Corrective effects of hepatotoxicity by hepatic Dyrk1a gene delivery in mice with intermediate hyperhomocysteinemia

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Hyperhomocysteinemia results from hepatic metabolism dysfunction and is characterized by a high plasma homocysteine level, which is also an independent risk factor for cardiovascular disease. Elevated levels of homocysteine in plasma lead to hepatic lesions and abnormal lipid metabolism. Therefore, lowering homocysteine levels might offer therapeutic benefits. Recently, we were able to lower plasma homocysteine levels in mice with moderate hyperhomocysteinemia using an adenoviral construct designed to restrict the expression of Dyrk1A, a serine/threonine kinase involved in methionine metabolism (and therefore homocysteine production), to hepatocytes. Here, we aimed to extend our previous findings by analyzing the effect of hepatocyte-specific Dyrk1A gene transfer on intermediate hyperhomocysteinemia and its associated hepatic toxicity and liver dysfunction. Commensurate with decreased plasma homocysteine and alanine aminotransferase levels, targeted hepatic expression of Dyrk1A in mice with intermediate hyperhomocysteinemia resulted in elevated plasma paraoxonase-1 and lecithin:cholesterol acyltransferase activities and apolipoprotein A–I levels. It also rescued hepatic apolipoprotein E, J, and D levels. Further, Akt/GSK3/cyclin D1 signaling pathways in the liver of treated mice were altered, which may help prevent homocysteine-induced cell cycle dysfunction. Dyrk1A gene therapy could be useful in the treatment of hyperhomocysteinemia in populations, such as end-stage renal disease patients, who are unresponsive to B-complex vitamin therapy.

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1. Introduction

Cystathionine beta synthase (CBS) deficiency is the most common inborn error of one-carbon metabolism and is the cause of classical homocystinuria, a condition characterized by high levels of plasma homocysteine (hcy) or severe hyperhomocysteinemia (hhcy)[1]. Elevated plasma hcy, or hhcy, is categorized by range as moderate (15 to 30 μM), intermediate (30 to 100 μM), and severe (above 100 μM). Hyperhomocysteinemia is associated with increased risk for congenital disorders, including neural tube closure defects, heart defects, cleft lip/palate, Down syndrome, and multi-system abnormalities in adults [2]. Hcy is a thiol-containing amino acid produced during metabolism of methionine (an essential amino acid supplied by dietary proteins) via the adenosylated compounds S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH). The metabolism of hcy occurs at the junc

Abbreviations: ALT, alanine aminotransferase; APO, apolipoprotein; CBS, cystathionine beta synthase; DCPPP, 2,6-dichlorophenolindophenol; hcy, homocysteine; HDLs, high-density lipoproteins; hcy, hyperhomocysteinemia; HPLC, high-performance liquid chromatography; KYN, kynurenic acid; LCAT, lecithin:cholesterol acyltransferase; NQO1, NAD(P)H:quinone oxidoreductase; PON-1, paraoxonase-1; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SAH/k, S-adenosylhomocysteine hydrolase; VLDL, very-low-density lipoprotein.

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increase in CBS activity [11]. Plasma hcy levels, in agreement with restoration of key enzymes of hcy metabolism, to hepatocytes of hhcy mouse models [9, 12]. Indeed, we have demonstrated the involvement of hepatic Dyrk1a levels on hepatic SAHH and CBS activities by a mechanism dependent of NAD(P)H: quinone oxidoreductase (NQO1) activity and pyridoxal phosphate respectively [10–12]. Injection of the specific adeno viral Dyrk1a gene transfer construct to mice with moderate hhcy produced decreased plasma hcy levels, in agreement with restoration of key enzymes of hcy metabolism, SAHH and CBS. Pyridoxal phosphate, the metabolically active form of vitamin B6 and cofactor of CBS, was elevated, consistent with the increase in CBS activity [11].

As the central organ of metabolism, many metabolic diseases originate in the liver; however, clinical manifestations can be extrahepatic. In the case of hhcy, the liver plays not only a central role in the metabolism of methionine and contributes the high levels of plasma hcy, but also produces and degrades lipoproteins. High-density lipoproteins (HDLs) exert potent protective effects, including the prevention and correction of endothelial dysfunction by their anti-oxidative and anti-inflammatory properties [13]. Commensurate with decreased plasma hcy levels, targeted hepatic expression of Dyrk1a by adeno viral gene transfer resulted in elevated activity of plasma paraoxonase-1 (PON-1), an HDL-associated protein that inactivates lipids in oxidized low-density lipoproteins, and increased plasma levels of apolipoprotein A–I (APO A–I), the major protein component of HDLs. Additionally, the Akt/GSK3 signaling pathways were rescued in the aorta of targeted mice, thus preventing hcy-induced endothelial dysfunction [11]. In the current study, we aimed to extend our previous findings by analyzing the effect of hepatocyte-specific Dyrk1a gene transfer on intermediate hhcy and on the associated hepatic toxicity by measuring plasma alamine aminotransferase (ALT) levels, and liver dysfunction by measuring lipid markers.

2. Materials and methods

2.1. Experimental animals

All procedures were carried out in accordance with internal guidelines of the French Agriculture Ministry for animal handling. Mice were maintained in a controlled environment with unlimited access to food and water on a 12 h light/dark cycle. Mice were fed a standard laboratory diet (CRM, Special Diets Services, Dietex, France Usine). This diet has a protein content of 19%, a methionine content of 2.700 mg kg$^{-1}$, a folic acid content of 4.41 mg kg$^{-1}$, and a vitamin B12 content of 0.082 mg kg$^{-1}$. The 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) [12]. Cbs$^{+/−}$ mice, on a C57BL6 background, were obtained by mating male Cbs$^{−/−}$ mice with female wild-type C57BL6 (Cbs$^{+/−}$) mice. DNA isolated from tail biopsies of 4-week-old mice was subjected to genotyping of the targeted Cbs allele using polymerase chain reaction (PCR) [14]. The E1E3E4-deleted adeno viral vector “AdDYRK1A” was constructed to induce hepatocyte specific overexpression of Dyrk1a as described previously [11]. Generation of the E1E3E4-deleted adeno viral vector “AdDYRK1A” and large-scale vector production were performed as described previously [15]. Before the experiments and to induce intermediate hhcy, female Cbs$^{+/−}$ mice, at 4 months of age, were maintained for three months on the standard diet supplemented with 0.5% l-methionine (Sigma-Aldrich, France) in drinking water. Mice were divided into two groups for the last month, with one group receiving injection in the retro-orbital sinus with an adeno viral vector AdDYRK1A to have 2 × 10$^{12}$ adeno viral particles/kg body weight and the second group receiving an equivalent dose of saline buffer [11]. Control mice, healthy control Cbs$^{+/−}$ mice also received an equivalent dose of saline buffer and were used as references to monitor hyperhomocysteinemic development. Five to twelve mice per experiment were used.

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

Upon euthanization of mice by Ketamine/Xylazine intraperitoneal injection, 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 [16]. Samples were treated with perchloric acid and kynurenic acid (KYN) was quantified by LC-MS/MS with CTC-PAL autosampler on an Agilent 1200 series system with a quaternary pump, and MS detection was performed on an API 3200 MS/MS spectrometer (ABSciex) operated with a Turbo Ion Spray source [17]. KYN was used as standard was purchased from Sigma-Aldrich. Data were acquired and processed using Analyst software (V. 1.4.2). Plasma APO A–I protein levels were measured by ELISA (E90519M, Uscl, Life Science Inc.) according to the manufacturer’s instructions. After the development of the colorimetric reaction, optical density (OD) at 450 nm was quantified by a microplate reader (Flex Station3, Molecular Device), and OD readings were converted to concentrations (µg/mL) on the basis of the standard curve obtained with APO A–I standard preparation. ALT was assayed using the Alanine Aminotransferase Activity Assay Kit (Sigma-Aldrich, France), based on the pyruvate generated.

2.3. Protein extraction and slot blot analysis

Liver protein extracts were prepared by homogenizing tissue in phosphate-buffered saline (PBS) with a cocktail of protease inhibitors (1 mM Pefabloc SC, 5 µg/mL E64, and 2.5 µg/mL Leupeptin). Homogenates were centrifuged at 12,500 × g for 15 min at 4 °C. Supernatants were then assayed for protein concentrations with the Coomassie (Bradford) Protein Assay reagent (Bio-Rad). Since specificity of each primary antibody used was previously validated by Western blotting, protein extracts (40 µg) under reducing conditions were subjected to slot blotting on nitrocellulose transfer membrane PROTRAN® (Whatman). The membrane was then blocked in 10% nonfat dry milk in Tris–saline buffer (1.5 mM Tris, 5 mM NaCl, 0.1% Tween-20) and probed overnight at 4 °C with one of the following antibodies: anti-APO D (1/1000; Santa Cruz Biotechnology, Tebu, France), anti-APO E (1/1000; Santa Cruz Biotechnology), anti-APO J (1/1000; Santa Cruz Biotechnology), anti-DYRK1A (1/250; Abnova Corporation, Tebu, France), anti-Akt (1/1000; Santa Cruz Biotechnology), anti-phospho-Akt1/2/3 (Ser 473; 1/1000; Santa Cruz Biotechnology), anti-GSK (1/2000, Santa Cruz Biotechnology), anti-phospho-GSK3 (ser21-9; 1/400, Cell Signaling, France), or anti-cyclin D1 (1/250, Cell Signaling). Horseradish peroxidase-conjugated secondary antibody and Western Blotting Luminol Reagent (Santa Cruz Biotechnology) were used to detect specific proteins. Digitized images of the immunoblots obtained using an LAS-
3000 imaging system (Fugi Photo Film Co., Ltd.) were used for densitometric measurements with an image analyzer (UnScan It software, Silk Scientific Inc.). Quantification of total proteins after Ponceau-S coloration was used as an internal control.

2.4. Enzyme activity assays

CBS activity assay was performed on 300 μg of protein extracts as described previously [18]. 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). Lecithin:cholesterol acyltransferase (LCAT) activity was assayed on 2 μL of plasma with an LCAT kit per manufacturer’s directions (Calbiochem/EMD-Millipore/Merck KGaA, Darmstadt, Germany). NAD(P)H:quinone oxidoreductase (NQO1) activity was assayed on 150 μg of protein extracts as described[19]. 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 μM 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 μmol of dicoumarol (Calbiochem, MERCK) used to specifically block NQO1. The reaction was started by adding 40 μM 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. 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 appearance of phenol at 270 nm every 10 s for 1 min using a spectrophotometer (Lambda XLS, PerkinElmer). SAHH activity was measured in the forward hydrolysis direction following the protocol described by Villanueva and Halsted[20] with some modifications. Protein extracts (300 μg) were incubated for 5 min at 37 °C in 1 mL of reaction mixture (452 mM NaCl, 8.9 mM KCl, 33 mM Na2HP04, 6.6 mM KH2PO4, 0.1% gelatin, 1.2 mM MT, 1.1 U adenosine deaminase, 0.45 U nucleoside phosphorylase, and 0.1 U xanthine oxidase). The SAHH reaction was started by adding 80 μM S-(5′-adenosyl)-l-homocysteine to the mixture. Following 5 min of incubation at 37 °C, SAHH-specific activity was detected by the production of formazan, which is detected by measuring the absorbance at 578 nm using a spectrophotometer (Lambda XLS, PerkinElmer).

2.5. Data analysis

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. A p value of 0.06–0.10 was considered to indicate a strong statistical tendency due to the small sample size. Correlations were determined by using Spearman’s rank correlation, as data were not normally distributed according to Shapiro–Wilk test.

3. Results

3.1. Dyrk1a overexpression reduces plasma hcy levels in mice with intermediate hhcy

To overexpress Dyrk1a specifically in the liver of mice, we used an E1E3E4-deleted adenoviral vector “AdDYRK1A”, which was constructed to induce hepatocyte-specific expression of Dyrk1a [11]. We injected “AdDYRK1A” into CBS-deficient mice (Cbs+/−) supplemented with
methionine in drinking water, a murine intermediate model of hhcy. Similar to a previous report [9], mean DYRK1A protein level was decreased in the liver of Cbs+/- mice supplemented with methionine when compared with wild-type (Cbs+/+) mice (Fig. 1a). However, after injection of AdDYRK1A to Cbs+/- mice supplemented with methionine, we observed an increase in DYRK1A protein levels when compared with Cbs+/- mice supplemented with methionine but uninjected (Fig. 1a).

To determine the effect of DYRK1A overexpression on plasma hcy levels in intermediate hhcy, we quantified plasma levels of hcy in Cbs+/- mice supplemented with methionine with or without Dyrk1a gene transfer. As expected, the hcy level in Cbs+/- mice supplemented with methionine was higher than that of Cbs+/- mice (Fig. 1b). Interestingly, Cbs+/- mice supplemented with methionine and injected with AdDYRK1A had a significantly lower plasma hcy level than uninjected Cbs+/- mice (Fig. 1b).

We next studied hepatic activities of the main enzymes implicated in hcy metabolism. Assays of hepatic CBS activity revealed, as expected, significantly lower activity in Cbs+/- mice supplemented with methionine than in Cbs+/- mice (Fig. 1c). Injection of AdDYRK1A resulted in a significant increase in CBS activity (Fig. 1c). A Spearman correlation test revealed a significant negative correlation between plasma hcy levels and hepatic CBS activity for hcy levels >5 μM (ρ = -0.861, p < 0.0013, Fig. 1d), and a significant positive correlation between liver Dyrk1a expression and CBS activity (ρ = 0.655, p < 0.01, Fig. 1e). Since hcy is lowered by its conversion into SAH, we also assayed SAHH activity in the liver of mice and found an increase after injection of AdDYRK1A (100.2 ± 9.7 versus 146.1 ± 18.1; p < 0.05; n = 8 for each). We previously reported that increased SAHH activity occurs concomitantly with an increase in NQO1 activity [10,11]. Therefore, we assayed hepatic NQO1 activity to confirm this finding in the intermediate hhcy model. After injection of AdDYRK1A, we observed a significant increase in NQO1 activity (100.3 ± 6.6 versus 136.6 ± 9.7; p < 0.01; n = 8 for each).

3.2. Dyrk1a overexpression reduces plasma ALT and increases kynurenic acid levels in mice with intermediate hhcy

Hepatocellular injury often results in an increase in serum ALT, and serum ALT levels are used as a marker for liver injury. To determine the effect of Dyrk1a overexpression on liver damage, we quantified plasma levels of ALT in Cbs+/- mice supplemented with methionine with or without Dyrk1a gene transfer. The mean ALT level in Cbs+/- mice supplemented with methionine was significantly higher than that of Cbs+/- mice (Fig. 2a). In contrast, in Cbs+/- mice supplemented with methionine and injected with AdDYRK1A, ALT level was significantly lower than in uninjected Cbs+/- mice (Fig. 2a).

Previous results have shown that low concentrations of hcy stimulate production of the tryptophan derivative KYNA, whereas high levels inhibit KYNA formation in vitro and in vivo [21]. Interestingly, KYNA might exert a protective role on multiple organs during heatstroke through anti-inflammatory mechanisms [22]. Here, we found that the plasma levels of KYNA were significantly lower in Cbs+/- mice supplemented with methionine but trended higher after injection with AdDYRK1A (Fig. 2b). Moreover, a Spearman correlation test revealed a significant positive correlation between plasma ALT and...
hcy levels (Fig. 2c) and a negative correlation between ALT and KYNA levels (Fig. 2d) ($\rho = 0.543, p < 0.01; \rho = -0.563, p < 0.05$, respectively). These results confirm the deleterious effect of hhcy on liver and support a beneficial effect of DYRK1A expression on rescuing the hhcy phenotype.

3.3. DYRK1A overexpression rescues enzymes involved in HDL metabolism in mice with intermediate hhcy

PON-1 is an HDL-associated protein that plays a major role in HDL-mediated protection against coronary artery disease [23], and hepatic
PON-1 is negatively correlated with plasma hcy levels [24]. PON-1 activity was therefore investigated in the context of hcy and Dyrk1a gene transfer rescue models. As expected, Cbs \(^{+/−}\) mice supplemented with methionine exhibited lower hepatic PON1 activity than Cbs\(^{+/+}\) mice (Fig. 3a). After injection of AdDYRK1A, we observed a significant increase in hepatic PON1 activity (Fig. 3a). Since PON1 is synthesized in the liver and is secreted into the serum, the activity of PON1 was also examined in plasma. Cbs\(^{+/−}\) mice supplemented with methionine had significantly lower plasma PON1 activity than Cbs\(^{+/+}\) mice (Fig. 3b). Injection of AdDYRK1A resulted in significantly higher plasma PON1 activity (Fig. 3b). A Spearman correlation test revealed a significant negative correlation between plasma PON1 activity and plasma hcy as well as ALT levels \((p = −0.631, p < 0.01; p = −0.576, p < 0.05\) respectively).

LCAT enzyme converts cholesterol into long-chain cholesteryl esters on HDLs and promotes cholesterol transport from tissues into HDLs. Thus, LCAT is a key enzyme in cholesterol homeostasis and transport regulation [25]. To further investigate the cholesterol-related effects of the Dyrk1a gene transfer in hhcy mice, we analyzed the LCAT activity. Cbs\(^{+/−}\) mice supplemented with methionine had significantly lower plasma LCAT activity than Cbs\(^{+/+}\) mice (Fig. 3c). Injection of AdDYRK1A resulted in significantly higher plasma LCAT activity (Fig. 3c). A Spearman correlation test revealed a significant negative correlation between plasma LCAT activity and plasma hcy levels \((p = −0.423, p < 0.05\) (Fig. 3d).

### 3.4. Dyrk1A overexpression rescues apolipoprotein levels in mice with intermediate hhcy

Because Cbs\(^{+/−}\) mice injected with AdDYRK1A displayed higher plasma LCAT activity, we analyzed plasma APO A-I levels and hepatic expression of apolipoproteins D (APO D) and J (APO J). Each of these apolipoproteins is a component of HDL. APO A-I, the major protein component of HDL, is a co-factor of LCAT. APO D may stabilize LCAT [26], APO J, or Clusterin, associates with HDL as one of its two main functions [27]. We previously found that plasma APO A-I levels are correlated with plasma hcy level and is increased in moderate hhcy mice injected with AdDYRK1A [10]. As in mice with moderate hhcy, mice with intermediate hhcy exhibited significantly lower circulating levels of APO A-I compared to Cbs\(^{+/−}\) mice (Fig. 4a). Further, AdDYRK1A injection resulted in a significant increase in plasma APO A-I levels (Fig. 4a). A Spearman correlation test revealed a significant negative correlation between plasma APO A-I and hcy levels \((p = −0.569, p < 0.02\) ). Consistent with the results for plasma APO A-I, Cbs\(^{+/−}\) mice supplemented with methionine also exhibited significantly lower plasma APO D and hepatic levels of APO D and APO J compared to Cbs\(^{+/−}\) mice (Fig. 4b, c, e), while AdDYRK1A injection resulted in a significant increase (Fig. 4b, c, e). A Spearman correlation test revealed a significant positive correlation between plasma and hepatic levels \((p = 0.567, p < 0.02\) ) (Fig. 4d).

APO E, on the other hand, is a polymorphic and pleiotropic apolipoprotein mainly synthesized by the liver and is a component of very low-density lipoprotein (VLDL) and HDL [28]. VLDLs are major contributors to atherosclerosis. Cbs\(^{+/−}\) mice supplemented with methionine exhibited significantly higher hepatic levels of APO E compared to Cbs\(^{+/+}\) mice (Fig. 4f). AdDYRK1A injection resulted in a significant decrease in hepatic APO E levels (Fig. 4f).

### 3.5. Dyrk1a overexpression rescues hepatic IkB levels in mice with intermediate hhcy

The second major function of APO J is to negatively modulate the NF-kB signaling pathway by stabilizing its inhibitor, IkB [27]. We therefore analyzed IkB protein expression in the liver of mice and, commensurate with the decreased levels of APO J, Cbs\(^{+/−}\) mice supplemented with methionine exhibited significantly lower hepatic levels of IkB compared to Cbs\(^{+/+}\) mice (Fig. 5). Moreover, AdDYRK1A injection resulted in a significant increase in IkB levels (Fig. 5).

### 3.6. Dyrk1a overexpression rescues hepatic Akt/GSK3 signaling pathways in mice with intermediate hhcy

Given the link between DYRK1A and the PI3K/Akt pathway [29,30], we analyzed the activation of Akt in the liver of mice. Consensurate with the decreased DYRK1A protein levels in the liver of Cbs\(^{+/−}\) mice supplemented with methionine, phospho-Akt levels were decreased (Fig. 6a). This decreased activation was rescued following injection of AdDYRK1A to Cbs\(^{+/−}\) mice supplemented with methionine (Fig. 6a). Akt phosphorylates GSK3 alpha and beta on inhibitory serine residues 21 and 9, respectively. No significant difference was found for GSK3 or phosphorylated GSK3 levels between Cbs\(^{+/+}\) mice and Cbs\(^{+/−}\) mice supplemented with methionine (Fig. 6b, c). However, significantly lower GSK3 (Fig. 6b) and higher phosphorylated GSK3 (Fig. 6c) levels were found in the liver of Cbs\(^{+/−}\) mice supplemented with methionine after injection of AdDYRK1A.

GSK3 phosphorylates cyclin D1 to trigger its degradation. Cyclin D1 levels were similar between Cbs\(^{+/+}\) mice and Cbs\(^{+/−}\) mice supplemented with methionine (Fig. 6d). However, injection of AdDYRK1A induced a significant increase in cyclin D1 level in Cbs\(^{+/−}\) mice supplemented with methionine (Fig. 6d).

### 3.7. Dyrk1a overexpression rescues brain DYRK1A expression in mice with intermediate hhcy

Many studies reported the link between DYRK1A overexpression and cognitive impairment [29]. Moreover, we also found an increase in DYRK1A protein expression in brain of hhcy mice [31,32]. Similar to our previous reports [31,32], mean DYRK1A protein level was increased in brain of Cbs\(^{+/−}\) mice when compared with wild-type (Cbs\(^{+/+}\) mice (Fig. 7). However, after injection of AdDYRK1A to Cbs\(^{+/−}\) mice supplemented with methionine, we observed a decrease in DYRK1A protein levels when compared with Cbs\(^{+/−}\) mice supplemented with methionine but un.injected (Fig. 7).
4. Discussion

We recently demonstrated decreased plasma hcy levels in mice with moderate hhcy after adenovirus-mediated Dyrk1a gene transfer to hepatocytes [11]. Here, we confirmed this positive effect of DYRK1A expression on plasma hcy levels in mice with intermediate hhcy. Not only the negative correlation between plasma hcy levels and hepatic CBS activity but also the positive correlation between liver Dyrk1a expression and CBS activity underline the role of Dyrk1a in one carbon metabolism [9,11,12]. Further, the positive correlation identified here between plasma hcy and ALT levels emphasizes the deleterious effect of hhcy on liver function and the beneficial effect of DYRK1A on hepatic hcy metabolism.

Although hcy is produced in the liver, hhcy is a risk factor for cardiovascular diseases [33], and lowering hcy levels could offer a viable approach to preventing cardiovascular diseases. Our findings demonstrate the link between hepatic and vascular functions. We found a negative correlation between ALT and KYNA levels. hcy lowered KYNA production in cultured bovine aortic endothelial cells [34]. Interestingly, KYNA exerts a protective effect against hcy-induced impairment of endothelial cells in vitro [35]. Vascular endothelial cells can also be impaired by lipoproteins, and changes in lipid metabolism and lipoproteins are often observed in cardiovascular diseases. Importantly, a correlation between serum hcy levels and lipids and lipoproteins has been observed [36]. A high concentration of LDL is found in patients with hhcy and primary hypertension, coupled to enhanced secretion of VLDL triglycerides [37]. VLDLs and LDLs are considered as one of the most important proatherogenic factors, and APO E is a major component of these particles. We found an increased hepatic APO E level in mice with intermediate hhcy, which was abolished after injection of AdDYRK1A. In the vasculature, VLDL and LDL particles are susceptible to oxidative modifications. The COMAC group studies have confirmed the existence of several proatherogenic factors, such as the disruption of lipids and lipoprotein balance, for which hhcy intensifies their negative effects on cardiovascular function [38]. Increased hepatic APO E level can also be explained as a compensatory protective effect. Indeed, APO E has a high affinity to LDL receptors on hepatic and extrahepatic cells. In mice, APO E deficiency causes accumulation in plasma of cholesterol-rich VLDL remnants for which prolonged circulation is atherogenic [39]. Conversely, the increased synthesis of hepatic APO E could increase the synthesis of APO E-rich VLDL, and those remnants could be cleared more rapidly due to their increased affinity for LDL receptor.

We found decreased hepatic APO D and plasma APO A–I levels in mice with intermediate hhcy, which was abolished after injection of AdDYRK1A. APO D and APO A–I are components of HDL particles. The observed decreases in APO D and APO A–I may be responsible for the reduced plasma LCAT activity also observed in mice with intermediate hhcy. Moreover, the decreased LCAT activity was abolished after injection of AdDYRK1A. Indeed, APO A–I is a cofactor of LCAT activity, and

Fig. 6. Effect of hepatic overexpression of Dyrk1a on phospho-Akt, GSK3, phospho-GSK3, and cyclin D1 levels in mice with intermediate hhcy. Phosphorylation of Akt (a), GSK3 alpha and beta expression (b), phosphorylation of GSK3 alpha and beta on ser 21 and 9 (c), and cyclin D1 expression (d) in the liver of wild-type (Cbs+/+) mice and Cbs+/- mice supplemented with methionine and injected (Cbs+/- Met/AdDYRK1A) or uninjected (Cbs+/- Met) with AdDYRK1A. GSK3 and cyclin D1 expression were determined by slot blotting, and values were obtained by normalization of images from GSK3 and cyclin D1 to total proteins colored with Ponceau-S. Relative protein expression was determined by normalization from p-Akt or p-GSK3 with that of total Akt or GSK3. Data were normalized to the mean of wild-type mice (Cbs+/-). Data correspond to the medians with interquartile ranges. n = number of mice.
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5. Conclusion

Injection of AdDYRK1A can ameliorate intermediate hhcy and its associated hepatic dysfunction. Thus, DYRK1A gene therapy could be useful in the treatment of hhcy in populations, such as end-stage renal disease patients, which are resistant to hhcy lowering by B-complex vitamin therapy. Although we found an increased SAHH activity after AdDYRK1A injection, we do not determine if this increase can modify the methylation status, which needs to be explored.

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