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Calpain activation is required for homocysteine-mediated hepatic degradation of inhibitor Ikappa B alpha

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ABSTRACT

Hepatic steatosis is a clinical feature observed in severe hyperhomocysteinemic patients. In mice, cystathionine beta synthase (CBS) deficiency, the most common cause of severe hyperhomocysteinemia, is also associated with steatosis, fibrosis and inflammation. Proinflammatory cytokines usually induce apoptosis. However, hyperhomocysteinemia does not increase apoptosis in liver of CBS-deficient mice compared to wild type mice. The aim of the study was to analyze the activation state of the NF-κB pathway in liver of CBS-deficient mice and to investigate its possible involvement in anti-apoptotic signals. We analyzed the level of IκBα in liver of CBS-deficient mice. A co-culture of primary hepatocytes and Kupffer cells was also used in order to investigate how IκBα degradation occurs in response to homocysteine. We found lower IκBα level not only in liver of CBS-deficient mice but also in hepatocyte/Kupffer cell co-culture. The homocysteine-mediated IκBα enhanced proteolysis occurred via calcium-dependent calpains, which was supported by an increased level of calpain activity and a reduced expression of calpastatin in liver of CBS-deficient mice. Intraperitoneal administration of the inhibitor PDTC normalized the expression of two genes induced by NF-κB activation, heme oxygenase-1 and cellular inhibitor of apoptosis 2. Moreover, PDTC administration induced an increase of caspase-3 activity in liver of CBS-deficient mice. Our results suggest that hyperhomocysteinemia induces calpain-mediated IκBα degradation which is responsible for anti-apoptotic signals in liver.

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Introduction

Homocysteine (Hcy), a thiol-containing amino acid, is an intermediate in the synthesis of cysteine from dietary methionine. Abnormally high plasma Hcy levels, defining hyperhomocysteinemia, can derive from nutritional or genetic factors [1]. Three different types of hyperhomocysteinemia are defined depending on plasma Hcy concentration: moderate (15–30 μM), intermediate (31–100 μM) and severe (>100 μM) [2]. A moderate increase of plasma Hcy level is well recognized as an independent risk factor for atherothrombosis in the coronary, cerebrovascular and peripheral arterial circulation [3–6]. Necropsies and biopsies of severe hyperhomocysteinemic patients not only revealed atherosclerosis and thrombosis but also hepatic steatosis [7,8].

Deficiency of cystathionine beta synthase (CBS), the enzyme catalyzing the condensation of Hcy and serine to cystathionine [9], is the most common disorder associated with severe hyperhomocysteinemia. Like severe hyperhomocysteinemic patients, CBS-deficient mice, a murine model of severe hyperhomocysteinemia, display abnormal hepatic histology with microvesicular lipid droplets [10]. An extensive study of the hepatic pathology observed in CBS-deficient mice demonstrated that hyperhomocysteinemia induces steatosis but also fibrosis and chronic inflammation [11]. Moreover, increased protein and lipid oxidation [11] indicates that CBS deficiency in mice also promotes oxidative stress. However, underlying molecular mechanisms of Hcy-induced hepatic pathology are still poorly understood.

In liver, apoptosis is the classical response to chronic inflammation since it is involved in replacement of injured hepatocytes. Robert et al. reported an activation of mitochondrial pro-apoptotic signals through increased Bax/Bcl-2 ratio in liver of CBS-deficient mice. However, they also demonstrated that there was no caspase-3 activation compared to wild type (Cbs +/+) mice, which shows that protective signals may counteract apoptotic signals [11]. Heme oxygenase-1 (HO-1) is an oxidative stress inducible protein, which has been shown to protect the liver from cytokine-induced apoptosis [12]. The human HO-1 gene promoter

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possesses a nuclear factor-kappaB (NF-κB)-binding motif [13] and HO-1 anti-apoptotic activity requires NF-κB activation in endothelial cells [14]. Rel/NF-κB proteins are a family of homo- and heterodimeric transcription factors. The most common transcription factor of this family is the p50/p65 heterodimer named NF-κB [15]. Classically, upon stimulation, IκBα degradation occurs in response to its phosphorylation on residues Ser32 and Ser36 by IκBα kinase (IKK) [16]. IKK phosphorylates IκBα which is degraded by the proteasome, thereby unmasking NF-κB nuclear localization signal. Then IκBα degradation indicated that NF-κB pathway is activated. NF-κB plays a central role in balancing apoptosis and survival [16], notably by governing inhibitors of apoptosis (IAP) expression [16–18]. Dela Peña et al. demonstrated that NF-κB activation is a very early step in steatohepatitis development, independently of tumor necrosis factor α overproduction [19]. NF-κB is known to be activated by Hcy in various cell types including vascular smooth muscle cells [20], macrophages [21] and endothelial cells [22]. Since HO-1 is upregulated in liver of CBS-deficient mice [23], we hypothesized that CBS deficiency might also activate the NF-κB hepatic pathway. The aim of this study was to demonstrate the involvement of NF-κB pathway in anti-apoptotic signals in liver of CBS-deficient mice. A co-culture of primary hepatocytes and Kupffer cells was also used in order to investigate how IκBα degradation could occur in response to Hcy.

Materials and methods

Mice models

Animal care was conducted in accordance with the French Agriculture and Forestry Ministry guidelines. We made every effort to minimize suffering and the number of animals used. Mice were housed in a controlled environment with unlimited access to food and water on 12-h light/dark cycle. Heterozygous CBS-deficient (Cbs +/−) mice were generously donated by Dr. N. Maeda (Department of Pathology, University of North Carolina, Chapel Hill, NC, USA). Cbs +/− mice, on a C57BL/6 background, and their Cbs +/+ littermates were used. Cbs +/− mice were produced by mating male Cbs +/− mice with female Cbs +/+ mice. Tail biopsies were performed on mice at 4 weeks of age and polymerase chain reaction was used for genotyping as previously described [10]. Age-matched male littermates were used for further analysis.

Pyrrolidine dithiocarbamate treatment

Mice were injected intraperitoneally with 150 μg/g of pyrrolidine dithiocarbamate (PDTC) dissolved at 25 μg/μL in 0.9% NaCl. Control mice were injected with 0.9% NaCl.

Samples collection and plasma total Hcy assay

At the time of sacrifice, blood samples were collected into tubes containing an 1/10 volume of 3.8% sodium citrate and placed on ice immediately. Plasma was isolated by centrifugation at 2500*g* for 15 min at 4 °C. Plasma total Hcy (tHcy) was assayed by using the fluorimetric high-performance liquid chromatography method described by Fortin and Genest [24]. Livers were harvested, immediately used or snap-frozen and stored at −80 °C until use.

Co-culture of primary hepatocytes and Kupffer cells

Hepatic cells and Kupffer cells were isolated from liver of 6 weeks old Cbs +/+ mice by collagenase perfusion as described [25]. If needed, Kupffer cell pellet was resuspended in the hepatocyte suspension. Hepatocytes with or without Kupffer cells were seeded at 0.8×10^6 cells into 6-well plates (Becton Dickinson) in medium M199 with Earle's salts, supplemented with 0.2% (wt/vol) bovine serum albumin, 2% (vol/vol) Ultroser G (Pall Life Sciences). After cells attachment for 4 h, medium was replaced by fresh medium M199 with Earle's salt, 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 (DMSO), the same volume of DMSO was added to controls.

Protein extraction

Liver protein extracts for Western blotting were prepared by homogenizing 100 mg of liver in 500 μL of PBS. Liver protein extracts for calpain activity assays were obtained by homogenizing 100 mg of liver in 500 μL of Tris buffer (100 mM Tris–HCl, 145 mM NaCl, pH 7.3, or Tris 25 mM, pH 7.5, respectively). Protein extracts from cultured hepatic cells were obtained by scrapping the cells in 300 μL of Tris buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP40, 0.1% SDS). All the extraction buffers were supplemented with protease inhibitors (1 mM Pefabloc SC, 5 μg/mL E64 and 2.5 μg/mL Leupeptin). Homogenates were centrifuged at 13,000g for 15 min at 4 °C. Supernatants were then assayed for protein concentrations with the Bio-Rad Protein Assay reagent (Bio-Rad).

Calpain activity assay

Calpain activity was measured using the fluorogenic peptide *N*-Succinyl-Leu-Tyr-7-amido-4-methylcoumarin as described [25], with 40 μg of liver extract.

Caspase-3 activity assay

Caspase-3 activity was measured with 80 μg of liver extract by using the caspase-3 colorimetric activity (Chemicon) following the supplier's instruction. Measurement of enzyme activity is based on detection of *p*-nitroalanine at 405 nm after incubation of liver extract with the caspase-3 specific substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroalanide.

Western blot analysis

Protein preparations were subjected to SDS electrophoresis on 12% acrylamide gels under reducing conditions and transferred to Hybond-C Extra membrane (GE Healthcare Europe GmbH). 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 primary antibody. The following primary antibodies were used, rabbit polyclonal antibodies to IκBα, Phospho-IκBα (Ser32) and calpastatin purchased from Cell Signaling Technology, and mouse monoclonal antibodies to β-actin, purchased from Sigma–Aldrich). Horseradish peroxidase-conjugated secondary antibodies and Western blotting luminol reagent (Santa Cruz Biotechnology) were used to detect specific proteins. β-Actin was used as an internal control. Blots were developed with a LAS-3000 imaging system (Fujifilm) and densitometry analysis of band intensities was performed with MultiGauge (Fujifilm).

RNA extraction and determination of mRNA levels

Total RNA were prepared from liver with the NucleoSpin RNA II kit (Macherey Nagel). Quantity and purity of the RNA were assessed by measuring absorbance at 260 and 280 nm. mRNA levels were analyzed by real-time quantitative reverse transcription-polymerase chain reaction (Q-PCR) as previously described [26] using the Light Cycler FastStart DNA Master SYBR Green I Kit

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