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Effect of hyperhomocysteinemia on the protein kinase DYRK1A in liver of mice

Julien Hamelet [a], Christophe Noll [a], Clémentine Ripoll [a], Jean-Louis Paul [b,c], Nathalie Janel [a,*,1], Jean-Maurice Delabar [a,1]

[a] EA 3508 – case 7104, Univ Paris Diderot, 75205 Paris cedex 13, France
[b] AP-HP, Hôpital Européen Georges Pompidou, Service de Biochimie, Paris, France
[c] Université Paris-Sud, UMR 1154-INRA, Faculté de Pharmacie, Châtenay-Malabry, France

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ABSTRACT

Hyperhomocysteinemia due to cystathionine beta synthase (CBS)-deficiency confers diverse clinical manifestations, notably liver diseases. Even if hyperhomocysteinemia in liver of CBS-deficient mice, a murine model of hyperhomocysteinemia, promotes mitochondrial oxidative stress and pro-apoptotic signals, protective signals may counteract these pro-apoptotic signals, leading to chronic inflammation. As DYRK1A, a serine/threonine kinase, has been described as a candidate antiapoptotic factor, we have analyzed the expression of DYRK1A in liver of CBS-deficient mice. We found that DYRK1A protein level was reduced in liver of CBS-deficient mice, which was not observed at the gene expression level. Moreover, the use of primary hepatocytes/Kupffer cells co-culture showed that degradation of DYRK1A induced by hyperhomocysteinemia requires calpain activation. Our results demonstrate a deleterious effect of hyperhomocysteinemia on DYRK1A protein expression, and emphasize the role of hyperhomocysteinemia on calpain activation.

Homocysteine (Hcy) is a thiol-containing amino acid produced during methionine metabolism via the adenosylated compounds *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH). Once Hcy is formed, it may be recycled to methionine after remethylation by two different pathways. The first one involves methionine synthase (MS), an enzyme that uses 5-methyltetrahydrofolate as the methyl donor, which is generated by 5,10-methylene tetrahydrofolate reductase (MTHFR) [1]. The second pathway involves the enzyme betaine-homocysteine methyltransferase (BHMT). Hcy may also undergo condensation with serine to form cystathionine, which is catalyzed by cystathionine beta synthase (CBS), the first enzyme involved in the transsulfuration pathway. Hcy can also turn back to SAH via reversal of the SAH hydrolase (SAHH) reaction [1]. Hyperhomocysteinemia, defined by elevated plasma Hcy level, is now recognized as an important vascular risk factor. It is associated with atherosclerosis in the coronary, cerebrovascular, and peripheral arterial circulation, even if the degree of hyperhomocysteinemia is moderate [2]. Patients with CBS deficiency also develop hepatic pathologies [3,4]. The basic steatosis of the liver in CBS-deficient mice, a murine model of severe hyperhomocysteinemia, has been described [5,6]. We also showed that CBS-deficient mice develop inflammation and fibrosis, concomitant with an enhanced expression of proinflammatory cytokines like TNFα [6]. Normally, the increased synthesis of several cytokines in the liver, particularly TNFα, is known to initiate apoptosis. However, even if we have found pro-apoptotic signals, the absence of caspase-3 activation, the absence of increased DNA fragmentation, and the absence of increased TUNEL-positive cells show that protective signals may counteract these pro-apoptotic signals, leading to chronic inflammation in liver of CBS-deficient mice [6]. Then we tried to establish relationships between hyperhomocysteinemia and alterations of signaling pathways. We showed an activation of extracellular signal-regulated protein kinase (ERK) pathways, a mitogen-activated protein kinase (MAPK) which is involved in cell survival signaling pathways, in hippocampus of CBS-deficient mice [7]. However, ERK kinase signaling pathway is not activated in liver of CBS-deficient mice [8]. DYRK1A, a serine/threonine kinase which is able to autophosphorylate on tyrosine residue [9], is composed of a kinase domain, a nuclear localization signal, a PEST region, and a repeat of histidines. The characterization of its transcriptional regulation by the transcription factor E2F1 suggests that DYRK1A may play a role in cell cycle regulation or apoptosis [10]. Chang et al. demonstrated that the raised expression of DYRK1A may serve as a candidate antiapoptotic factor [11]. It has also been demonstrated that DYRK1A, upon NGF stimulation, prolongs the kinetics of ERK activation by facilitating the formation of a Ras/B-Raf/MEK1 multiprotein complex [12]. Therefore, we aimed to analyse the expression of DYRK1A in liver of CBS-deficient mice.

* Corresponding author. Fax: +33 1 57 27 83 54.
E-mail address: nathalie.janel@univ-paris-diderot.fr (N. Janel).
[1] These authors share senior authorship.

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Materials and methods

Mice and genotyping. Mice were maintained in a controlled environment with unlimited access to food and water on 12 h light/dark cycle. All procedures were carried out in accordance with internal guidelines of the French Agriculture Ministry for animal handling. 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) [5]. $Cbs^{+/-}$ mice, on a C57BL/6 background were obtained by mating male $Cbs^{+/-}$ mice with female wild type C57BL/6 ($Cbs^{+/+}$) mice. DNA isolated from 4 week-aged mice tail biopsies was subjected to genotyping of the targeted CBS allele using polymerase chain reaction (PCR) assay [5].

Preparation of serum samples, tissue collection, and plasma total Hcy assay. At the time of sacrifice, blood samples were collected into tubes containing a 1/10 volume of 3.8% sodium citrate, placed on ice immediately. Plasma was isolated by centrifugation at 2500*g* for 15 min at 4 °C. Liver was harvested, snap-frozen and stored at −80 °C until use. Plasma total Hcy (tHcy) was assayed by using the fluorimetric high-performance liquid chromatography method described by Fortin and Genest [13].

Primary hepatocytes/Kupffer cells co-culture. Hepatic cells were isolated from livers of 6 weeks-old $Cbs^{+/+}$ mice by collagenase perfusion via the portal vein at a flow rate of 5 mL/min. First, livers were washed for 5 min with Hank's balanced salt solution (HBSS, Invitrogen, Cergy, France) containing 50 mM Hepes and 0.5 mM EGTA, pH 7.4. Then, washing buffer were replaced for 10 min by HBSS (Invitrogen, Cergy, France) containing 0.025% collagenase, 50 mM Hepes, and 7 mM $CaCl_2$. Hepatic cells were dispersed in HBSS, filtered through a 250 μm nylon gauze (Dutscher Brumath, France) and pelleted by centrifugation at 60*g* for 3 min. Supernatant was collected and centrifuged at 1000*g* for 10 min to pellet Kupffer cells [14]. Hepatocyte-enriched pellet was resuspended in medium M199 with Earle salts (Invitrogen, Cergy, France) with 100 μg/mL of streptomycin, 100 U/mL of penicillin and centrifugated at 60*g* for 3 min. Cell viability was assessed on hepatocyte suspension by the trypan blue exclusion test and was always higher than 60%. Then, Kupffer cells pellet was resuspended in the hepatocyte suspension for co-culture. Cells were seeded and after cell attachment for 4 h, medium was replaced by fresh medium M199 with Earle salts containing only antibiotics and cells were maintained in culture overnight. For all pharmacological treatments, drugs were added 1 h prior to Hcy. When drugs were dissolved in dimethyl sulfoxide, the same volume of dimethyl sulfoxide was added to controls.

Western blot analysis. Proteins preparations were subjected to SDS electrophoresis on 7.5% acrylamide gels under reducing conditions and transferred to Hybond-C Extra membrane (GE Healthcare Europe GmbH, Saclay, France). After transfer, membranes were 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 DYRK1A antibody (1/500) (Abnova Corporation, Tebu, France). Horseradish peroxidase-conjugated secondary antibody and Western blotting luminol reagent (Santa Cruz Biotechnology, Tebu, France) were used to detect specific proteins. β-actin (1/4000) (Sigma–Aldrich, France) was used as an internal control. Digitized images of the immunoblots obtained using a LAS-3000 imaging system (Fuji Photo Film Co., Ltd.) were used for densitometric measurements with an image analyzer (Multi Gauge software, Fuji Photo Film Co., Ltd.).

RNA extraction and determination of mRNA levels. Total RNA was prepared from mice liver by the guanidinium thiocyanate procedure. The quantity and purity of the RNA was assessed by measuring absorbance at 260 and 280 nm. Reverse transcription was carried out on 2 μg total RNA as described by the manufacturer (Ambion, UK). The mRNA levels were assessed by real-time quantitative reverse transcription-polymerase chain reaction (Q-PCR). cDNA (0.4 μL) was diluted with PCR mix (Light Cycler FastStart DNA Master SYBR Green I Kit, Roche Diagnostics) containing a final concentration of 3 mM $MgCl_2$ and 0.5 μM of primers in a final volume of 10 μL. The primers were designed by Primer 3 software. The primers pairs were selected to yield a single amplicon based on dissociation curves. The peptidylprolyl isomerase B (PPIB) mRNA was used as an endogenous control. Similar results were obtained by normalizing to superoxide dismutase-1 (SOD1) expression levels [15]. Primer sequences are 5′ATCCGACGCACCA GCATC3′ (left primer) and 5′AATTGTAGACCC TTGGCCTGGT3′ (right primer) for DYRK1A, 5′GGATTTGGCTAC AAAAACAGCAA3′ (left primer) and 5′ACCAGGCCCGTAGTGCTTC3′ (right primer) for PPIB, 5′TGGGGACAATACACAAGGCTGT3′ (left primer) and 5′TTTCCAC CTTTGCCCAAGTCA3′ (right primer) for SOD1. Q-PCR was performed on total RNA isolated from liver of individual mice in a Lightcycler system (Roche Diagnostics). The thermal cycler parameters were as follows: hold for 8 min at 95 °C for one cycle followed by amplification of cDNA for 40 cycles with melting for 5 s at 95 °C, annealing for 5 s at 65 °C and extension for 10 s at 72 °C. Each reaction was performed in duplicate. Subsequent assay efficiency calculations were carried out in Light Cycler Relative Quantification Software (Roche Diagnostics). As the efficiency of the target gene and the control genes were comparable, ΔΔCp analysis of the results allows to assess the ratio of the target mRNA versus control mRNA [16].

Calpain activity assay. Calpain activity was measured using the fluorogenic peptide *N*-Succinyl-Leu-Tyr-7-Amido-4-Methylcoumarin as described by Kohli et al [17]. Briefly, 40 μg of liver extract in a final volume of 40 μL was added to 160 μL of 50 μM *N*-Succinyl-Leu-Tyr-7-Amido-4-Methylcoumarin dissolved in dimethyl sulfoxide and Tris buffer (100 mM Tris–HCl, 145 mM NaCl at pH 7.3). Proteolysis of the substrate was monitored for 30 min at room temperature with a FluoStar Galaxy plate reader (excitation: 380 nm, emission: 460 nm; BMG Labtechnologies, Champigny sur Marne, France) either in presence of 10 mM Ca^{2+} or 10 mM EGTA to determine calcium-independent activity, thus excluding cathepsin activity. Calpain activities data are expressed as percentage of $Cbs^{+/-}$ mice.

Proteasome activity assay. Fluorogenic peptides *N*-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin (40 μM), Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (50 μM), and *Z*-Leu-Leu-Glu β-naphthylamide (400 μM) were used to assay, respectively, chymotrypsin-like, trypsin-like and caspase-like activities of the proteasome. Forty micrograms of liver extract were incubated for 30 min at 37 °C with the appropriate substrate in 200 μL 25 mM Tris, pH 7.5. Fluorescence appearance was monitored in a FluoStar Galaxy plate reader (excitation: 380 nm, emission: 460 nm for Amido-4-Methylcoumarin-linked substrates and excitation: 320 nm, emission: 420 nm for naphthylamide-linked substrate; BMG Labtechnologies). Activities were measured in absence or in presence (50 μM) of proteasome inhibitor MG132, the difference between the two values was attributed to proteasome activity.

Data analysis. The results are expressed as means ± SEM. Since plasma Hcy level and DYRK1A gene and protein expression were not normally distributed according to the Shapiro–Wilk's test, statistical analysis was done with Mann–Whitney–Wilcoxon's test followed by Holm's correction for multiple tests. Correlation between plasma Hcy level and DYRK1A protein expression was determined after log transformation of the not normally distributed variables by using the non-parametric Spearman's rank correlation test. Data were analyzed using R software (http://www.R-project.org) and considered significant when $p \leqslant 0.05$.