Amino Acids (2010) 39:1563–1569
DOI 10.1007/s00726-010-0627-y

ORIGINAL ARTICLE

Identification and origin of *N*ε-homocysteinyl-lysine isopeptide in humans and mice

Rafał Głowacki · Edward Bald · Hieronim Jakubowski

Received: 22 March 2010 / Accepted: 8 May 2010 / Published online: 30 May 2010

Abstract Homocysteine (Hcy) metabolites, Hcy-thiolactone and *N*-Hcy-proteins, have been linked to the pathology of human cardiovascular and neurodegenerative diseases. Hcy-thiolactone is generated in an error-editing reaction in protein biosynthesis when Hcy is selected in place of methionine by methionyl-tRNA synthetase. *N*-Hcy-protein, in which Hcy is linked via isopeptide bond to ε-amino group of a protein lysine residue, forms in a post-translational reaction of Hcy-thiolactone with proteins. Here, we identify a novel metabolite, *N*ε-Hcy-Lys, in human and mouse plasma, and show that this metabolite is elevated in genetic (cystathionine β-synthase deficiency in humans and mice, methylenetetrahydrofolate reductase deficiency in mice) or dietary (high Met diet in mice) deficiencies in Hcy metabolism. We also show that *N*ε-Hcy-Lys is generated by proteolytic degradation of *N*-Hcy-protein in mouse liver extracts. Our data indicate that free *N*ε-Hcy-Lys is an important pathology-related component of Hcy metabolism in humans and mice.

Keywords Cystathionine β-synthase deficiency · Methylenetetrahydrofolate reductase deficiency · Dietary hyperhomocysteinemia · *N*-Hcy-protein turnover · *N*ε-Homocysteinyl-lysine

R. Głowacki · E. Bald
Department of Environmental Chemistry, Łódź University, 90-236 Łódź, Poland

H. Jakubowski
Institute of Bioorganic Chemistry, Polish Academy of Sciences, 61-704 Poznań, Poland

H. Jakubowski (✉)
Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, International Center for Public Health, 225 Warren Street, Newark, NJ 07101-1709, USA
e-mail: jakubows@umdnj.edu

Introduction

Homocysteine (Hcy), a sulfur-containing amino acid, is an intermediate metabolite that forms from the essential dietary protein amino acid methionine (Met). Genetic or nutritional deficiencies in Hcy metabolism lead to hyperhomocystenemia (Mudd et al. 2001), which is a risk factor for cardiovascular and neurodegenerative disorders such as dementia and Alzheimer's disease. In mammals, levels of Hcy are regulated by remethylation to Met, catalyzed by Met synthase and betaine-Hcy methyltransferase, and transsulfuration to cysteine, the first step of which is catalyzed by cystathionine β-synthase (CBS) (Mudd et al. 2001). In all organisms Hcy is also metabolized to the thioester Hcy-thiolactone in an error-editing reaction in protein biosynthesis when Hcy is selected in place of Met by methionyl-tRNA synthetase (Jakubowski and Goldman 1993). Hcy-thiolactone, known to be cytotoxic in experimental animals and cell cultures, is detrimental mostly because of its ability to form isopeptide bonds with protein lysine residues (Jakubowski 1997, 2000; Jakubowski et al. 2000), which impairs or alters the protein's function (Jakubowski 1999; Glowacki and Jakubowski 2004; Perla-Kajan et al. 2007; Sauls et al. 2006). *N*-Homocysteinylation also increases protein's susceptibility to oxidative damage (Glowacki and Jakubowski 2004) via a thiyl radical mechanism (Sibrian-Vazquez et al. 2010). That protein *N*-homocysteinylation may be linked to human pathology such as atherothrombosis is suggested by the accumulation of pro-thrombotic *N*-Hcy-fibrinogen observed in CBS-deficient patients (Jakubowski et al. 2008) and an

autoimmune response manifested by the accumulation of IgG auto-antibodies against *N*-Hcy-protein observed in coronary heart disease and stroke patients (Jakubowski 2005).

Originally discovered ex vivo in cultured human fibroblasts and endothelial cells, protein *N*-homocysteinylation is now known to occur in vivo in humans and mice. Both Hcy-thiolactone (Chwatko and Jakubowski 2005; Chwatko et al. 2007; Jakubowski 2002) and *N*-Hcy-protein (Jakubowski 2000; Jakubowski et al. 2008, 2009) have been identified as constituents of the blood and shown to be elevated in nutritional or genetic hyperhomocysteinemia. While *N*-linked Hcy occurs in each individual protein examined (Jakubowski 2002), the highest amounts, 0.5 mol *N*-linked Hcy/mol protein, are present in human ferritin (Jakubowski 2008). In normal human blood, about 70% of circulating Hcy is *N*-linked to blood proteins, mostly hemoglobin (Hb) and albumin (Jakubowski 2002). Human clinical studies show that plasma *N*-Hcy-protein levels are associated with the risk of coronary heart disease (Yang et al. 2006), whereas plasma Hcy-thiolactone levels are associated with the development and progression of diabetic macrovasculopathy (Gu et al. 2008). Immunohistochemical studies show that *N*-Hcy-protein accumulates within atherosclerotic lesions in aortas of *ApoE−/−* mice fed with a normal chow diet and the accumulation increases in the animals fed with a hyperhomocysteinemic high Met diet (Perla-Kajan et al. 2008).

Although *N*-Hcy-proteins are expected to be metabolized, there is no evidence for this. Because turnover of post-translationally modified proteins yields modified amino acids (e.g., methylated arginine and methylated lysine residues; Wang et al. 2009), one can predict that *N*-Hcy-protein would yield *Nε*-Hcy-Lys isopeptide among products of its hydrolysis. The present work was undertaken to test this prediction.

Materials and methods

Human plasma

We used plasma samples from our previous studies of apparently healthy individuals ($n = 30$) and renal disease patients ($n = 5$) from the Łódź region of Poland, and Dutch patients with homocystinuria caused by mutations in the CBS gene ($n = 28$) (Jakubowski et al. 2008). CBS activity in fibroblasts derived from these patients was <2.5% of the control CBS activity from unaffected individuals. Patients were initially diagnosed at ages 2–54 on the basis of clinical manifestations of CBS deficiency (ectopia lentis, Marfanoid appearance, osteoporosis, severe hyperhomocysteinemia and hypermethioninemia). Five CBS-deficient patients survived a vascular event before diagnosis. All patients were on Hcy-lowering treatment (vitamin B6) following diagnosis. Blood samples were taken into Vacutainer EDTA tubes for the preparation of plasma as previously described (Jakubowski et al. 2008; Chwatko et al. 2007). All specimens were stored at −80°C.

Mouse plasma

We used plasma samples from our previous studies of wild type C57BL/6J mice fed a standard chow diet or a high methionine diet, and *Cbs*- and *Mthfr*-deficient mice fed a standard chow diet (Jakubowski et al. 2009). Mouse blood was collected in plastic tubes containing 10 mM EDTA and the plasma was separated by centrifugation (4°C, 2,000×*g*, 5 min) and stored at −80°C.

Preparation of *N*-Hcy-hemoglobin

N-Hcy-Hb was prepared as previously described (Jakubowski 1999; Glowacki and Jakubowski 2004). Human Hb (Sigma-Aldrich) was dissolved at 50 mg/mL in 0.1 M sodium phosphate buffer, pH 7.4, 0.2 mM EDTA, and incubated with 2 molar excess of Hcy-thiolactone. Low molecular weight compounds were removed by ultra-filtration using 10 kDa cut-off devices (Millipore). The resulting *N*-Hcy-Hb contained 0.75 mol Hcy/mol protein.

Proteolytic degradation of *N*-Hcy-hemoglobin

Frozen liver tissues from 2-month-old C57BL/6 mice were disintegrated by grinding with dry ice using mortar and pestle pre-chilled to −80°C. The mixture was transferred to a 5 mL plastic test tube (Falcon) and further homogenized by sonication (two 10-s strokes with chilling on ice for 5 min in between) in ten volumes of ice-cold 20 mM K-Hepes, pH 7.4, 0.1 M NaCl, 2 mM DTT, 2 mM $CaCl_2$, 0.2 mM EDTA. Extracts were clarified by centrifugation at 12,000×*g* for 10 min at 4°C. Reaction mixtures (100 µL) containing 10 mg/mL *N*-Hcy-Hb, 0.1 M K-Hepes buffer, pH 7.4, 1 mM DTT, and 10 µL mouse liver protein extract were incubated at 25°C for 16 h. Controls without protein extract or substrate were also included. Reaction mixtures were assayed for *Nε*-Hcy-Lys.

Preparation of *Nε*-Hcy-Lys isopeptide

Nε-Hcy-Lys isopeptide has been originally identified as a product of facile reaction of Hcy-thiolactone with lysine and the kinetics of its formation have been reported (Jakubowski 1999, 2000). *Nε*-Hcy-Lys was recently obtained by chemical synthesis (Sibrian-Vazquez et al. 2010). In the present work, we have prepared pure *Nε*-Hcy-Lys isopeptide as follows.

L-Lysine (0.78 g, 5 mmol) (Sigma-Aldrich) and D, L-Hcy-thiolactone hydrochloride (0.93 g, 5 mmol) (Lancaster, England) were dissolved in 100 mL 0.2 M sodium phosphate buffer, pH 7.4, 0.2 mM EDTA and allowed to react for 24 h at room temperature. The reaction product, *N*ε-Hcy-Lys, was purified by HPLC using a reverse phase X Bridge Prep C18 preparative column (19 × 100 mm, 5 µm) from Waters (Milford, MA, USA). After sample application, the column was eluted with a linear gradient from 0 to 40% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 25 mL/min for 30 min and A_{215} of the effluent was monitored. Fractions containing *N*ε-Hcy-Lys isopeptide, a predominant product eluting at 2 min, were collected and the solvent was evaporated under vacuum to leave a white powder. ^{1}H NMR spectrum was recorded using a Varian Mercury-300 spectrometer; chemical shifts δ in ppm; coupling constants J in Hz. ^{1}H NMR (300 MHz, D_2O): $\delta = 4.10$ (t, $J = 6.6$ Hz, 1H, C*H*NH_2), 4.04 (t, $J = 6.6$ Hz, 1H, C*H*NH_2), 3.32–3.17 (m, 2H, CH_2NHC(O)), 2.61 (dt, $J = 7.2$ Hz, $J = 2.4$ Hz, 2H, CH_2SH), 2.24–2.11 (m, 2H, CH_2CH_2SH), 2.03–1.85 (m, 2H, HOOCCH(NH_2)CH_2), 1.64–1.55 (m, 3H, CH_2CH_2NHC(O)), S*H*), 1.51–1.36 (m, 2H, CH_2CH_2CH_2NHC(O)).

Determination of *N*ε-Hcy-Lys

The *N*ε-Hcy-Lys assay is based on the procedure used previously for the determination of plasma thiols (Glowacki and Bald 2009). Plasma or a reaction mixture (50 µL) was diluted with 100 µL 0.2 M sodium phosphate buffer, pH 7.4, disulfide bonds were reduced by treatment with 10 µL 0.25 M tris(2-carboxyethyl)phosphine (TCEP) (Merck, Darmstadt, Germany) for 10 min, and derivatized with 10 µL 0.1 M 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT), prepared as previously described (Bald et al. 2004), for 3 min at room temperature. The reaction mixture was treated with 50 µL of 3 M perchloric acid, the precipitated protein removed by centrifugation (10 min, 12,000×*g*), and 20 µL of the supernatant was injected onto a C18 HPLC column. Without TCEP treatment, *N*ε-Hcy-Lys values were about 80% lower, indicating that bulk of the plasma *N*ε-Hcy-Lys exists as oxidized disulfide(s).

HPLC

HPLC analyses were carried out using a C18 reverse phase column (Zorbax SB-C18, 4.6 × 150 mm, 5 µm) from Agilent Technologies and a Hewlett-Packard 1100 series instrument consisting of a quaternary pump, autosampler, thermostated column compartment, vacuum degaser, and a diode-array UV–vis detector. The instrument was controlled by a Vectra computer running Hewlett-Packard ChemStation for LC 3D system software. Solvents A (0.07 M trichloroacetic acid in water, adjusted to pH 2.23 with 1 M NaOH) and B (neat acetonitrile) were used as eluents and the flow rate was 1.2 mL/min. After sample application, the column was eluted for 3 min with 11% solution B, followed by a linear gradient from 11 to 35% B for 9 min, and then from 35 to 11% B (v/v) for 3 min.

The effluent was monitored at A_{355}, the UV absorption maximum for the thiol-CMQT derivative. For each sample, the identity of the eluted material as *N*ε-Hcy-Lys was confirmed by its co-migration with an authentic *N*ε-Hcy-Lys standard, by the characteristic UV spectrum of its CMQT derivative (recorded during the HPLC runs), and by its thiol reagent reactivity. The detection limit was 1 pmol of *N*ε-Hcy-Lys. The inter-assay and intra-assay variations were 4.3 and 7.5%, respectively. Average spike recovery was 99% (range from 97 to 101%) for *N*ε-Hcy-Lys monitored in plasma matrix across all concentrations used (0.1–10 µM).

Determination of *N*-Hcy-protein and tHcy

Plasma *N*-Hcy-protein (Jakubowski 2008) and tHcy (Bald et al. 2004) were assayed as previously described.

Results

*N*ε-Hcy-Lys isopeptide assay

Previous experiments established that Hcy-thiolactone reacts about three times faster with the ε-amino group than with the α-amino group of lysine (second-order rate constants are 3.8 and 1.2 M^{-1} h^{-1}, respectively; Jakubowski 1999, 2000) and that incubation of Hcy-thiolactone with lysine affords *N*ε-Hcy-Lys isopeptide as a major product that migrates slower than Hcy on TLC plates (Jakubowski 1997). We found that the rate of *N*ε-Hcy-Lys formation increased about twofold when pH increased from 6.0 to 7.4 and did not significantly change between pH 7.4 and 9.0 whereas the yield increased 1.7-fold as the pH increased from 6.0 to 8.0 and then somewhat decreased (to a value of 1.4-fold relative to that observed at pH 6.0) (not shown). *N*ε-Hcy-Lys was purified from reaction mixtures by preparative HPLC and its structure confirmed by NMR (see "Materials and methods"). The thiol group of *N*ε-Hcy-Lys can be reversibly oxidized to a disulfide form, which does not move from the origin of the TLC plates (Jakubowski 1997). The thiol reactivity was exploited to develop an *N*ε-Hcy-Lys assay. Towards this end, we have used the thiol-specific reagent CMQT (Glowacki and Bald 2009; Bald et al. 2004) which undergoes facile reaction also with *N*ε-Hcy-Lys to give derivative with characteristic UV

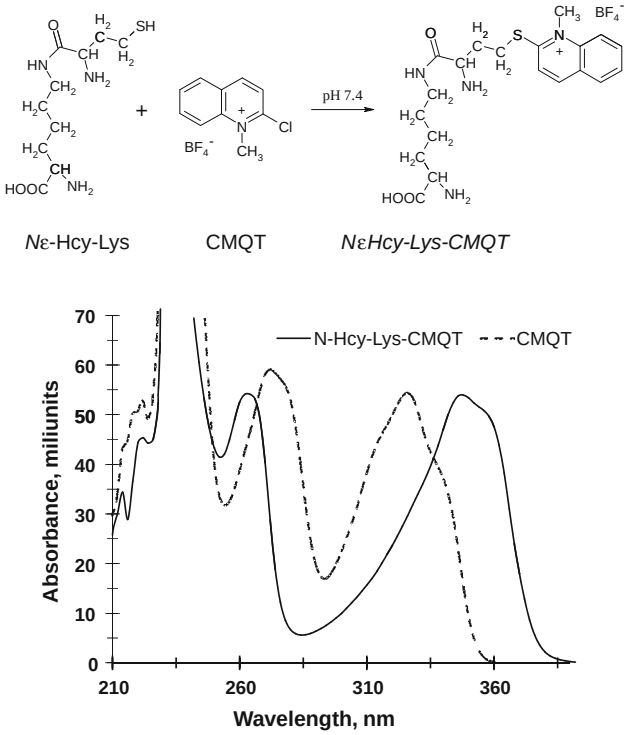

Fig. 1 Derivatization of *N*ε-Hcy-Lys with CMQT. *Upper panel* Derivatization reaction equation. *Lower panel* Absorption spectra of CMQT (*broken line*) and CMQT-derivatized *N*ε-Hcy-Lys peaks recorded during HPLC run (*solid line*)

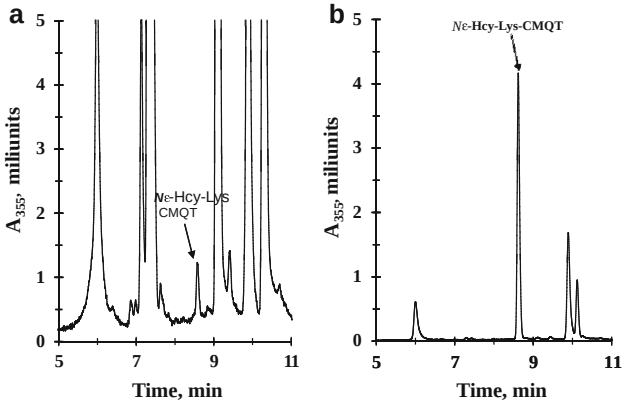

Fig. 2 Reverse phase HPLC determination of *N*ε-Hcy-Lys derivatized with CMQT. **a** Human plasma found to contain 0.75 μM *N*ε-Hcy-Lys. **b** CMQT-derivatized *N*ε-Hcy-Lys standard, 4 μM. *N*ε-Hcy-Lys 2-*S*-quinolinium derivative elutes at 8.6 min, indicated by *arrows*. 2-*S*-Quinolinium derivatives of Hcy and Cys elute at 7.3 and 9.2 min, respectively

absorption spectrum (maximum at 355 nm) (Fig. 1). The *N*ε-(Hcy-S-CMQT)Lys derivative was stable for at least 24 h at room temperature. An example of HPLC analysis of *N*ε-Hcy-Lys content in human plasma is shown in Fig. 2. The *N*ε-Hcy-Lys assay was linear in the concentration range 0.1–10 μM.

Levels of *N*ε-Hcy-Lys in normal mouse and human plasma

Plasma concentrations of *N*ε-Hcy-Lys in wild type C57BL/6 mice varied from <0.1 to 0.56 μM with an average of 0.40 ± 0.20 μM (Table 1). Plasma concentrations of *N*-Hcy-protein and tHcy in these mice were 1.4 ± 0.5 and 9.2 ± 3.2 μM, respectively. Concentrations of *N*ε-Hcy-Lys in normal human plasma were <0.1 μM (Table 2).

Genetic or nutritional hyperhomocysteinemia elevates plasma *N*ε-Hcy-Lys levels in mice

Effects of genetic hyperomocysteinemia on *N*ε-Hcy-Lys levels were examined in *Mthfr*-deficient and *Cbs*-deficient mice, which have previously been shown to have >10-fold elevated protein *N*-linked Hcy levels relative to wild type mice (Jakubowski et al. 2009). Plasma *N*ε-Hcy-Lys concentrations were significantly higher in homozygous $Mthfr^{-/-}$ mice than in heterozygous $Mthfr^{+/-}$ or wild type animals (Table 1). A single examined homozygous $Cbs^{-/-}$ mouse had 5–10 times more plasma *N*ε-Hcy-Lys (1.98 μM) and *N*-Hcy-protein (17.8 μM) than a heterozygous $Cbs^{+/-}$ mouse (0.20 μM *N*ε-Hcy-Lys and 1.8 μM *N*-Hcy-protein) or wild type mice (Table 1). There was a linear relationship between free *N*ε-Hcy-Lys and protein *N*-linked Hcy (Fig. 3).

Nutritional hyperhomocysteinemia was induced by feeding mice a 1.5% Met diet, which has previously been shown to cause ~12-fold increase in plasma *N*-Hcy-protein levels relative to wild type animals (Jakubowski et al. 2009). We found that plasma *N*ε-Hcy-Lys levels increased ~10-fold in wild type mice fed a 1.5% Met diet relative to animals fed a standard chow diet (Table 1).

Hyperhomocysteinemia increases plasma *N*ε-Hcy-Lys levels in humans

Individuals with CBS deficiency (Mudd et al. 2001; Jakubowski et al. 2008) or renal disease (Perna et al. 2006) are known to have elevated plasma Hcy and *N*-Hcy-protein levels. We found that in CBS-deficient patients plasma *N*ε-Hcy-Lys levels varied from <0.1 to 2.52 μM with a mean value of 0.56 ± 0.69 μM, significantly higher than in healthy individuals (Table 2). It should be noted that CBS-deficient patients were on Hcy-lowering therapy (see "Methods"); thus these *N*ε-Hcy-Lys concentrations represent minimal values. Because patients are treated immediately after ascertainment of CBS deficiency, untreated patients were not available for these studies.

Table 1 Plasma Nε-Hcy-Lys, *N*-Hcy-protein, and tHcy in mice

Genotype (*n*) and diet	Nε-Hcy-Lys (μM)	*N*-Hcy-protein (μM)[a]	tHcy (μM)[a]
Wild type ($n = 6$)	0.40 ± 0.20	1.4 ± 0.5	9.2 ± 3.2
Wild type ($n = 3$), high Met, 8 weeks	4.13 ± 4.86	11.7 ± 3.8	109 ± 53
$Mthfr^{-/-}$ ($n = 4$)	0.88 ± 0.35	10.4 ± 8.7	87.5 ± 44.1
$Mthfr^{+/-}$ ($n = 3$)	0.42 ± 0.00	1.7 ± 1.3	15.9 ± 2.7

Number of mice plasma samples (*n*) is indicated in the parentheses. Data are expressed as mean ± SD

[a] Data from Jakubowski et al. (2009)

Table 2 Plasma Nε-Hcy-Lys, *N*-Hcy-protein, and tHcy in human subjects

Subjects (*n*)	Nε-Hcy-Lys (μM)	*N*-Hcy-protein (μM)	tHcy (μM)
Healthy ($n = 30$)	<0.1	0.79 ± 0.44	8.5 ± 3.6
Renal disease patients ($n = 5$)	0.17 ± 0.06	1.1 ± 0.5	23.3 ± 7.6
CBS-deficient patients ($n = 29$)[a]	0.56 ± 0.69	3.2 ± 2.2[b]	47.1 ± 56.9[b]

Number of subjects (*n*) is indicated in parentheses. Data are expressed as mean ± SD

[a] CBS-deficient patients were on an Hcy-lowering therapy

[b] Data from Jakubowski et al. (2008)

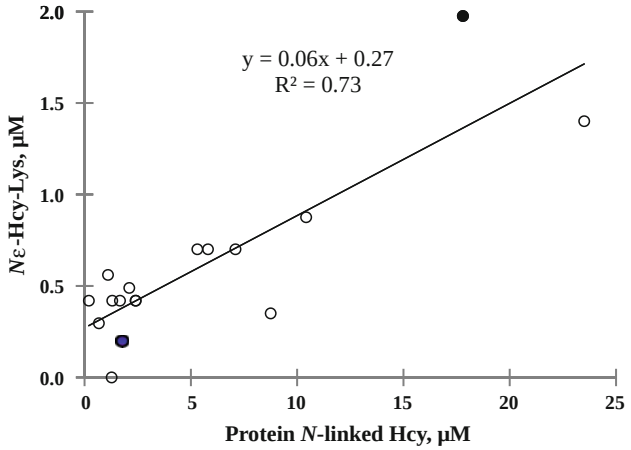

Fig. 3 The relationship between plasma Nε-Hcy-Lys and protein *N*-linked Hcy in $Mthfr^{-/-}$, $Mthf^{+/-}$, $Mthfr^{+/+}$ (*empty circle*), $Cbs^{-/-}$, $Cbs^{+/-}$ (*filled circle*) mice

We also found that in renal disease patients, plasma Nε-Hcy-Lys levels varied from 0.11 to 0.27 μM with a mean value of 0.17 ± 0.06 μM, significantly higher than in healthy individuals (Table 2). Consistent with previous observations (Perna et al. 2006), plasma *N*-Hcy-protein and tHcy in these patients were 1.1 ± 0.5 and 23.3 ± 7.6 μM, also significantly higher than in healthy individuals (Table 2).

Proteolytic degradation of *N*-Hcy-hemoglobin in mouse liver extracts affords Nε-Hcy-Lys

To determine whether Nε-Hcy-Lys can arise by proteolytic degradation of *N*-Hcy-protein, we incubated *N*-Hcy-Hb with mouse liver extracts and assayed the reaction mixtures for Nε-Hcy-Lys. We found that free Nε-Hcy-Lys appeared in complete reaction mixtures containing *N*-Hcy-Hb and mouse liver extract (Fig. 4a). Control experiments showed that free Nε-Hcy-Lys was absent both in mouse liver extract and in *N*-Hcy-Hb preparations (Fig. 4b, c). Similar results were obtained with liver extracts prepared from three individual C57BL/6 mice.

Discussion

The data presented in this communication show that (1) Nε-Hcy-Lys is a novel metabolite present in humans and mice, (2) plasma Nε-Hcy-Lys levels are significantly elevated in genetically or nutritionally induced hyperhomocysteinemia, and (3) free Nε-Hcy-Lys is a product of *N*-Hcy-protein turnover. These data provide evidence that Nε-Hcy-Lys is a pathology-related component of Hcy metabolism in humans and mice.

Hcy is known to exist in the circulation in several molecular forms (Jakubowski 2005; Mudd et al. 2000). The bulk of plasma Hcy exists in oxidized forms such as homocystine and mixed disulfides with albumin, globulins (Hcy-S-S-protein), and cysteine (Hcy-S-S-Cys), whereas free reduced Hcy is a minor form. Hcy recovered by treatments of biological samples with reducing agents is called "total" Hcy (tHcy) (Mudd et al. 2000). In addition to tHcy, other important Hcy forms are present in plasma: Hcy-thiolactone and *N*-Hcy-protein, well-documented in humans and mice in previous work of ours (Jakubowski 2000, 2002a, b, 2008; Glowacki and Jakubowski 2004; Jakubowski et al. 2008, 2009; Chwatko and Jakubowski

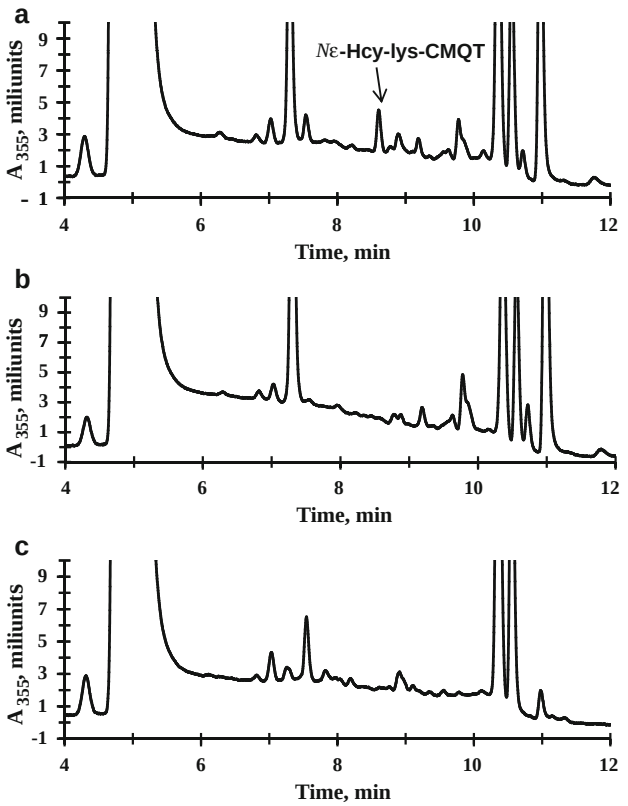

Fig. 4 Reverse phase HPLC analyses of *N*-Hcy-hemoglobin degradation in mouse liver extracts. HPLC traces were obtained with **a** complete reaction mixture containing *N*-Hcy-hemoglobin and mouse liver extract, **b** *N*-Hcy-hemoglobin, and **c** mouse liver extract. *Nε*-Hcy-Lys, eluting at 8.6 min, is present only in complete reaction mixture, indicated by an *arrow* in **a**

2005a, b; Chwatko et al. 2007; Perla-Kajan et al. 2008) and others (Perna et al. 2006; Zang et al. 2009; Daneshvar et al. 2003; Uji et al. 2002), as well as *Nε*-Hcy-Lys discovered in plasma in the present work. Plasma *Nε*-Hcy-Lys comprises 0.7–1.2 and 0.7–4.4% of plasma tHcy or 15.5–17.5 and 8.5–35% of plasma *N*-Hcy-protein in humans (renal disease or CBS-deficient patients) and mice, respectively (calculated from the values shown in Tables 1 and 2 according to the formulas 100 × [*Nε*-Hcy-Lys]/[tHcy] or 100 × [*Nε*-Hcy-Lys]/[*N*-Hcy-protein]). In healthy human subjects *Nε*-Hcy-Lys comprises <1.2% of plasma tHcy or <12.7% of plasma *N*-Hcy-protein (Table 2). Thus, plasma *Nε*-Hcy-Lys levels are higher in mice than in humans, most likely reflecting higher Hcy-thiolactone (Chwatko et al. 2007) and *N*-Hcy-protein (Jakubowski et al. 2008) levels in mice compared with humans. Our present data show that, similar to other Hcy metabolites, *Nε*-Hcy-Lys levels are elevated under pathological conditions, i.e., in human CBS deficiency, as wells as in mouse Cbs or Mthfr deficiency.

It is well established that *Nε*-Hcy-Lys occurs in a protein-bound form, *N*-Hcy-protein (Jakubowski et al. 2008, 2009), which is generated by a chemical reaction of protein lysine residues with Hcy-thiolactone (Jakubowski 1997, 1999, 2000). Free *Nε*-Hcy-Lys can thus arise by proteolytic degradation of *N*-Hcy-protein. Indirect evidence for proteolytic degradation of *N*-Hcy-protein is provided by the discovery of anti-*N*-Hcy-protein IgG autoantibodies (Jakubowski 2005), which specifically recognize *Nε*-Hcy-Lys epitopes, and whose formation can only be initiated by proteolytic degradation of *N*-Hcy-protein to antigenic peptides that are then displayed on the cell surface. Our present results provide direct evidence showing that free *Nε*-Hcy-Lys arises by proteolytic degradation of *N*-Hcy-protein in mouse liver extracts. Free *Nε*-Hcy-Lys can also arise by a reaction of free lysine with Hcy-thiolactone. However, because the concentration of free lysine (0.18 mM in human plasma) is some 250 times lower than the concentration of protein lysine residues (∼50 mM in plasma), the reaction of Hcy-thiolactone with protein lysine residues greatly predominates over the reaction with free lysine. Thus, *N*-Hcy-protein is the major source of free *Nε*-Hcy-Lys while the contribution of the reaction of free lysine with Hcy-thiolactone to the pool of free *Nε*-Hcy-Lys is expected to be very small.

Metabolic pathway leading to *Nε*-Hcy-Lys is illustrated by the following scheme:

Hcy → Hcy-thiolactone → *N*-Hcy-protein → *Nε*-Hcy-Lys. The pathway is initiated by the conversion of Hcy to Hcy-thiolactone catalyzed by methionyl-tRNA synthetase (Jakubowski and Goldman 1993). Hcy-thiolactone spontaneously reacts with protein lysine residues (Jakubowski 1997, 1999, 2000; Jakubowski et al. 2000) generating *N*-Hcy-protein (Jakubowski et al. 2008, 2009; Jakubowski 2002). Proteolytic degradation of *N*-Hcy-protein affords *Nε*-Hcy-Lys. Because its levels are elevated in genetic and nutritional hyperhomocysteinemia (Tables 1, 2), we conclude that *Nε*-Hcy-Lys is linked to Hcy-related pathology.

Accumulating evidence suggests that *N*-Hcy-protein can be toxic. For example, human clinical studies show that plasma *N*-Hcy-protein levels are associated with the risk of coronary heart disease (Yang et al. 2006). Furthermore, in vitro studies show that *N*-Hcy-low-density lipoprotein and *N*-Hcy-albumin, similar to other *N*-Hcy-proteins, have the propensity to aggregate (Jakubowski 1999) and induce cell death in cultured human endothelial (Ferretti et al. 2004) and murine endothelioma cells (Paoli et al. 2010), respectively. Further studies are required to determine whether free *Nε*-Hcy-Lys isopeptide itself is toxic and whether it could serve as a predictor of heart or brain disease.

Acknowledgments This work was supported in part by grants from the American Heart Association (0855919D) and the Ministry of Science and Higher Education, Poland (NN 401 230634, N401 065 32/1504, POIG.01.03.01-00-097/08).

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