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ORIGINAL ARTICLE

Metabolism and neurotoxicity of homocysteine thiolactone in mice: protective role of bleomycin hydrolase

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Abstract Genetic or nutritional disorders in homocysteine (Hcy) metabolism elevate Hcy-thiolactone and cause heart and brain diseases. Hcy-thiolactone has been implicated in these diseases because it has the ability to modify protein lysine residues and generate toxic *N*-Hcy-proteins with auto-immunogenic, pro-thrombotic, and amyloidogenic properties. Bleomycin hydrolase (Blmh) has the ability to hydrolyze L-Hcy-thiolactone (but not D-Hcy-thiolactone) to Hcy in vitro, but whether this reflects a physiological function has been unknown. Here, we show that $Blmh^{-/-}$ mice excreted in urine 1.8-fold more Hcy-thiolactone than wild-type $Blmh^{+/+}$ animals ($P = 0.02$). Hcy-thiolactone was elevated 2.3-fold in brains ($P = 0.004$) and 2.0-fold in kidneys ($P = 0.047$) of $Blmh^{-/-}$ mice relative to $Blmh^{+/+}$ animals. Plasma *N*-Hcy-protein was elevated in $Blmh^{-/-}$ mice fed a normal (2.3-fold, $P < 0.001$) or hyperhomocysteinemic diet (1.5-fold, $P < 0.001$), compared with $Blmh^{+/+}$ animals. More intraperitoneally injected L-Hcy-thiolactone was recovered in plasma in $Blmh^{-/-}$ mice than in wild-type $Blmh^{+/+}$ animals (83.1 vs. 39.3 μM, $P < 0.0001$). In $Blmh^{+/+}$ mice injected intraperitoneally with D-Hcy-thiolactone, D,L-Hcy-thiolactone, or L-Hcy-thiolactone, 88, 47, or 6.3%, respectively, of the injected dose was recovered in plasma. The incidence of seizures induced by L-Hcy-thiolactone injections (3,700 nmol/g body weight) was higher in $Blmh^{-/-}$ than in $Blmh^{+/+}$ mice (93.8 vs. 29.5%, $P < 0.001$). Using the *Blmh* null mice, we provide the first direct evidence that a specific Hcy metabolite, Hcy-thiolactone, rather than Hcy itself, is neurotoxic in vivo. Taken together, our findings indicate that Blmh protects mice against L-Hcy-thiolactone toxicity by metabolizing it to Hcy and suggest a mechanism by which Blmh might protect against neurodegeneration associated with hyperhomocysteinemia and Alzheimer's disease.

Keywords Bleomycin hydrolase · Homocysteine thiolactone · Brain · Seizures · Alzheimer's disease

Abbreviations

Blmh	Bleomycin hydrolase
Cbs	Cystathionine β-synthase
Hcy	Homocysteine
tHcy	Total Hcy
i.p.	Intraperitoneal
Mthfr	Methylenetetrahydrofolate reductase

Introduction

Homocysteine (Hcy) is generated during metabolism of the essential dietary protein amino acid methionine (Met). Hcy

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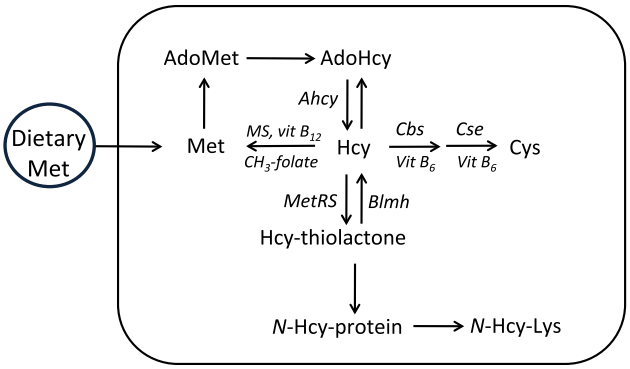

Fig. 1 Schematic representation of mammalian homocysteine metabolism. Met, methionine; AdoMet, S-adenosylmethionine; Hcy, homocysteine; AdoHcy, S-adenosylhomocysteine; Cys, cysteine; MS, Met synthase; Ahcy, AdoHcy hydrolase; Cbs, cystathionine β-synthase; Cse, cystathionine γ-lyase; MetRS, methionyl-tRNA synthetase; *N*-Hcy-protein, protein containing Hcy linked to ε-amino group of a lysine residue; *N*-Hcy-Lys, an isopeptide containing Hcy linked to ε-amino group of lysine; Blmh, bleomycin hydrolase

levels are regulated by remethylation to Met and transsulfuration to cysteine (Fig. 1). Genetic or nutritional deficiencies in these pathways cause hyperhomocysteinemia and lead to heart and brain pathologies (Mudd et al. 2001).

Hcy is also metabolized to the thioester Hcy-thiolactone in an error-editing reaction in protein biosynthesis when Hcy is erroneously selected in place of methionine by methionyl-tRNA synthetase (Jakubowski 1990, 2011; Jakubowski and Goldman 1993) (Fig. 1). The flow through the Hcy-thiolactone pathway greatly increases in genetic or nutritional hyperhomocysteinemia. Hcy-thiolactone is chemically reactive and has a propensity to form isopeptide bonds with protein lysine residues (*N*-Hcy-protein) (Jakubowski 1997, 1999, 2000a; Jakubowski et al. 2000). This reaction impairs or alters the protein's structure/function, causes protein damage (Jakubowski 1999; Perla-Kajan et al. 2007; Glowacki and Jakubowski 2004) by a thiyl radical mechanism (Sibrian-Vazquez et al. 2010), generates amyloid-like structures (Paoli et al. 2010), and contributes to multiple human pathologies (Jakubowski 2008a), including an autoimmune response (Undas et al. 2004), atherosclerosis (Perla-Kajan et al. 2008), thrombosis (Sauls et al. 2006; Jakubowski et al. 2008), and Alzheimer's disease (Suszynska et al. 2010). Proteolytic degradation of *N*-Hcy-protein generates the isopeptide *N*-Hcy-Lys (Glowacki et al. 2010) (Fig. 1).

Hcy-thiolactone (Chwatko et al. 2007), *N*-Hcy-protein (Jakubowski et al. 2008, 2009), and *N*-Hcy-Lys (Glowacki et al. 2010) have been identified as constituents of the blood and shown to be elevated in genetic or dietary hyperhomocysteinemia in humans and mice. Human clinical studies show that plasma *N*-Hcy-protein and Hcy-thiolactone are associated with a risk of coronary heart disease (Yang et al. 2006) and the development and progression of diabetic vascular damage (Gu et al. 2008), respectively, whereas anti-*N*-Hcy-protein auto-antibody titers predict stroke (Undas et al. 2004) and coronary heart disease (Undas et al. 2005). *N*-Hcy-Lys isopeptide is elevated in patients with acute myocardial infarction (Zabczyk et al. 2011). Injections of Hcy-thiolactone cause acute seizures and lethality in mice (Sprince et al. 1969; Spence et al. 1995) and rats (Folbergrova 1997; Rasic-Markovic et al. 2009).

Bleomycin hydrolase (Blmh), named for its ability to hydrolyze the anticancer drug bleomycin, ubiquitously expressed in various human and rat organs (Bromme et al. 1996; Kamata et al. 2007), has been studied in the context of cancer therapy (Bromme et al. 1996), Alzheimer's disease (Papassotiropoulos et al. 2000; Lefterov et al. 2001; Kajiya et al. 2006), protein breakdown (Kamata et al. 2009), and Hcy toxicity (Suszynska et al. 2010; Zimny et al. 2006). We found that Blmh is an Hcy-thiolactonase that protects against Hcy toxicity in yeast (Zimny et al. 2006). More recently, we found that Hcy-thiolactonase activity of Blmh is decreased in brains of Alzheimer's disease patients (Suszynska et al. 2010). As expected, $Blmh^{-/-}$ mice are more sensitive to bleomycin toxicity than wild-type animals and, unexpectedly, prone to tail dermatitis (Schwartz et al. 1999). However, whether Blmh participates in Hcy-thiolactone metabolism in vivo in mammals is unknown.

The present work was undertaken to examine a hypothesis that Blmh metabolizes Hcy-thiolactone in vivo in mice using a *Blmh* knockout model. We also studied how the inactivation of the *Blmh* gene affects neurotoxicity of Hcy-thiolactone in mice.

Materials and methods

Mice and diet

Knockout $Blmh^{-/-}$ mice on the C57BL/6J genetic background (Schwartz et al. 1999) and wild-type $Blmh^{+/+}$ littermates were maintained on a rodent chow (LabDiet 5010, Purina Mills International, St. Louis, MO). The Blmh genotype was confirmed by PCR analysis (Schwartz et al. 1999). $Blmh^{-/-}$ mice produced smaller litter size and exhibited tail dermatitis, which often led to shorter tails and facilitated identification. To induce hyperhomocysteinemia 4-week-old mice were provided 1% Met in drinking water for 2–8 weeks (Zhou et al. 2001; Velez-Carrasco et al. 2008). Equal numbers of males and females were used in each experiment. Animal procedures were approved by the

Institutional Animal Care and Use Committee at the New Jersey Medical School.

Mouse plasma and urine

The blood was collected by cheek vein puncture into plastic Eppendorf tubes containing 10 mM EDTA and chilled on ice. Plasma was separated by centrifugation (4°C, 4,000×*g*, 10 min) and stored at −80°C. Urine was collected at about 2-h intervals for a period of 24 h; each portion was chilled on ice and stored at −80°C.

Hcy-thiolactone and Hcy turnover

L-Hcy-thiolactone was dissolved in PBS and injected intraperitoneally (i.p.) into 4–12 weeks old mice (40–600 nmol/g body weight). The size of Hcy-thiolactone dose, as well as mice age and sex did not affect the rate of L-Hcy-thiolactone clearance. The mice were bled 5, 10, 20 and 30 min (for Hcy-thiolactone assays) or 10, 20, 30, 45, 60, 75, and 90 min (for Hcy assays) post-injection. Plasma samples were stored with 10 mM EDTA at −80°C. Hcy-thiolactone and Hcy were assayed by HPLC.

N-Hcy-protein turnover

Mice were fed 1% Met in drinking water for 6 days and then shifted to plain water. Alternatively, mice were injected i.p. with L-Hcy-thiolactone to increase *N*-Hcy-protein levels. Because lower doses of L-Hcy-thiolactone did not result in a significant increase in plasma *N*-Hcy-protein, a dose of 2,850 nmol L-Hcy-thiolactone/g body weight was used. This dose was nontoxic. Plasma samples were collected on EDTA at 2, 4, 8, 12, 24, 36, and 48 h post-shift or post-injection, and frozen at −80°C. *N*-Hcy-protein levels in each plasma sample were assayed by HPLC.

Hcy-thiolactone toxicity

L-Hcy-thiolactone was dissolved in PBS and injected i.p. into 4–5 weeks old mice (3,700 nmol/g body weight). Mice were placed on the top of a plastic cage and observed for behavioral manifestations for 90 min. This was assessed by the incidence and latency of seizures and death (Sprince et al. 1969; Spence et al. 1995).

Hcy-thiolactone, *N*-Hcy-protein, and Hcy assays

Hcy-thiolactone, *N*-Hcy-protein, and total Hcy were assayed by HPLC-based methods with post-column derivatization and fluorescence detection as previously described (Chwatko and Jakubowski 2005a; Jakubowski 2008b).

Results

$Blmh^{-/-}$ mice excrete more Hcy-thiolactone than $Blmh^{+/+}$ animals

To determine whether Blmh participates in Hcy-thiolactone metabolism, we studied $Blmh^{-/-}$ mice and their $Blmh^{+/+}$ littermates. To facilitate Hcy-thiolactone measurements, mice were provided 1% Met in drinking water for 8 weeks, which induced hyperhomocysteinemia (Zhou et al. 2001; Velez-Carrasco et al. 2008). Plasma tHcy in $Blmh^{-/-}$ and $Blmh^{+/+}$ mice fed Met-supplemented drinking water was elevated 5.7- and 10.4-fold (to 39 ± 19 and 77 ± 45 μM, respectively), from a basal level of 6.8 ± 2.2 and 7.4 ± 2.2 μM, respectively, in mice that drank a non-supplemented water. The consumption of Met-supplemented water (3.1 ml/mouse) was not affected by the *Blmh* genotype. Because Hcy-thiolactone is efficiently cleared by the kidneys, and its concentrations are ~100-fold higher in urine than in plasma (Chwatko et al. 2007; Chwatko and Jakubowski 2005b), we quantified Hcy-thiolactone in 24-h urine. As shown in Table 1, $Blmh^{-/-}$ mice excreted 1.8-fold more Hcy-thiolactone than $Blmh^{+/+}$ animals. Urinary tHcy levels were somewhat lower in $Blmh^{-/-}$ than in $Blmh^{+/+}$ mice, but the difference was not statistically significant (Table 1).

Inactivation of the *Blmh* gene elevates brain and kidney Hcy-thiolactone

Because levels of Blmh protein vary between different tissues (Kamata et al. 2007), the inactivation of *Blmh* gene might affect Hcy-thiolactone accumulation in a tissue-specific manner. Indeed, we found that Hcy-thiolactone levels were significantly elevated in brain and kidney of $Blmh^{-/-}$ mice, compared with wild-type $Blmh^{+/+}$ animals (Table 2). Hcy-thiolactone levels in heart, liver, lung, spleen, and plasma were not significantly affected by the inactivation of *Blmh* gene (Table 2). Because the increase in Hcy-thiolactone levels could be due to the increase in Hcy levels, we assayed brain tHcy and found that it was similar in $Blmh^{-/-}$ and $Blmh^{+/+}$ mice, 46.4 ± 10.9 and 49.8 ± 3.1 pmol/mg tissue, respectively. Thus, the increase in Hcy-thiolactone observed in $Blmh^{-/-}$ mice is caused by inactivation of the *Blmh* gene, and not by its effect on Hcy metabolism.

Metabolic conversion of Hcy-thiolactone to Hcy is impaired in $Blmh^{-/-}$ mice

To further explore Hcy-thiolactone metabolism and toxicity, we used the i.p. injection model, which has been extensively used to define mechanism of seizures in mice

Table 1 Urinary Hcy-thiolactone and total Hcy in *Blmh*$^{-/-}$ and wild-type *Blmh*$^{+/+}$ mice

Genotype (*n*)	Urinary Hcy-thiolactone (nmol/24 h)	Urinary Hcy (nmol/24 h)	Urine volume (ml/24 h)	Mouse body weight (g)
Blmh$^{-/-}$ (4)	2.4 ± 0.7*	670 ± 334	0.86 ± 0.14	21.3 ± 0.9
Blmh$^{+/+}$ (9)	1.3 ± 0.4	817 ± 231	0.58 ± 0.14	21.4 ± 0.7

The mice were fed a 1% Met-supplemented drinking water for 8 weeks. Consumption of Met-supplemented water (3.1 ml/mouse) was not affected by the genotype. Plasma total Hcy levels in *Blmh*$^{-/-}$ and *Blmh*$^{+/+}$ mice were 39 ± 19 and 77 ± 45 μM, respectively

* Significantly increased versus *Blmh*$^{+/+}$: *t* test $P = 0.02$

Table 2 Tissue levels of Hcy-thiolactone in *Blmh*$^{-/-}$ and wild-type *Blmh*$^{+/+}$ mice

Genotype (*n*)	Hcy-thiolactone (pmol/mg tissue)						
	Brain	Heart	Kidney	Liver	Lung	Spleen	Plasma
Blmh$^{-/-}$ (5)	0.76 ± 0.27*	0.11 ± 0.04	1.02 ± 0.35*	<0.05	0.14 ± 0.01	0.22 ± 0.34	0.095 ± 0.082
Blmh$^{+/+}$ (6)	0.33 ± 0.15	0.10 ± 0.02	0.50 ± 0.27	<0.05	0.22 ± 0.13	0.12 ± 0.14	0.112 ± 0.078

* Significantly increased versus *Blmh*$^{+/+}$: *t* test $P = 0.004$

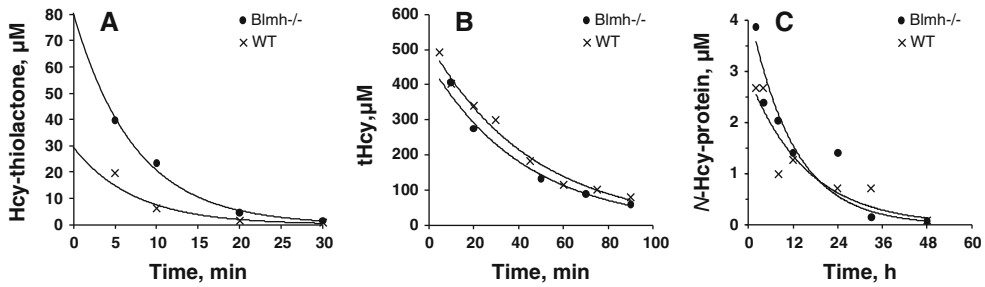

Fig. 2 Kinetics of plasma Hcy-thiolactone (**a**), total Hcy (**b**), and *N*-Hcy-protein (**c**) turnover in mice. For Hcy-thiolactone (**a**) and total Hcy (**b**) turnover experiments, mice were injected i.p. with 600 nmol L-Hcy-thiolactone/g body weight. For *N*-Hcy-protein (**c**) turnover experiments, 2,850 nmol L-Hcy-thiolactone/g body weight L-Hcy-thiolactone was used. Metabolites were analyzed at indicated times post-injection and data points were fitted to an exponential equation $[A^t] = [A^0] \cdot e^{-k \cdot t}$, where k is a first-order rate constant, $[A^t]$ is metabolite concentration measured at time t, and $[A^0]$ is metabolite concentration extrapolated to time zero. Representative kinetics obtained for individual knockout *Blmh*$^{-/-}$ (*filled circle*) and wild-type *Blmh*$^{+/+}$ (*multi symbol*) mice are shown

(Sprince et al. 1969; Spence et al. 1995) and rats (Spence et al. 1995; Folbergrova 1997; Rasic-Markovic et al. 2009). We injected L-Hcy-thiolactone i.p. into *Blmh*$^{-/-}$ and wild-type *Blmh*$^{+/+}$ mice, and followed the kinetics of Hcy-thiolactone and Hcy in plasma. In these experiments, we used a non-toxic dose of 600 nmol L-Hcy-thiolactone/g body weight, but similar results were obtained with doses as low as 40 nmol/g body weight.

Representative kinetics of plasma Hcy-thiolactone in individual *Blmh*$^{-/-}$ and *Blmh*$^{+/+}$ mice following i.p. injection with L-Hcy-thiolactone are illustrated in Fig. 2a, and averages of calculated kinetic indices for Hcy-thiolactone are shown in Table 3. The highest Hcy-thiolactone levels were observed at 5 min post-injection. Although identical doses were injected per each mouse, significantly more plasma Hcy-thiolactone was present in *Blmh*$^{-/-}$ than in *Blmh*$^{+/+}$ mice at early post-injection times (2.1-fold, $P < 0.0001$; Table 3). Assuming that the i.p.-injected Hcy-thiolactone, being mostly neutral at physiological pH [p$K_a = 6.7$, ref. (Jakubowski 2006)], distributes uniformly throughout the body and that blood constitutes 8% of the mouse body weight, plasma Hcy-thiolactone, extrapolated to time zero (HTL^0, Table 3), represented 14.4 and 5.9% ($P < 0.0001$) of the dose injected into *Blmh*$^{-/-}$ and *Blmh*$^{+/+}$ mice, respectively. These results show that, during its transit from the i.p. cavity to the bloodstream, Hcy-thiolactone is metabolized less efficiently in *Blmh*$^{-/-}$ mice compared with *Blmh*$^{+/+}$ animals.

At later post-injection times, plasma Hcy-thiolactone gradually decreased, approaching a basal level at 30 min post-injection. The decay of Hcy-thiolactone in plasma followed exponential kinetics (Fig. 2a) with a half-life of ~5 min both in *Blmh*$^{-/-}$ and *Blmh*$^{+/+}$ mice (Table 3). These half-lives are ~10 times shorter than Hcy-thiolactone

Table 3 Turnover of Hcy-thiolactone and Hcy in mouse plasma

Genotype	HTL^0 (μM)	HTL $t_{0.5}$ [min] (*n*)	Hcy^0 (μM)	Hcy $t_{0.5}$ [min] (*n*)
$Blmh^{-/-}$	83.1 ± 25.3*	5.1 ± 0.8 (8)	387 ± 91*	31.8 ± 6.2 (4)
$Blmh^{+/+}$	39.0 ± 13.9	5.0 ± 0.9 (15)	524 ± 136	26.2 ± 2.6 (5)

L-Hcy-thiolactone was injected i.p. at a dose of 600 nmol/g body weight. Plasma Hcy-thiolactone (HTL) and total Hcy were assayed at time points up to 30 min (for HTL) or 90 min (for Hcy). Half-lives ($t_{0.5} = 0.69/k$) and plasma concentrations at time zero (HTL^0, Hcy^0) were calculated from plasma concentrations at time t (HTL^t, Hcy^t) according to the equation $[HTL^t] = [HTL^0]\cdot e^{-k\cdot t}$ or $[Hcy^t] = [Hcy^0]\cdot e^{-k\cdot t}$, where k is a first-order rate constant

* Significantly different versus $Blmh^{+/+}$: t test $P < 0.0001$ (for HTL^0), P=0.021 (for Hcy^0)

Table 4 Turnover of Hcy-thiolactone in mouse serum

Genotype (*n*)	Hcy-thiolactone half-life (min)
$Blmh^{-/-}$ (8)	73 ± 7*
$Blmh^{+/+}$ (8)	55 ± 8

Serum was supplemented with [^{35}S]Hcy-thiolactone (Jakubowski 2007), incubated at 37°C, and the remaining thiolactone assayed at time intervals of 0.25–5 h as described previously (Perla-Kajan and Jakubowski 2010). Half-lives were calculated as in Table 3

* Significantly increased versus wild type: t test $P < 0.001$

Table 5 Turnover of D-, D,L-, and L-Hcy-thiolactone in the mouse

Hcy-thiolactone stereoisomer	HTL recovered in plasma (% injected dose)	HTL $t_{0.5}$ (min)
L-	6.3 ± 0.2	5.0 ± 0.9
D-	88 ± 3	5.7 ± 0.3
D,L-	47 ± 4	5.5 ± 0.2

Wild-type C57BL/6J mice were injected i.p. with L- ($n = 6$), D- ($n = 3$), or D,L-Hcy-thiolactone ($n = 6$) at a dose of 150 nmol/g body weight. Plasma Hcy-thiolactone (HTL) levels were assayed at time points up to 30 min post-injection. Plasma concentrations at time zero (HTL^0) and half-lives ($t_{0.5} = 0.69/k$) were calculated from plasma concentrations at time t (HTL^t) according to the equation $[HTL^t] = [HTL^0]\cdot e^{-k\cdot t}$, where k is a first-order rate constant. Hcy-thiolactone recovery calculations are based on assumptions that the i.p.-injected Hcy-thiolactone, being mostly neutral [pK_a = 6.7, Ref. (Jakubowski 2006)], distributes uniformly throughout the body and that blood constitutes 8% of the mouse body weight

half-lives of 73 and 55 min measured in vitro in serum from $Blmh^{-/-}$ and $Blmh^{+/+}$ mice, respectively (Table 4). Somewhat longer in vitro Hcy-thiolactone half-life in serum from $Blmh^{-/-}$ mice could be due to lower levels of the serum Hcy-thiolactonase/Pon1 (Jakubowski 2000b) in these mice. Similar in vivo plasma Hcy-thiolactone half-lives in $Blmh^{-/-}$ and $Blmh^{+/+}$ mice suggest that Blmh does not contribute to Hcy-thiolactone clearance from the blood and are consistent with the absence of Blmh in plasma (Bromme et al. 1996; Kamata et al. 2007).

Kinetics of plasma total Hcy in individual $Blmh^{-/-}$ and $Blmh^{+/+}$ mice i.p.-injected with L-Hcy-thiolactone are illustrated in Fig. 2b and averages of calculated kinetic parameters are shown in Table 3. In wild-type mice fed a standard chow diet, plasma total Hcy increased from a basal level of ~7 (Table 5) to ~500 μM at 5 min post-injection (Fig. 2b; Table 3). This shows that, during its transit from the i.p. cavity to the bloodstream, Hcy-thiolactone was quickly hydrolyzed to Hcy. Post-injection plasma total Hcy concentration gradually declined with first-order kinetics and a half-life of 26.2 min. In contrast, post-injection plasma total Hcy was significantly lower in $Blmh^{-/-}$ mice compared with wild-type $Blmh^{+/+}$ animals (Table 3). This shows that the hydrolytic turnover of Hcy-thiolactone is impaired in $Blmh^{-/-}$ mice.

Half-lives of plasma total Hcy turnover in $Blmh^{-/-}$ and $Blmh^{+/+}$ mice were similar, 31.8 and 26.2 min, respectively (Table 3). Overall, the in vivo clearance of Hcy in mouse plasma was about five times slower than the in vivo clearance of Hcy-thiolactone.

D-Hcy-thiolactone is metabolized less efficiently than L-Hcy-thiolactone in wild-type mice

Blmh is highly stereoselective and hydrolyzes the L-stereoisomer of Hcy-thiolactone, while the D-steroisomer is not hydrolyzed (Zimny et al. 2006; Jakubowski 2007). Thus, comparative studies of D-Hcy-thiolactone versus L-Hcy-thiolactone clearances in mice might provide additional evidence that Blmh participates in Hcy-thiolactone clearance in vivo. Toward this end, wild-type mice were i.p. injected with D-, D,L-, or L-Hcy-thiolactone at a dose of 150 nmol/g body weight. We found that 88 and 47% of the injected dose of D- and D,L-Hcy-thiolactone, respectively, was recovered in mouse plasma (Table 5). These amounts are 14- and 7-fold higher, respectively, than the amount of plasma Hcy-thiolactone recovered in mice injected with L-Hcy-thiolactone (6.3% of the i.p.-injected dose). However, half-lives of plasma D-, D,L-, or L-Hcy-thiolactone were similar (Table 5). These findings provide evidence that stereoselective Hcy-thiolactone-hydrolyzing enzyme(s), such as Blmh, are responsible for the metabolism of Hcy-thiolactone during its transition from the i.p. cavity to the bloodstream in mice. In contrast, clearance of Hcy-thiolactone from the bloodstream is non-stereospecific.

Table 6 Plasma *N*-Hcy-protein and total Hcy levels in $Blmh^{-/-}$ and wild-type $Blmh^{+/+}$ mice

Time [weeks] (8 mice/group)	*N*-Hcy-protein (μM)		*N*-Hcy-protein/Hcy		Hcy (μM)	
	$Blmh^{-/-}$	$Blmh^{+/+}$	$Blmh^{-/-}$	$Blmh^{+/+}$	$Blmh^{-/-}$	$Blmh^{+/+}$
0	2.8 ± 0.8*	1.2 ± 0.4	0.47 ± 0.25*	0.17 ± 0.05	6.8 ± 2.2	7.4 ± 2.2
2	7.3 ± 2.0*	4.9 ± 1.4	0.16 ± 0.12*	0.04 ± 0.02	40 ± 30†	169 ± 101
8	8.4 ± 2.8*	5.4 ± 2.9	0.24 ± 0.08*	0.09 ± 0.03	39 ± 19†	77 ± 45

The mice were fed 1% Met-supplemented drinking water for 2 and 8 weeks

* Significantly increased versus $Blmh^{+/+}$: *t* test $P < 10^{-3}$

† Significantly decreased versus $Blmh^{+/+}$: *t* test $P < 10^{-6}$

Elevated plasma *N*-Hcy-protein in $Blmh^{-/-}$ mice

N-Hcy-protein is known to accumulate predominantly in plasma and the accumulation increases in hyperhomocysteinemia (Jakubowski et al. 2009). To determine whether Blmh has the ability to protect against protein N-homocysteinylation in vivo, we assayed plasma *N*-Hcy-protein in $Blmh^{-/-}$ and $Blmh^{+/+}$ mice. We found that, $Blmh^{-/-}$ mice fed a standard chow diet accumulated significantly more *N*-Hcy-protein than $Blmh^{+/+}$ animals (Table 6). Providing mice with 1% Met in drinking water for 2 and 8 weeks increased plasma tHcy 6- to 20-fold and plasma *N*-Hcy-protein 3- to 4-fold. Under these hyperhomocysteinemic conditions, significantly (1.5-fold) more *N*-Hcy-protein accumulated in $Blmh^{-/-}$ compared with wild-type $Blmh^{+/+}$ animals (Table 6). However, it should be noted that *N*-Hcy-protein accumulation is proportional to Hcy concentration (Jakubowski et al. 2000, 2009; Jakubowski 2002) and that $Blmh^{-/-}$ mice had two- to fourfold lower plasma total Hcy than $Blmh^{+/+}$ animals. Thus, the 1.5-fold increase in *N*-Hcy-protein reflects both the effect of the *Blmh* gene inactivation and of the difference in Hcy levels. After normalizing to identical Hcy levels, the effect of the *Blmh* gene inactivation on *N*-Hcy-protein levels was greater: the *N*-Hcy-protein/Hcy ratio was 2.7- to 4.0-fold higher in $Blmh^{-/-}$ mice than in wild-type $Blmh^{+/+}$ animals (Table 6).

Because it also has an aminopeptidase activity (Schwartz et al. 1999), Blmh can participate in protein turnover (Kamata et al. 2009). To test whether impaired protein degradation could contribute to increased accumulation of *N*-Hcy-protein in $Blmh^{-/-}$ mice, we studied the kinetics of plasma *N*-Hcy-protein in $Blmh^{-/-}$ and $Blmh^{+/+}$ mice following i.p. injection with L-Hcy-thiolactone. Examples of the analyses for individual mice are shown in Fig. 2c. Plasma *N*-Hcy-protein levels were the highest at 2 h post-injection and then gradually decreased with exponential kinetics. Half-lives of *N*-Hcy-protein were similar in $Blmh^{-/-}$ and $Blmh^{+/+}$ animals, 10.3 ± 3.3 ($n = 5$) and 10.2 ± 1.4 h ($n = 5$), respectively. Similar results were obtained with mice that were fed Met-supplemented drinking water to elevate *N*-Hcy-protein (Jakubowski et al. 2009) and then shifted to plain water to observe *N*-Hcy-protein decay. Overall, the clearance of plasma *N*-Hcy-protein was about 120 and 24 times slower than the clearance of plasma Hcy-thiolactone and total Hcy (Table 3), respectively.

Blmh protects against Hcy-thiolactone toxicity in mice

L-Hcy-thiolactone i.p. are known to be toxic to rodents (Sprince et al. 1969). In C3H mice, LD_{50} and LD_{10} are reported to be 2,540 and 2,390 nmol/g body weight, respectively (Spence et al. 1995). We found that i.p. injections of L-Hcy-thiolactone at a dose of 3,700 nmol/g body weight induced seizures in 29.5% and death in 2.3% of wild-type C57BL/6J mice (Table 7), while doses from 40 to 2,850 nmol L-Hcy-thiolactone/g mouse body weight did not induce seizures or death.

To determine whether Blmh has the ability to protect against Hcy-thiolactone toxicity, $Blmh^{-/-}$ and $Blmh^{+/+}$ mice were injected i.p. with 3,700 nmol L-Hcy-thiolactone/g body weight and behavioral manifestations were

Table 7 Blmh protects against neurotoxicity of Hcy-thiolactone in mice

Genotype (*n*)	Incidence of seizures, % (*n*)	Incidence of death, %	Seizure latency period, min	Death latency period, min
$Blmh^{-/-}$ (32)	93.8 (30)*	46.9 (15)*	33.1 ± 10.1†	46.6 ± 15.1
$Blmh^{+/+}$ (44)	29.5 (13)	2.3 (1)	41.2 ± 10.8	61

L-Hcy-thiolactone was injected i.p. (3,700 nmol/g body weight) and the mice were monitored for 90 min

* Significantly increased versus $Blmh^{+/+}$: Fisher exact test $P < 0.001$

† Significantly decreased versus $Blmh^{+/+}$: *t* test $P = 0.012$

recorded. Essentially all mice became somnolescent within 5–10 min post-injection. Convulsions, characterized by spontaneous tonic-clonic, grand-mal seizures (kangaroo position, extension of fore and hind limbs and tail, status epilepticus), and running fits occurred within 50 min. The incidence of seizures was significantly increased in $Blmh^{-/-}$ mice compared with $Blmh^{+/+}$ animals (93.8 vs. 29.5%, $P < 0.001$) (Table 7). Seizure latency (i.e., time to first seizure) was significantly decreased for $Blmh^{-/-}$ mice compared with $Blmh^{+/+}$ animals (33.1 vs. 41.2 min, $P = 0.012$) (Table 7). Only one mouse out of 44 $Blmh^{+/+}$ mice (2.3%) died (at 61 min) after L-Hcy-thiolactone injection. The incidence of lethality was significantly increased for $Blmh^{-/-}$ mice (to 46.9%, $P < 0.001$). These data show that Blmh protects against the toxicity of i.p.-injected Hcy-thiolactone.

Brain Hcy-thiolactone levels assayed 90 min post-injection were higher in $Blmh^{-/-}$ than in $Blmh^{+/+}$ mice (1.8 ± 0.6 vs. 1.1 ± 0.1 μM, $P = 0.049$). Brain Hcy levels assayed 90 min post-injection in $Blmh^{-/-}$ and $Blmh^{+/+}$ mice were not significantly different (550 ± 233 vs. 660 ± 182 μM).

Discussion

Although Blmh has the ability to hydrolyze L-Hcy-thiolactone to Hcy in vitro (Zimny et al. 2006), its physiological role in mammals is not fully understood. The present work using the Blmh knockout mice provides the first direct evidence that (1) Blmh metabolizes L-Hcy-thiolactone to Hcy in vivo in mice, and (2) that a specific Hcy metabolite, Hcy-thiolactone, rather than Hcy itself is neurotoxic in vivo. These findings suggest a mechanism by which Blmh can protect against neurodegeneration associated with hyperhomocysteinemia and Alzheimer's disease.

Our results also indicate that Blmh significantly contributes to Hcy-thiolactone metabolism mainly in the brain and the kidney and that these two organs are major sources of urinary Hcy-thiolactone excreted by $Blmh^{-/-}$ mice. Modest and tissue-specific increases in Hcy-thiolactone levels in $Blmh^{-/-}$ mice suggest that, in addition to Blmh, other enzyme(s), which remain to be identified, contribute to Hcy-thiolactone turnover in mice.

Experiments with mice injected i.p. with Hcy-thiolactone provide additional evidence that Blmh metabolizes Hcy-thiolactone in vivo. For example, our finding that $Blmh^{-/-}$ mice produce significantly less plasma Hcy from i.p.-injected L-Hcy-thiolactone than $Blmh^{+/+}$ animals suggests that the $Blmh^{-/-}$ mice are deficient in their ability to hydrolyze L-Hcy-thiolactone. Furthermore, our present findings that significantly more of the i.p.-injected L-Hcy-thiolactone ends up in the blood of $Blmh^{-/-}$ mice, compared with wild-type $Blmh^{+/+}$ animals, indicate that Blmh present in tissues separating the i.p. cavity from the bloodstream contributes to L-Hcy-thiolactone metabolism in vivo. That Blmh is responsible for Hcy-thiolactone metabolism is also supported by our findings that 88, 47, and 6.3% of the i.p.-injected dose of D-Hcy-thiolactone, D,L-Hcy-thiolactone, and L-Hcy-thiolactone was recovered in the plasma in wild-type mice, consistent with the known L-stereospecificity of Blmh (Zimny et al. 2006).

Our present data suggest that Blmh protects against protein N-homocysteinylation by hydrolyzing Hcy-thiolactone in vivo. But, due to its aminopeptidase activity (Suszynska et al. 2010; Bromme et al. 1996), Blmh could also participate in *N*-Hcy-protein degradation. However, this possibility is unlikely because *N*-Hcy-protein turnover was not affected by *Blmh* genotype (Fig. 1c). Thus, our data indicate that the increased accumulation of *N*-Hcy-protein in $Blmh^{-/-}$ mice is mostly due to a less efficient hydrolysis of Hcy-thiolactone in these animals.

There seems to be an apparent discrepancy between the measurable effect of *Blmh* gene inactivation on plasma *N*-Hcy-protein and the lack of any effect on plasma Hcy-thiolactone. While the reason for this discrepancy is not clear, high inter-individual variability in plasma Hcy-thiolactone observed previously in humans (Chwatko et al. 2007; Chwatko and Jakubowski 2005a) and now in mice might be a contributing factor. It should be noted that small differences in Hcy-thiolactone levels (5–10%), which could not be detected due to a high inter-individual variability in plasma Hcy-thiolactone levels, would be compounded over time to much greater differences in *N*-Hcy-protein levels. For instance, a 5% difference in Hcy-thiolactone levels would be compounded to $(1.05)^{50} = 11$-fold difference in *N*-Hcy-protein levels after 150 h [equivalent to fifty 3-h N-homocysteinylation reaction cycles (Jakubowski 1999)]. This explanation is consistent with results showing that the concentration of *N*-Hcy-protein (Jakubowski et al. 2009) is much higher than the concentration of Hcy-thiolactone in mouse plasma (Chwatko et al. 2007).

Hcy-thiolactone is toxic in experimental animals and cell cultures, and a preponderance of evidence strongly suggests that it is involved in the pathology of hyperhomocysteinemia (Jakubowski 2008a). For example, Hcy-thiolactone is elevated in cystathionine β-synthase (Cbs)- or methylenetetrahydrofolate reductase (Mthfr)-deficient patients and in mice fed a pro-atherogenic high-Met diet (Chwatko et al. 2007). Protein modified by Hcy-thiolactone—*N*-Hcy-protein—accumulates in Cbs- or Mthfr-deficient patients (Jakubowski et al. 2008) and in Cbs-, Mthfr-, or Pcft-deficient mice (Jakubowski et al. 2009). Furthermore, *N*-Hcy-protein accumulates in atherosclerotic lesions in aortas of $ApoE^{-/-}$ mice and the accumulation

increases in mice fed a high-Met-diet (Perla-Kajan et al. 2008). Chronic infusions of Hcy-thiolactone or Hcy-thiolactone-supplemented diet produce atherosclerotic changes in baboons (Harker et al. 1974) or rats (Endo et al. 2006).

Acute injections of Hcy-thiolactone are known to cause epileptic seizures in mice (Spence et al. 1995) and rats (Sprince et al. 1969). An underlying mechanism may involve inhibition of Na^+/K^+-ATPase, critical for normal brain function, by Hcy-thiolactone, which would contribute to the seizures. Indeed, the Na^+/K^+-ATPase activity was reported to be diminished by acute injections of Hcy-thiolactone, but not Hcy (Rasic-Markovic et al. 2009). The in vivo sensitivity to injections of Hcy-thiolactone, but not Hcy, suggests that Na^+/K^+-ATPase might be targeted for N-homocysteinylation by Hcy-thiolactone in the brain. This suggestion is supported by our present findings showing that the inactivation of the *Blmh* gene, which impairs Hcy-thiolactone disposition, increases the incidence and the latency of seizures. Our data provide the first evidence that Blmh protects against the toxicity of i.p.-injected Hcy-thiolactone in mice.

Hyperhomocysteinemia due to Cbs or Mthfr deficiency results in neurological abnormalities in humans, manifested by mental retardation and seizures (Mudd et al. 2001). However, how hyperhomocysteinemia leads to these neurological abnormalities is not known. In particular, it is not known whether Hcy itself or any of its metabolites is responsible for the neurotoxicity of hyperhomocysteinemia. Although specific mechanisms were assigned to individual metabolites, such as Hcy itself, AdoHcy, homocysteic acid (Smith 2008; Selhub et al. 2010), or Hcy-thiolactone (Suszynska et al. 2010), it was not possible to unequivocally link the observed neurotoxicity with a specific metabolite. This would require an experimental model that allows manipulation of one metabolite and not the others. Such model has been developed in our present study.

Using a mouse model with a genetic deficiency in Hcy-thiolactone disposition, $Blmh^{-/-}$, allowed us to examine the role of Blmh in Hcy-thiolactone metabolism and in the pathology caused by acute hyperhomocysteinemia. Findings of our present work, showing that inactivation of *Blmh* gene greatly increases the susceptibility to Hcy-thiolactone toxicity in mice, provide the first direct evidence that a specific Hcy metabolite—Hcy-thiolactone—is toxic in vivo. Although in our experimental Hcy-thiolactone toxicity model Hcy was also generated, Hcy levels were decreased by the inactivation of *Blmh* gene. Thus, in this model neurotoxicity can be assigned to Hcy-thiolactone, but not to Hcy.

It should be noted that the concentrations of Hcy-thiolactone used in the i.p. injection experiments greatly exceed physiological concentrations. Such high concentrations, required due to very efficient metabolism of Hcy-thiolactone in the mouse, caused extreme neurological manifestations within 30–60 min. Although much lower Hcy-thiolactone concentrations occur in pathological hyperhomocysteinemia (Chwatko et al. 2007), it is possible that under chronic exposure even small amounts of damage caused by Hcy-thiolactone can accumulate to significant levels over an extended period of time that is usually required for the development of brain damage, similar to that observed in Alzheimer's disease (Smith et al. 2010). A recent finding that Hcy-thiolactonase activity of Blmh is reduced in brains of Alzheimer patients suggests that diminished functional Blmh activity contributes to the pathology of Alzheimer's disease (Suszynska et al. 2010).

In conclusion, by showing that the *Blmh* gene inactivation increases the accumulation of and the susceptibility to Hcy-thiolactone toxicity in mice, our findings identify novel functions of the Blmh protein.

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Conflict of interest The authors declare that they have no conflict of interest.

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