorimetric method, using 170 μ g of protein extracts and acetaldehyde as the substrate. The absorbance was measured at 450 nm after incubation for 5 min at room temperature and then every 2–3 min up to 30 min.

2.7. Protein extraction and analysis

Protein samples were prepared by homogenizing liver in 500 μ L phosphate-buffered saline with a cocktail of proteases inhibitors. Protein concentrations were detected with the Bio-Rad Protein Assay reagent (Bio-Rad). To assess the relative amount of proteins, we used a slot blot method previously developed [24]. Protein preparations were blotted on Hybond-C Extra membrane (GE Healthcare Europe GmbH) using Bio-Dot SF Microfiltration Apparatus (Bio-Rad). After transfer, membranes were saturated by incubation in 5% w/v nonfat milk powder in Tris-saline buffer (1.5 mM Tris base, pH 8; 5 mM NaCl; 0.1% Tween-20), and incubated overnight at 4 °C with an antibody directed against ADH1 (1/1000, Acris Antibodies, San Diego, CA), ADH4 (1/1000, Abgent, San Diego, CA), CYP2E1 (1/10,000, rabbit antiserum against CYP2E1 kindly provided by Isabelle De Waziers) and DYRK1A (1/500, Abnova corporation, Tebu, France). Binding of the primary antibody was detected by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Tebu, France). Ponceau-S coloration (Sigma-Aldrich, France) was used as an internal control. Digitized images of the immunoblots obtained using an LAS-3000 imaging system (Fuji Photo Film Co., Ltd.) were used for densitometric measurements with an image analyzer (UnScan It software, Silk Scientific Inc.).

2.8. Isolation of Total RNA and analysis by qPCR

Total RNAs were extracted using the RNeasy mini kit (Qiagen, Les Ulis, France) and reverse transcription was performed using the cDNA high-capacity archive kit (Applied Biosystems) as previously described [25]. Gene-specific primers used for the real-time PCR were designed using the OLIGO Explorer software (Molecular Biology Insights, Inc., Cascade, CO, USA), and are described in Supporting Table S1. Quantitative real-time PCR was carried out in a 10 μ L reaction volume containing 40 ng of cDNA, 300 nM of each primer and Absolute QPCR SYBR Green (Abgene, Villebon sur Yvette, France) using an ABI Prism 7900 Sequence Detector system (Applied Biosystems). PCR cycles consisted of the following steps: Taq activation (15 min, 95 °C), denaturation (15 s, 95 °C) and annealing and extension (1 min, 60 °C). The threshold cycle (Ct) was measured as the number of cycles for which the reporter fluorescent emission first exceeds the background. The relative amounts of mRNA were estimated using the $2^{-\Delta\Delta Ct}$ method with GAPDH and HPRT1 for normalization.

2.9. Blood alcohol concentration

Mice were sacrificed at 90 min after a single dose of ethanol (2 g/kg) administered by intraperitoneal injection. Whole blood was collected,

and centrifuged at 3000 rpm at 4 °C for 20 min. Serum was isolated and blood alcohol concentration was measured using the Enzychrom Ethanol Assay Kit (BioAssay Systems, USA).

2.10. Data analysis

For multiple pairwise comparisons between genotypes and treatments, statistical analysis was done with one-way ANOVA followed by Fisher post-hoc test using Statview software. The results are expressed as medians with interquartile ranges. Data were considered significant when $p < 0.05$. Correlations were determined by using Spearman's rank correlation, as data were not normally distributed according to Shapiro-Wilk test.

3. Results

3.1. Effects of alcohol administration on liver histology, liver triglyceride content, and plasma ALT levels

To investigate histopathological features present in liver of mice, hematoxylin and eosin staining were used. Livers from mice without alcohol administration showed normal histological features. Livers from Cbs +/- mice showed foci of peribulbar mononuclear inflammatory infiltrate around the vessels after alcohol administration (Cbs +/- 5%). This histological feature was not detected in liver of CTL (CTL 5%) and TgDyrk1A (TgDyrk1A 5%) mice after alcohol administration (Fig. 1A). In addition to inflammation, CTL (CTL 5%), Cbs +/- (Cbs +/- 5%) and TgDyrk1A (TgDyrk1A 5%) mice after alcohol administration possessed cytoplasmic macrovesicular lipid droplets, which are more extensive in liver of CTL and Cbs +/- mice (Fig. 1A). Lipid droplets consist mainly of triglycerides, we therefore measured liver triglyceride content. Liver triglyceride contents were significantly increased in CTL (CTL 5%) and Cbs +/- (Cbs +/- 5%), but not in TgDyrk1A (TgDyrk1A 5%) after alcohol administration compared to mice on diet without ethanol (CTL, Cbs +/-, TgDyrk1A) (Fig. 1B). Note that liver triglyceride contents were significantly higher in CTL (CTL 5%) and Cbs +/- (Cbs +/- 5%) compared TgDyrk1A (TgDyrk1A 5%) after alcohol administration (Fig. 1B).

Hepatocellular injury often results in an increase in serum ALT level that is used as a marker for liver injury. To determine the effect of alcohol administration on liver damage, we quantified plasma levels of ALT. Plasma ALT levels were significantly decreased in TgDyrk1A compared to CTL mice with (TgDyrk1A 5% vs CTL 5%) or without (TgDyrk1A vs CTL) alcohol administration (Fig. 1). On the contrary, the mean plasma ALT levels in Cbs +/- mice (Cbs +/- 5%) were significantly higher than that of CTL mice (CTL 5%) after alcohol administration (Fig. 1). Note that CTL, TgDyrk1A, and Cbs +/- mice after alcohol administration (Cbs +/- 5%, TgDyrk1A 5%, CTL 5%) also showed an increased plasma ALT levels compared to mice on diet without ethanol (Cbs +/-, TgDyrk1A, CTL) (Fig. 1C).

3.2. Effects of alcohol administration on hcy metabolism

We also analyzed the effect of alcohol administration on plasma hcy levels, reflecting mainly liver production. TgDyrk1A mice (TgDyrk1A 5%) showed decreased plasma hcy levels compared to CTL mice (CTL 5%) after administration of alcohol (Fig. 2A). On the contrary, alcohol consumption increased plasma hcy levels in Cbs +/- mice (Cbs +/- 5%) compared to CTL mice with alcohol administration (CTL 5%) and Cbs +/- mice (Fig. 2A).

CTL, TgDyrk1A, and Cbs +/- mice after alcohol administration (Cbs +/- 5%, TgDyrk1A 5%, CTL 5%) also showed a decreased CBS activity compared to mice on diet without ethanol (Cbs +/-, TgDyrk1A, CTL) (Fig. 2B). However, TgDyrk1A mice (TgDyrk1A 5%) showed an increased hepatic CBS activity compared to CTL mice (CTL 5%) after alcohol administration (Fig. 2B). On the contrary, Cbs

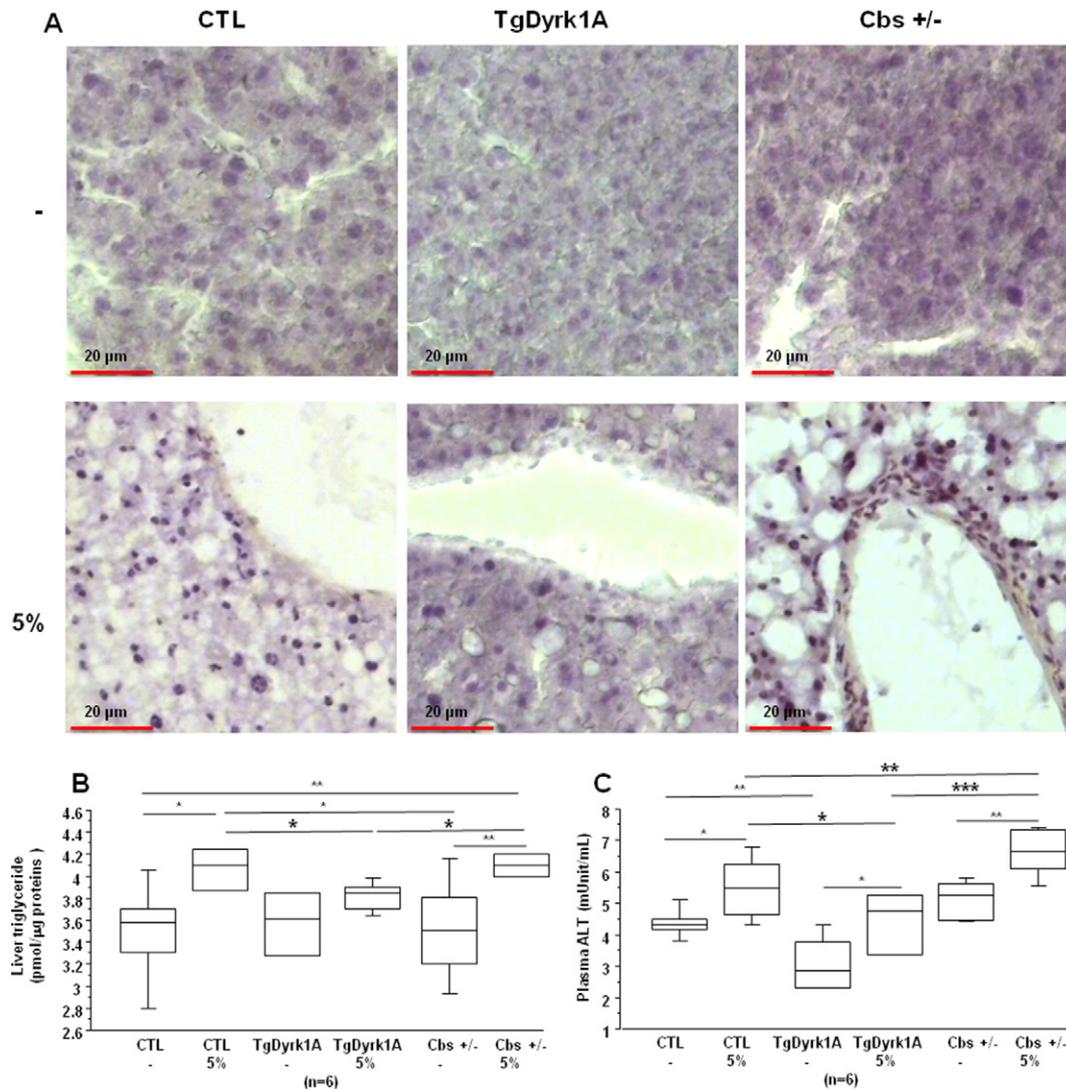


Fig. 1. Liver histology assessed by hematoxylin and eosin staining (A), liver triglyceride (B) and plasma ALT (C) levels in control (CTL), mBACtgDyrk1A transgenic (TgDyrk1A) and CBS-deficient (Cbs +/-) mice fed a Lieber–DeCarli liquid diet with (5%) or without (–) ethanol. Statistical analysis was done with one-way ANOVA followed by Fisher post-hoc test. Data correspond to the medians with interquartile ranges. n = number of mice. *p < 0.05; **p < 0.005; ***p < 0.0005. Scale bars: 20 μm.

+/- mice (Cbs +/- 5%) showed a decreased hepatic CBS activity compared to CTL mice (CTL 5%) after alcohol administration (Fig. 2B). We observed a positive correlation between plasma hcy and ALT levels for mice on Lieber–DeCarli liquid diet with (5%) ($p < 0.02$; $r = 0.621$) or without (–) ($p < 0.01$; $r = 0.635$) ethanol (Fig. 2C). In addition, there is a negative correlation between plasma hcy levels and hepatic CBS activity for mice on Lieber–DeCarli liquid diet with (5%) ($p < 0.007$; $r = -0.723$) or without (–) ($p < 0.04$; $r = -0.527$) ethanol (Fig. 2D).

3.3. Effects of alcohol administration on hepatic XMEs

To investigate the effect of alcohol administration on hepatic XMEs, we analyzed PON1 as phase I and NQO1 as phase II XMEs. The mean hepatic PON1 activity after alcohol administration in CTL mice (CTL 5%), TgDyrk1A mice (TgDyrk1A 5%) and Cbs +/- mice (Cbs +/- 5%) were significantly higher than that of mice on control diet (CTL, TgDyrk1A and Cbs +/- mice respectively) (Fig. 3A). The mean hepatic NQO1 activity after alcohol administration in Cbs +/- mice (Cbs +/- 5%) was significantly higher than that of Cbs +/- mice (Cbs +/-) (Fig. 3B). However, no effect was found for CTL (CTL 5%) and TgDyrk1A mice (TgDyrk1A 5%) after alcohol administration compared to mice on diet without alcohol administration (CTL and TgDyrk1A respectively) (Fig. 3B). Taken together, alcohol consumption causes an activation of

hepatic PON1 activity in the three groups of mice, but only in Cbs +/- mice for hepatic NQO1 activity.

3.4. Effects of alcohol administration on hepatic alcohol metabolizing enzymes

To investigate the effect of alcohol administration on hepatic alcohol metabolizing enzymes, we first analyzed CYP2E1 expression, since this phase I XME is involved in ethanol metabolism. Alcohol exposure increased hepatic CYP2E1 protein levels in the three groups of mice (CTL 5%, Cbs +/- 5% and TgDyrk1A 5%) (Fig. 4A). On the contrary, alcohol administration decreased hepatic Cyp2e1 mRNA levels in the three groups of mice (CTL 5%, Cbs +/- 5% and TgDyrk1A 5%) (Table 1).

The mean hepatic ALDH activity in Cbs +/- mice with (Cbs +/- 5%) or without (Cbs +/-) alcohol administration was significantly higher than that of CTL mice (CTL) (Fig. 4B). However, no effect was found for the three groups of mice after chronic alcohol administration compared to the three groups of mice on diet without alcohol administration (Fig. 4B). Moreover, any effect was found at the mRNA expression level (Table 1).

Regarding hepatic ADH activity, we observed a decreased hepatic ADH activity in CTL mice (CTL 5%) after alcohol administration compared to CTL mice (CTL) (Fig. 4C). Moreover TgDyrk1A mice after

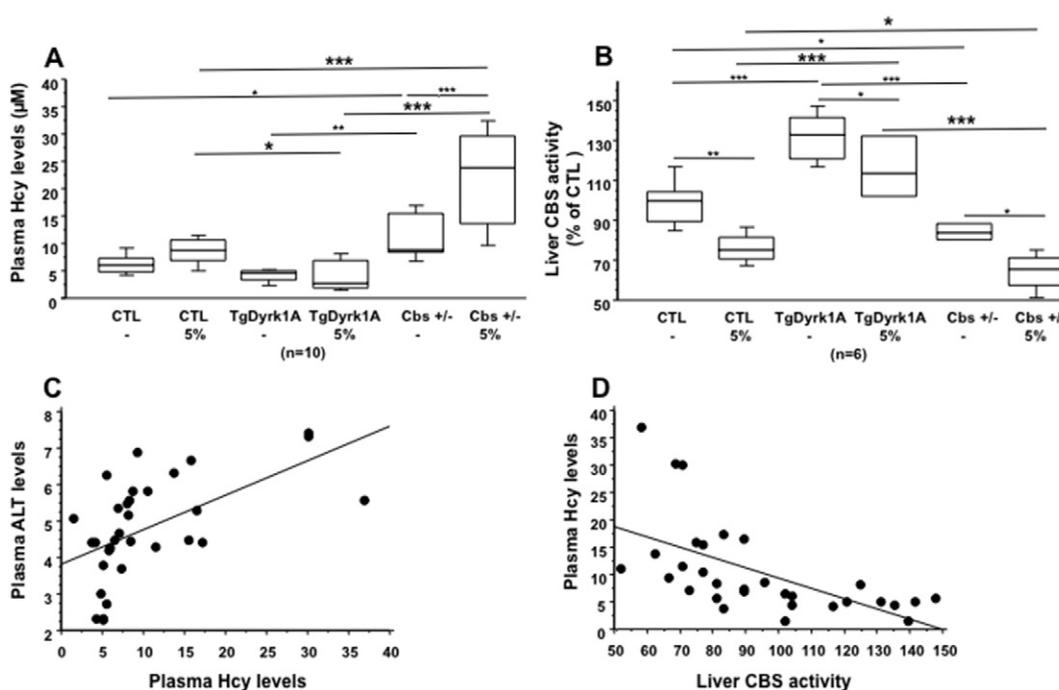


Fig. 2. Plasma hcy levels (A) and hepatic CBS activity (B) in control (CTL), mBACtgDyrk1A transgenic (TgDyrk1A) and CBS-deficient (Cbs +/-) mice fed a Lieber-DeCarli liquid diet with (5%) or without (-) ethanol. Statistical analysis was done with one-way ANOVA followed by Fisher post-hoc test. Data of CBS activity were normalized to the mean of control (CTL) mice. Data correspond to the medians with interquartile ranges. n = number of mice. *p < 0.05; ***p < 0.0005. (C) Levels of plasma ALT were positively correlated with plasma hcy for mice on Lieber-DeCarli liquid diet with (5%) (p < 0.02; r = 0.621) or without (-) (p < 0.01; r = 0.635) ethanol. (D) Levels of plasma hcy and hepatic CBS activity were negatively correlated for mice on Lieber-DeCarli liquid diet with (5%) (p < 0.007; r = -0.723) or without (-) (p < 0.04; r = -0.527) ethanol.

chronic alcohol administration (TgDyrk1A 5%) showed the same mean hepatic ADH activity than that of CTL mice (CTL) (Fig. 4C). In contrast, we showed that Cbs +/- mice with (Cbs +/- 5%) or without (Cbs +/-) alcohol administration have a significantly reduction compared to CTL and TgDyrk1A mice (CTL and TgDyrk1A) (Fig. 4C). Given that ADH1 and ADH4 are the major ADH in ethanol oxidation, we also analyzed ADH1 and ADH4 protein and mRNA levels. Alcohol administration decreased *Adh1* and *Adh4* levels in the three groups of mice (CTL 5%, Cbs +/- 5% and TgDyrk1A 5%) compared to control mice (CTL) (Table 1). While expression of ADH1 protein was similar for the three groups of mice (data not shown), TgDyrk1A mice with alcohol administration (TgDyrk1A 5%) showed an increased hepatic ADH4 protein level compared to CTL and Cbs +/- on diet supplemented with alcohol (CTL 5%, Cbs +/- 5%) (Fig. 4D). Similar results were observed for TgDyrk1A mice (TgDyrk1A) compared to CTL and Cbs +/- on control diet (CTL, Cbs +/-) (Fig. 4D). We also observed a positive correlation between hepatic ADH activity and ADH4 protein levels for mice on Lieber-DeCarli liquid diet with (5%) (p < 0.04; r = 0.421) or without (-) (p < 0.03; r = 0.586) ethanol (Fig. 4E).

3.5. Effects of alcohol administration on hepatic Dyrk1A level

To determine the effect of alcohol administration on Dyrk1A level, protein expression was examined in liver of mice. A decreased hepatic Dyrk1A level in CTL mice after alcohol exposure (CTL 5%) and Cbs +/- mice with (Cbs +/- 5%) or without (Cbs +/-) alcohol administration was shown compared to control mice (CTL) (Fig. 5A). On the contrary, TgDyrk1A mice with (TgDyrk1A 5%) or without (TgDyrk1A) alcohol administration showed an increased hepatic Dyrk1A level compared to CTL with (CTL 5%) or without (CTL) alcohol administration respectively (Fig. 5A). We observed a negative correlation between serum ALT and hepatic Dyrk1A level for mice on Lieber-DeCarli liquid diet with (5%) (p < 0.01; r = -0.462) or without (-) (p < 0.03; r = -0.558) ethanol (Fig. 5B), and between plasma hcy and hepatic Dyrk1A level for mice on Lieber-DeCarli liquid diet with (5%) (p < 0.01; r = -0.462) or without (-) (p < 0.0002; r = -0.689) ethanol (Fig. 5C). A positive correlation was observed between hepatic CBS activity and Dyrk1A levels for mice on Lieber-DeCarli liquid diet with (5%) (p < 0.009; r = 0.669) or without (-) (p < 0.008; r = 0.682)

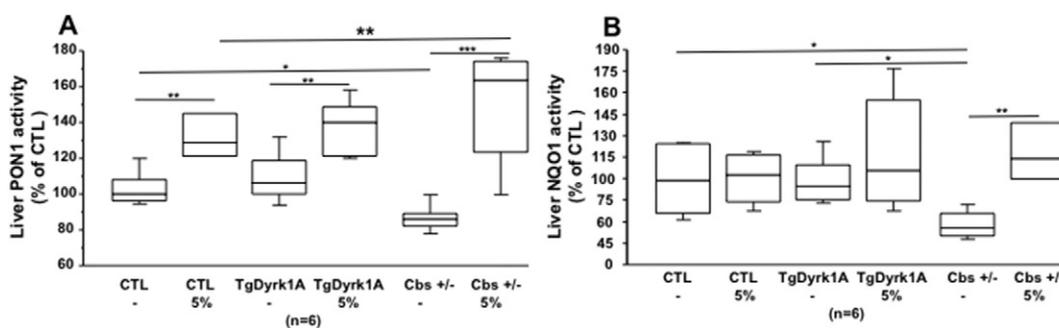


Fig. 3. Hepatic PON1 (A) and NQO1 (B) activity in control (CTL), mBACtgDyrk1A transgenic (TgDyrk1A) and CBS-deficient (Cbs +/-) mice fed a Lieber-DeCarli liquid diet with (5%) or without (-) ethanol. Statistical analysis was done with one-way ANOVA followed by Fisher post-hoc test. Data were normalized to the mean of control (CTL) mice. Data correspond to the medians with interquartile ranges. n = number of mice. *p < 0.05; **p < 0.005; ***p < 0.0005.

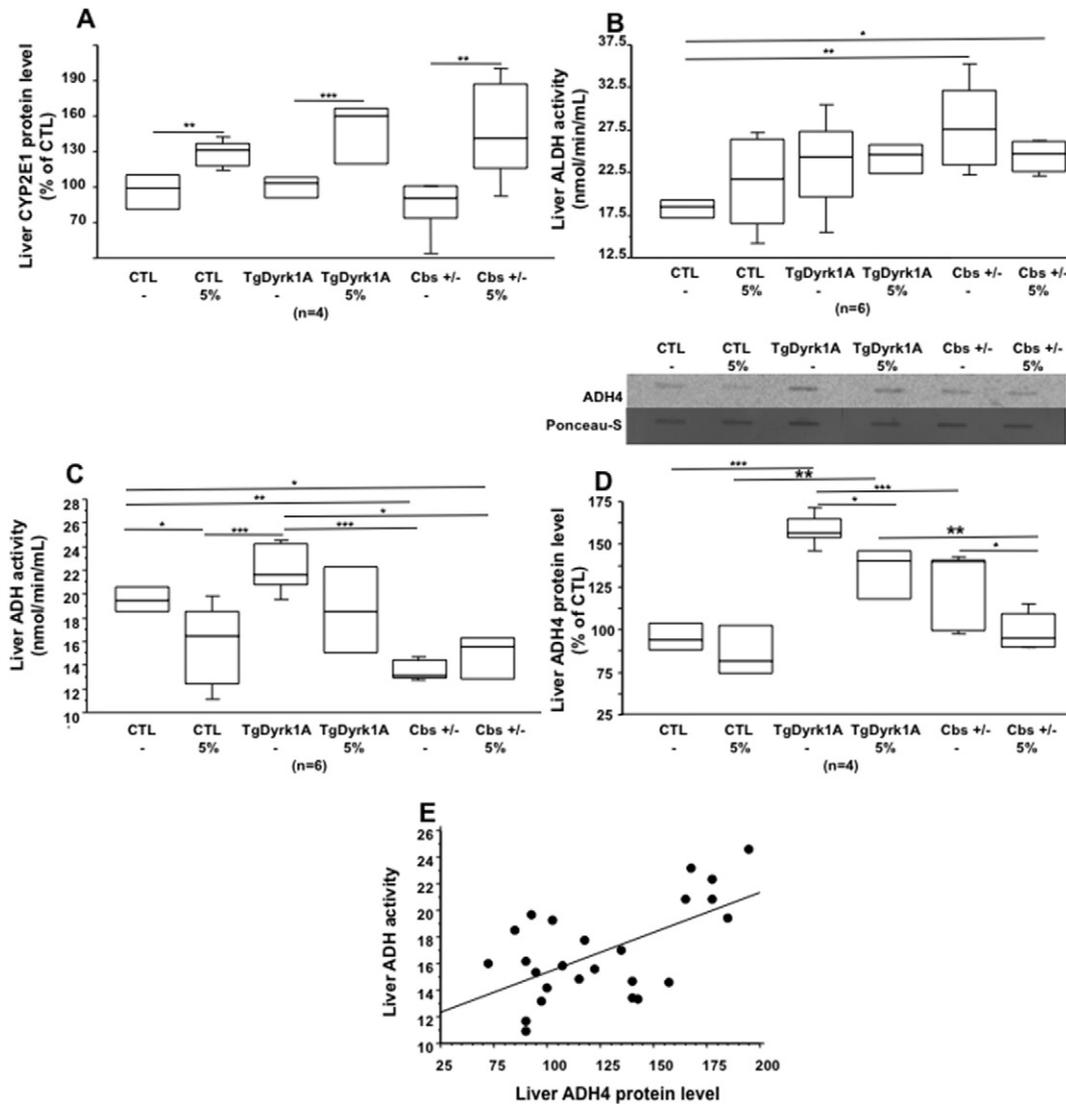


Fig. 4. Hepatic CYP2E1 protein level (A), ALDH activity (B), ADH activity (C) and ADH4 protein level (D) in control (CTL), mBACTgDyrk1A transgenic (TgDyrk1A) and CBS-deficient (Cbs +/-) mice fed a Lieber–DeCarli liquid diet with (5%) or without (–) ethanol. CYP2E1 and ADH4 levels were determined by slot blotting, and values were obtained by normalization of images from CYP2E1 or ADH4 to total proteins marked with Ponceau-S. Statistical analysis was done with one-way ANOVA followed by Fisher post-hoc test. Data of CYP2E1 and ADH4 protein levels were normalized to the mean of control (CTL) mice. Data correspond to the medians with interquartile ranges. n = number of mice. * $p < 0.05$; ** $p < 0.005$. (E) Levels of hepatic ADH activity and ADH4 proteins were positively correlated for mice on Lieber–DeCarli liquid diet with (5%) ($p < 0.04$; $r = 0.421$) or without (–) ($p < 0.03$; $r = 0.586$) ethanol.

ethanol (Fig. 5D), between ADH activity and Dyrk1A level for mice on Lieber–DeCarli liquid diet with (5%) ($p < 0.03$; $r = 0.362$) or without (–) ($p < 0.007$; $r = 0.726$) ethanol (Fig. 5E), and between ADH4 and Dyrk1A protein level for mice on Lieber–DeCarli liquid diet with (5%) ($p < 0.05$; $r = 0.545$) or without (–) ($p < 0.05$; $r = 0.524$) ethanol (Fig. 5F).

3.6. Blood ethanol concentration in mice

In order to analyze the effect of Dyrk1A on ethanol metabolism, alcohol concentration in blood 90 min after a single dose of ethanol (2 g/kg) administered by intraperitoneal injection was measured. We observed a significant increase in blood of Cbs +/- mice compared to CTL and TgDyrk1A mice, and, on the contrary, a significant decrease in blood of TgDyrk1A mice compared to CTL and Cbs +/- mice (Fig. 6).

4. Discussion

The biochemical liver function tests showed mild elevation of plasma ALT levels in hhcy mice and in control mice, the level being higher

in hhcy mice after chronic alcohol administration. ALT and AST are highly concentrated in the liver. AST is also represented in the heart, kidneys, brain and red blood cells, and ALT has low concentration in skeletal muscle and kidney. Therefore, an increase in serum ALT levels is more specific for liver damage [26]. The ALT levels are in agreement with histopathological features present in liver of hhcy mice. Inflammation was detected in liver of hhcy after chronic alcohol administration, while liver triglyceride content was higher in control and hhcy mice, but not in mice overexpressing Dyrk1A after alcohol administration. In contrast, mice overexpressing Dyrk1A have a decreased plasma ALT level compared to control mice after chronic alcohol administration. Dyrk1A has been previously found to have a positive effect on NRF2, a factor implicated in anti-inflammatory response [14]. These results underline the hepatoprotective effect of Dyrk1A on chronic alcohol consumption in mice. The positive correlation between plasma ALT and hcy levels also demonstrates the deleterious effect of hepatic impairment on hhcy in chronic alcoholism. Moderate hhcy is common in chronic alcoholism, mainly in those with liver damage, suggesting that liver impairment is the most important mechanism for the elevated plasma hcy found in these patients [27]. Ethanol consumption resulted in a significant

Table 1

Quantitative PCR analysis of Aldh2, Adh1, Adh4 and Cyp2e1 in liver of control (CTL), CBS-deficient (Cbs +/-) and mBACtgDyrk1A transgenic (TgDyrk1A) fed a Lieber–DeCarli liquid diet with (5%) or without (–) ethanol.

	Aldh2	Adh1	Adh4	Cyp2e1
CTL	1.04 ± 0.24	1.02 ± 0.16	1.02 ± 0.15	1.01 ± 0.15
–	(n = 4)	(n = 4)	(n = 4)	(n = 4)
CTL	0.77 ± 0.11	0.48 ± 0.12**	0.25 ± 0.05*,\$\$,#	0.39 ± 0.14**,#
5%	(n = 7)	(n = 7)	(n = 7)	(n = 7)
Cbs +/-	0.88 ± 0.07	0.68 ± 0.16	0.89 ± 0.37	0.61 ± 0.19
–	(n = 5)	(n = 5)	(n = 5)	(n = 5)
Cbs +/-	1.03 ± 0.24	0.66 ± 0.21*	0.27 ± 0.07*,\$\$	0.43 ± 0.12*
5%	(n = 6)	(n = 6)	(n = 6)	(n = 6)
TgDyrk1A	0.89 ± 0.11	0.66 ± 0.09*	1.13 ± 0.47	0.93 ± 0.30
–	(n = 5)	(n = 6)	(n = 6)	(n = 6)
TgDyrk1A	0.68 ± 0.18	0.61 ± 0.25**	0.17 ± 0.07*,\$#	0.34 ± 0.06*,\$#
5%	(n = 4)	(n = 4)	(n = 4)	(n = 4)

Statistical analysis was done with one-way ANOVA followed by Bonferroni-Dunn post-hoc test. Data were normalized to the mean of control (CTL) mice. Results are means ± SEM. n = number of mice.

- * p < 0.005 vs CTL.
- ** p < 0.001 vs CTL.
- \$\$ p < 0.001 vs Cbs +/-.
- # p < 0.005 vs TgDyrk1A.

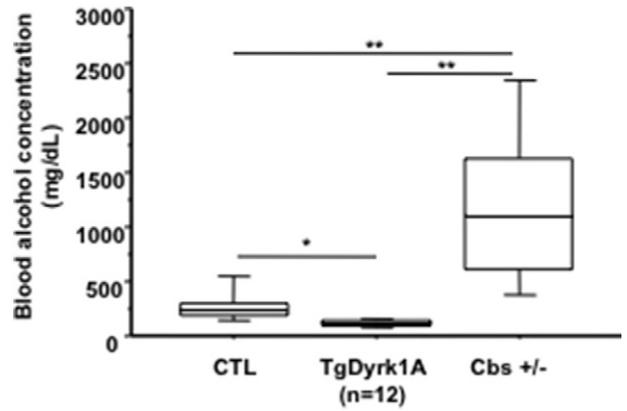


Fig. 6. Blood alcohol concentration in control (CTL), mBACtgDyrk1A transgenic (TgDyrk1A) and CBS-deficient (Cbs +/-) mice 90 min after a single dose of ethanol (2 g/kg) administered by intraperitoneal injection. n = number of mice. *p < 0.005; **p < 0.0005.

disturbance in hepatic sulfur amino acid metabolism closely associated with chronic liver injury, resulting in hhcy [9]. This dysregulation was attributed to abnormality in the enzymes involved in the metabolic reactions including CBS [28]. Pyridoxal-5'-phosphate is one of the cofactor

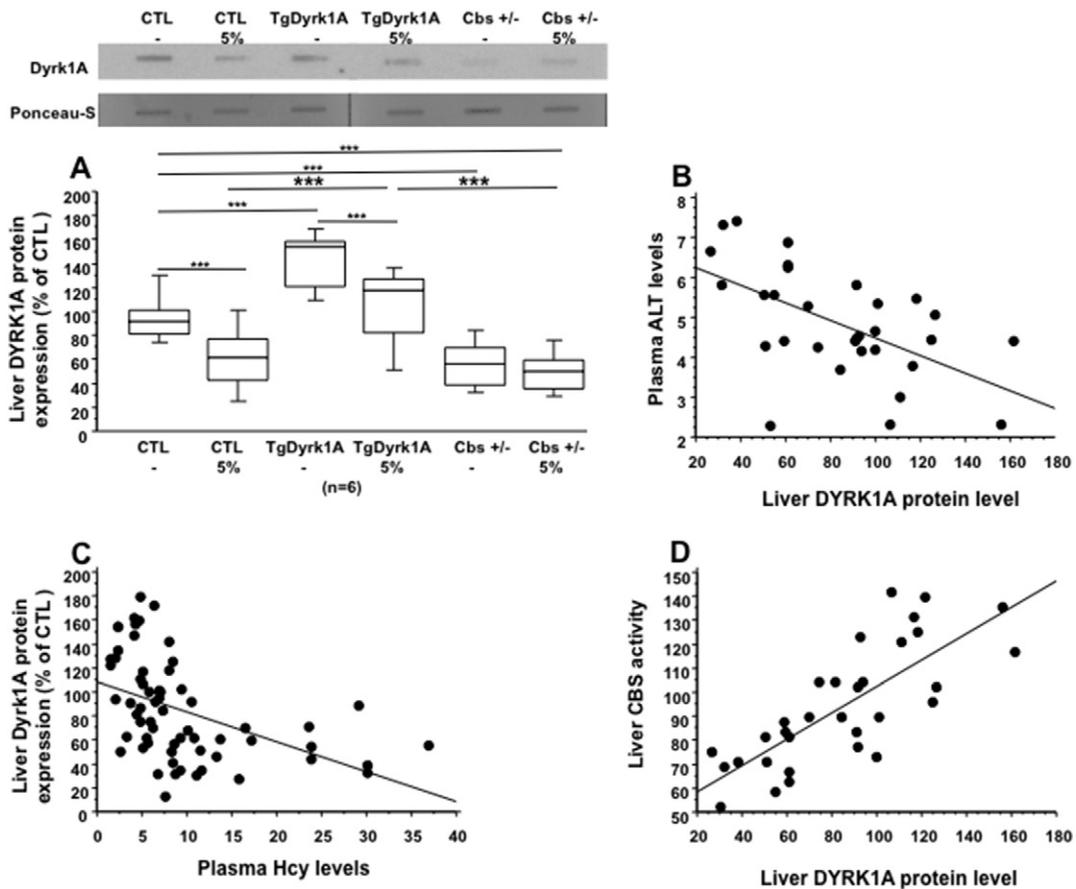


Fig. 5. Hepatic Dyrk1A protein level (A) in control (CTL), mBACtgDyrk1A transgenic (TgDyrk1A) and CBS-deficient (Cbs +/-) mice fed a Lieber–DeCarli liquid diet with (5%) or without (–) ethanol. Dyrk1A level was determined by slot blotting, and values were obtained by normalization of images from Dyrk1A to total proteins marked with Ponceau-S. Statistical analysis was done with one-way ANOVA followed by Fisher post-hoc test. Data were normalized to the mean of control (CTL) mice. Data correspond to the medians with interquartile ranges. n = number of mice. **p < 0.005; ***p < 0.0005. (B) Levels of plasma ALT were negatively correlated with hepatic Dyrk1A proteins for mice on Lieber–DeCarli liquid diet with (5%) (p < 0.01; r = –0.462) or without (–) (p < 0.03; r = –0.558) ethanol. (C) Levels of plasma hcy and hepatic Dyrk1A proteins were negatively correlated for mice on Lieber–DeCarli liquid diet with (5%) (p < 0.01; r = –0.462) or without (–) (p < 0.0002; r = –0.689) ethanol. (D) Levels of hepatic CBS activities and Dyrk1A proteins were positively correlated for mice on Lieber–DeCarli liquid diet with (5%) (p < 0.009; r = 0.669) or without (–) (p < 0.008; r = 0.682) ethanol. (E) Levels of hepatic ADH activities were positively correlated with hepatic Dyrk1A proteins for mice on Lieber–DeCarli liquid diet with (5%) (p < 0.03; r = 0.362) or without (–) (p < 0.007; r = 0.726) ethanol. (F) Levels of hepatic ADH4 and Dyrk1A proteins were positively correlated for mice on Lieber–DeCarli liquid diet with (5%) (p < 0.05; r = 0.545) or without (–) (p < 0.05; r = 0.524) ethanol.

required for CBS function. Deleterious effect of ethanol oxidation on pyridoxal-5'-phosphate metabolism is mediated at least in part by acetaldehyde which displaces this coenzyme from protein binding. The deleterious effect of ethanol on hepatic pyridoxal-5'-phosphate metabolism has been demonstrated to be mediated by acetaldehyde, thereby enhancing its degradation [29]. Here, we found that administration of alcohol increased plasma hcy level by decreasing the rate of catabolism of hcy in control and hhcy mice, but not in mice overexpressing Dyrk1A. We also observed a significant negative correlation between plasma hcy levels and hepatic CBS activity. Thus, it can be suggested a hepatoprotective effect of Dyrk1A via an improvement of impaired sulfur amino acid metabolism.

We previously demonstrated a positive correlation between the liver CBS activity and liver Dyrk1A protein expression [17]. Here we observed, not only the positive correlation between hepatic Dyrk1A levels and CBS activity, but also a negative correlation between plasma hcy and hepatic Dyrk1A levels. By the use of an adenoviral construct designed to restrict expression of Dyrk1A to hepatocytes, we previously found an elevation of liver pyridoxal-5'-phosphate content, which is consistent with an increase in liver CBS activity and a decrease in plasma hcy levels in hhcy [30]. In addition, there is a negative correlation between serum ALT and hepatic Dyrk1A levels. Taken together, these results highlight the beneficial effect of Dyrk1A overexpression on the deleterious effect of alcohol on hepatic hcy metabolism. It is interesting to note that after chronic administration of alcohol, mice overexpressing Dyrk1A showed a protein amount comparable to untreated control mice. However, deleterious effect of alcohol consumption on hepatic Dyrk1A levels was also found.

It has been reported that intracellular Ca^{2+} levels are unquestionably involved in the processes of cellular injury and death. Elevated intracellular Ca^{2+} levels activate a family of Ca^{2+} -regulated cysteine proteases called calpains, including calpain 1 and calpain 2, which mediate regulatory cleavage of specific substrates involved in apoptosis and necrosis [31]. We previously demonstrated that calpains are activated in liver of hhcy mice [15,32] and are able to degrade Dyrk1A [15]. Previous studies found that chronic alcohol consumption increased the level of calpain 1 and calpain 2 expression [33,34], and increased calpain 2 protein expression was associated with decreased CBS activity [34]. PON1, a phase I XME, is altered in liver of hhcy mice due to CBS deficiency [10–12]. PON1 is a Ca^{2+} -dependent HDL-associated ester hydrolase that catalyzes the hydrolysis of organophosphates, aromatic carboxylic acid esters, and carbamates. We found an increased PON1 activity after chronic alcohol administration in the three groups of mice. Moderate alcohol consumption has been found to be associated with an increase in serum PON1 activity and HDL cholesterol in normal volunteers during 3 weeks as compared to non-alcohol consumption [35]. Light ethanol feeding caused an increase in PON1 activity in both serum and liver of rats compared with pair-fed control rats [36]. Moreover, even if PON1 enzymatic activity is decreased, probably as a consequence of inactivation by lipid peroxides and/or to HDL structural alterations coexisting in alcoholic liver disease, serum PON1 concentration is significantly increased in chronic alcohol abusers with liver diseases, probably as a response to enhanced oxidative stress [37]. We also found an increased CYP2E1 protein level after chronic alcohol administration in the three groups of mice, but this result was not found at the mRNA level. Cytochrome P450 family is also a member of phase I XMEs. Previous results have demonstrated that alcohol intake induces the activity and expression of CYP2E1 enzyme, notably in hhcy pigs [6,38]. The catalytic activity of CYP2E1 enzyme requires oxygen activation, resulting in oxidative stress in liver [39]. Acute and chronic alcohol exposure leads to enhance reactive oxygen species generation and a concomitant reduction of antioxidant levels, culminating in oxidative stress. To compensate for these oxidative stress insults, higher animals have evolved physiological defense mechanisms, including antioxidant proteins and phase II XMEs [40]. Induction of phase II enzymes renders cells more resistant to the potential subsequent

challenges of greater stress. We found an increase of NQO1 activity only in hhcy mice after chronic alcohol administration, suggesting a greater effect of alcohol in liver of hhcy mice than that of control mice and mice overexpressing Dyrk1A. Studies have shown that silencing of heme oxygenase 1 and NQO1 results in increased expression of inflammatory cytokines in vitro [41], whereas induction of these enzymes protects against excess proinflammatory responses [41]. In vitro and in vivo results provide evidence that ethanol-induced oxidative stress activates the expression of heme oxygenase 1 and NQO1 in liver cells [42], and suggest that therapeutic modalities to induce phase II detoxification enzymes utilizing selective activators of NRF2 may be beneficial in preventing alcohol mediated liver inflammation and injury. The greater deleterious effect of alcohol on liver function in hhcy mice was also demonstrated by the analysis of alcohol metabolizing enzymes. ADH activity was reduced when the liver damage progressed to cirrhosis [43]. We observed a significant positive correlation between hepatic ADH activity and protein Dyrk1A levels. In this sense, ethanol concentration in blood was increased in hhcy mice with lower hepatic Dyrk1A level, and interestingly, it was decreased in transgenic mice with higher hepatic Dyrk1A level. A positive correlation was also found between hepatic Dyrk1A protein level and ADH4 protein level. It is not clear on the mechanism of protein Dyrk1A effect on ADH4 protein level. Immuno-precipitation experiments using mass spectrometry have recently identified ADH4 as a partner of Dyrk1A (Janel, unpublished data). Increased ADH activity by increased Dyrk1A level could be caused by increased ADH4 protein stability. There are four major classes of ADH. Most members of ADH family are present in liver, and ADH4 is the major hepatic ADH. Patients with alcoholic cirrhosis are at higher risk for hepatocellular carcinoma. Expression level of ADH4 was found to be markedly reduced in hepatocellular carcinoma and significantly associated with survival [44].

5. Conclusion

Our study reveals on the one hand a role of Dyrk1A in ethanol metabolism through ADH activity and more precisely ADH4 protein level, and on the other hand a deleterious effect of chronic alcohol administration on hepatic Dyrk1A level. Whether polymorphism(s) of Dyrk1A exist(s) in the general population and influence(s) the ability to liver to metabolize alcohol needs to be investigated.

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Author contributions

KA and NJ designed study; MR, BL, EB, FB, CB and MM performed research; JLP, JMD, HR, KA and NJ analyzed data; KA and NJ wrote the paper.

Conflict of interest statement

None.

Transparency document

The [Transparency document](#) associated with this article can be found, in the online version.

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