

Genetic or nutritional disorders in homocysteine or folate metabolism increase protein N-homocysteinylation in mice

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ABSTRACT Genetic disorders of homocysteine (Hcy) or folate metabolism or high-methionine diets elevate plasma Hcy and its atherogenic metabolite Hcy-thiolactone. In humans, severe hyperhomocysteinemia due to genetic alterations in cystathionine β -synthase (*Cbs*) or methylenetetrahydrofolate reductase (*Mthfr*) results in neurological abnormalities and premature death from vascular complications. In mouse models, dietary or genetic hyperhomocysteinemia results in liver or brain pathological changes and accelerates atherosclerosis. Hcy-thiolactone has the ability to form isopeptide bonds with protein lysine residues, which generates modified proteins (N-Hcy-protein) with autoimmunogenic and prothrombotic properties. Our aim was to determine how N-Hcy-protein levels are affected by genetic or nutritional disorders in Hcy or folate metabolism in mice. We found that plasma N-Hcy-protein was elevated 10-fold in mice fed a high-methionine diet compared with the animals fed a normal commercial diet. We also found that inactivation of *Cbs*, *Mthfr*, or the proton-coupled folate transporter (*Pcft*) gene resulted in a 10- to 30-fold increase in plasma or serum N-Hcy-protein levels. Liver N-Hcy-protein was elevated 3.4-fold in severely and 11-fold in extremely hyperhomocysteinemic *Cbs*-deficient mice, 3.6-fold in severely hyperhomocysteinemic *Pcft* mice, but was not elevated in mildly hyperhomocysteinemic *Mthfr*-deficient animals, suggesting that mice have a capacity to prevent accumulation of N-Hcy-protein in their organs. These findings provide evidence that N-Hcy-protein is an important metabolite associated with Hcy pathophysiology in the mouse.—Jakubowski, H., Perla-Kaján, J., Finnell, R. H., Cabrera, R. M., Wang, H., Gupta, S., Kruger, W. D., Kraus, J. P., and Shih, D. M. Genetic or nutritional disorders in homocysteine or folate metabolism increase protein N-homocysteinylation in mice. *FASEB J.* 23, 000–000 (2009)

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IN MAMMALS SUCH AS MICE AND HUMANS, homocysteine (Hcy) is formed from the dietary methionine (Met) (1). 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). The remethylation requires betaine or vitamin B₁₂ and 5-methyltetrahydrofolate, generated by 5,10-methylenetetrahydrofolate reductase (MTHFR) (which requires riboflavin), whereas the transsulfuration requires vitamin B₆. The requirements for cofactors are met entirely from the absorption of dietary B vitamins and folates in the small intestine.

Hcy is also metabolized to the thioester Hcy-thiolactone in an error-editing reaction in protein biosynthesis when Hcy is mistakenly selected in place of Met by methionyl-tRNA synthetase (2–5). The flow through the Hcy-thiolactone pathway increases when the remethylation or transsulfuration reaction is impaired by genetic alterations of enzymes, such as CBS (6, 7) or MTHFR (7, 8), by an inadequate supply of cofactors, e.g., 5-methyltetrahydrofolate (6, 9, 10), or by a high-Met diet (7).

Although there is a preponderance of data showing that total Hcy (tHcy) is a risk factor for cardiovascular disease in the general population, the question of causality remains unanswered, and the results of Hcy-

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lowering clinical trials have fallen short of expectations (11). In contrast, there is less debate regarding the toxicity of Hcy or its metabolite in patients with hyperhomocysteinemia caused by the inborn errors of metabolism (1). Furthermore, studies of genetic and nutritional hyperhomocysteinemia in mouse models provide additional support for a causal role of Hcy or its metabolite in atherosclerosis (12).

Among the pathological manifestations of human CBS deficiency, which include mental retardation, ectopia lentis, and osteoporosis, vascular complications remain the major cause of morbidity and mortality in untreated CBS-deficient patients (1); in these patients cerebrovascular incidents are 8 times more frequent than myocardial infarctions (13). Hcy lowering by cofactor supplementation improves vascular outcomes in CBS-deficient patients. Specifically, untreated CBS-deficient patients have 1 vascular event per 25 patient-years (13), whereas treated CBS-deficient patients have only 1 vascular event per 263 patient-years (relative risk 0.091, $P < 0.001$) (14). Hcy-lowering therapy started early in life also prevents neurological abnormalities from human MTHFR deficiency (15, 16). These findings suggest that Hcy or its metabolite plays a causal role in atherothrombosis and brain disease.

However, mechanisms by which CBS or MTHFR deficiency leads to specific clinical manifestations are not clear. Because of parallel changes in the concentrations of Hcy metabolites under most clinical and experimental circumstances, it has not been possible to assign the observed toxicity to a specific Hcy metabolite. Hcy itself, S-adenosyl-Hcy, and Hcy-thiolactone remain potential candidates, and possible mechanisms have been assigned to each (12, 17–19).

One hypothesis suggests that the metabolic conversion to Hcy-thiolactone contributes to Hcy-associated pathological changes (6). Hcy-thiolactone could be detrimental because of its ability to form N-Hcy-protein in which Hcy is N-linked by an isopeptide bond to a protein lysine residue (4, 19). In humans, N-Hcy-protein accumulation has been linked to an autoimmune response and atherothrombosis (8, 17, 18). Plasma Hcy-thiolactone and N-Hcy-protein, originally discovered in human fibroblasts and endothelial cells (6, 9, 10, 20), occur in the human body (7, 10, 21–23) and are greatly elevated under conditions predisposing to atherothrombosis, such as hyperhomocysteinemia caused by mutations in the CBS or MTHFR gene in humans (7, 23). Recent human clinical studies show that plasma N-Hcy-protein levels are associated with a risk of coronary heart disease (24), whereas plasma Hcy-thiolactone levels are associated with the development and progression of diabetic macrovasculopathy (25). In ApoE-deficient mice, N-Hcy-protein accumulates in atherosclerotic lesions, and the accumulation increases in the animals fed a high-Met diet (26).

The purpose of the current research was to extent the hypothesis that Hcy-thiolactone is a toxic metabolite and that one of its potential mechanisms of toxicity is the formation of N-Hcy-protein. With this in mind, in

the present study we examined effects of dietary (high-Met diet) or genetic modifications in *Cbs* (27), *Mthfr* (28), and proton-coupled folate transporter (*Pcft*) (29) genes on N-Hcy-protein levels in mice and their relationships to Hcy.

MATERIALS AND METHODS

Mice

Wild-type mice (6–9 mo old, strain C57BL/6J; The Jackson Laboratory, Bar Harbor, ME) were fed a control diet containing 0.5% Met or a high-Met isocaloric diet containing 1.5% Met (TD.04352 or TD.04353, respectively; Harlan Teklad, Madison, WI, USA). One group of mice ($n=8$) was fed the 1.5% Met diet for 2 wk followed by a control 0.5% Met diet for 4 wk. Another group of mice ($n=8$) was fed the 1.5% Met diet for 8 and 18 wk.

Cbs-deficient (27) and *Mthfr*-deficient mice (28) were created as described previously. *Cbs*-deficient mice are on a C57BL6 background, whereas *Mthfr*-deficient mice are on a mixed BALB/cAnNCrIBR background. The mice were fed a standard chow diet. Animals between 15 and 20 wk (*Cbs*-deficient) or 6 and 14 mo (*Mthfr*-deficient) of age were used in experiments.

Transgenes harboring wild-type human CBS (*Tg-hCBS*), S466L (*Tg-S466L*), or I278T (*Tg-I278T*) variant were used to rescue the neonatal lethality associated with homozygosity for *Cbs*^{-/-} (30, 31). In these animals, the human CBS is under control of the zinc-inducible metallothionein promoter, which allows one to rescue the neonatal lethality phenotype of *Cbs*^{-/-} in mice by supplementing the drinking water of pregnant dams with 25 mM zinc. Zinc water is replaced by plain water after weaning. All transgenic mice are on a C57BL/6J strain background. The transgenic mice were fed a standard chow diet (Teklad 2018SX; Harlan Teklad).

The murine *Pcft* gene was disrupted in embryonic stem (ES) cells by homologous recombination. A replacement vector was designed to delete a 2.9-kb fragment of *Pcft* gene containing exons 1–3 of the wild-type locus. The deleted sequence was replaced by pKO Scrambler targeting vector carrying LacZ/Neo. The targeting vector was electroporated into 129SvEvBrd-derived ES cells for homologous recombination. The ES medium was replaced with M15 medium containing gentamicin G418 (350 µg/ml) 24 h after electroporation. ES cells carrying the disrupted gene were identified by Southern blot analysis using 5' internal and 3' external probes. Briefly, 7–8 days after electroporation and selection in G418 media, individual colonies were picked and expanded, and genomic DNA was isolated. Five positive clones containing 5' 9.3-kb *Apa*I and 3' 12.3-kb *Eco*RI inserts were identified by Southern blot. ES cell clones that were characterized by correct homologous recombination of the targeting vector and a single integration event were selected for blastocyst injections. The chimeric mice were obtained and bred to C57BL/6J albino mice to generate F1 heterozygous animals. DNA samples were isolated from the tails of ES cell-derived animals and were analyzed for the presence of a disrupted *Pcft* by Southern blot analysis. Germline transmission by chimeras of disrupted *Pcft* was identified from the *Pcft*-derived clone 1D1. These progeny were intercrossed to generate F2 wild-type, heterozygous, and homozygous mutant progeny. For genotyping, DNA was isolated and subjected to PCR analysis using primers specific to exon 3 (28-mer), intron 3 (29-mer), neo (neo3a), and intron 3 (rev). Primers sequences were 5'-CCCAACTCCAAAGCGCAGGTTTCAT-3' (28-

TABLE 1. Plasma N-Hcy-protein, Hcy-thiolactone, and tHcy levels in wild-type C57BL/6J mice

Diet (8 mice/group)	NHcy-protein (μM)	Hcy-thiolactone (nM)	tHcy (μMm)
Control, 0 wk	1.89 \pm 0.70	5.74 \pm 2.42	3.45 \pm 0.30
High Met, 2 wk	22.1 \pm 13.5	79.4 \pm 11.9	126 \pm 76
High Met, 2 wk; control, 4 wk	3.60 \pm 1.11	4.47 \pm 0.97	5.47 \pm 2.83

mer) and 5'-TCCAGATGGGAAAGAAGAGGTCTA-3' (29-mer) for wild-type *Pcft* and 5'-GCAGCGCATCGCCTTCTATC-3' (neo3a) and 5'-CTTGACCACAACCTGTCCATGTGC-3' (rev) for the mutant allele carrying neo. The *Pcft*-deficient mice were fed a standard chow diet; 6- to 8-wk-old animals were used in the experiments.

Determination of N-Hcy-protein, Hcy-thiolactone, and tHcy

Plasma N-Hcy-protein (32), Hcy-thiolactone (21), and tHcy (22) were assayed as described previously. Serum and tissue tHcy for *Tg-S466L*, *Tg-I278T*, and *Tg-hCBS* animals were measured as described previously (30, 31). Tissue N-Hcy-protein and tHcy for other mice were assayed by similar procedures. In brief, 100–150 mg of frozen mouse liver or heart tissue was pulverized with dry ice in a mortar prechilled to -80°C , suspended in 10 vol of 20 mM sodium phosphate buffer (pH 7.4), 0.1 M NaCl, 0.5 mM EDTA, and 2 mM DTT, and further disintegrated by sonication (two strokes of 15 s, 70 W power, on wet ice). Crude extracts were clarified by centrifugation, and aliquots containing 0.5 mg of protein were used for N-Hcy-protein (32) and tHcy assays (21).

Statistics

Each variable was calculated as mean \pm sd. The two-sample *t* test was used to compare the variables between the groups. The Pearson correlation coefficient *r* was used to evaluate simple linear relationships between variables. The level of statistical significance was set at $P < 0.05$.

RESULTS

High-Met diet increases plasma N-Hcy-protein levels in mice

We found that plasma N-Hcy-protein levels increased 11.7-fold in C57BL/6J mice fed a high-Met diet relative to the animals fed a normal chow diet. The increase in plasma N-Hcy-protein reflected 36- and 14-fold increases in plasma tHcy and Hcy-thiolactone levels (Table 1). After replacing the high-Met diet with a normal chow diet, plasma Hcy-thiolactone and tHcy returned to normal values, whereas plasma N-Hcy-protein, although decreased 6-fold, remained 2-fold higher than that in mice fed only a normal chow diet (Table 1). In mice fed a high-Met diet for 8 and 18 wk, plasma N-Hcy-protein levels remained elevated 6.1- and 8.2-fold, respectively (not shown).

Plasma N-Hcy-protein levels are elevated in *Cbs*-deficient mice

Plasma N-Hcy-protein levels were 8.1-fold higher in *Cbs*^{-/-} mice than in *Cbs*^{+/+} animals (Table 2). In transgenic *Tg-S466L* mice, plasma N-Hcy-protein levels were 6.4 to 12.8-fold higher in *Tg-S466L Cbs*^{-/-} than in *Tg-S466L Cbs*^{+/-} or *Tg-S466L Cbs*^{+/+} animals (Table 2). Significantly more plasma N-Hcy-protein was present in *Tg-S466L Cbs*^{+/-} than in *Tg-S466L Cbs*^{+/+} animals.

Plasma tHcy concentrations in *Cbs*^{-/-} mice were severely elevated relative to those of the wild-type *Cbs*^{+/+} animals (Table 2), as expected. In *Tg-S466L* mice, plasma tHcy concentrations were also severely elevated in the *Tg-S466L Cbs*^{-/-} mice compared with those in the *Tg-S466L Cbs*^{+/-} or *Tg-S466L Cbs*^{+/+} animals (Table 2).

Serum and liver N-Hcy-protein levels are elevated in *Tg-hCBS* and *Tg-I278T Cbs*-deficient mice

In transgenic *Tg-I278T* mice, serum N-Hcy-protein levels were 6.3- to 10.6-fold higher in homozygous *Tg-I278T Cbs*^{-/-} than in heterozygous *Tg-I278T Cbs*^{+/-} or homozygous *Tg-I278T Cbs*^{+/+} animals (Table 3). Liver N-Hcy-protein levels were 11.5-fold higher in *Tg-I278T Cbs*^{-/-} than in *Tg-I278T Cbs*^{+/-} and *Tg-I278T Cbs*^{+/+} animals (Table 3).

Serum tHcy concentrations were extremely elevated (54- to 143-fold) in the *Tg-I278T Cbs*^{-/-} mice compared with *Tg-I278T Cbs*^{+/-} and *Tg-I278T Cbs*^{+/+} animals (Table 3). Liver tHcy concentrations were elevated 51- to 75-fold, to a lower degree than serum tHcy (Table 3), as previously reported (31).

In transgenic *Tg-hCBS* mice, plasma N-Hcy-protein levels were 4.2-fold higher in homozygous *Tg-hCBS Cbs*^{-/-} than in heterozygous *Tg-hCBS Cbs*^{+/-} animals (Table 3). Liver N-Hcy-protein levels were 3.4-fold higher in homozygous *Tg-hCBS Cbs*^{-/-} than in heterozygous *Tg-hCBS Cbs*^{+/-} animals (Table 3).

There was a 3.3-fold greater accumulation of N-Hcy-protein in *Tg-I278T Cbs*^{-/-} mice than in *Tg-hCBS Cbs*^{-/-} mice (Table 3). There were significant linear relationships between N-Hcy-protein and tHcy in the serum and in the liver (Fig. 1).

Plasma but not liver or heart N-Hcy-protein levels are elevated in *Mthfr*-deficient mice

Plasma N-Hcy-protein levels were significantly higher in homozygous *Mthfr*^{-/-} than in heterozygous *Mthfr*^{+/-}

TABLE 2. Plasma N-Hcy-protein and tHcy levels in *Cbs*-deficient mice

Genotype	NHcy-protein (μM)	tHcy (μM)
<i>Cbs</i> ^{-/-} (n=3)	15.3 \pm 2.6	204 \pm 62
<i>Cbs</i> ^{+/+} (n=8)	1.89 \pm 0.7	3.45 \pm 0.30
<i>Tg-S466L Cbs</i> ^{-/-} (n=4)	12.8 \pm 4.5	201 \pm 46
<i>Tg-S466L Cbs</i> ^{+/-} (n=4)	2.00 \pm 0.63	5.65 \pm 0.21
<i>Tg-S466L Cbs</i> ^{+/+} (n=4)	1.01 \pm 0.26	3.25 \pm 0.49

TABLE 3. Serum and liver N-Hcy-protein and tHcy levels in *Tg-I278T* and *Tg-hCBS* mice

Genotype	N-Hcy-protein		tHcy	
	Plasma (μM)	Liver (pmol/mg of protein)	Plasma (μM)	Liver (pmol/mg of protein)
<i>Tg-I278T Cbs</i> ^{-/-} (n=4)	16.6 \pm 4.1	1857 \pm 452	272 \pm 50	5680 \pm 1777
<i>Tg-I278T Cbs</i> ^{+/-} (n=2)	2.62 \pm 1.73	161 \pm 19	5.0 \pm 2.6	110
<i>Tg-I278T Cbs</i> ^{+/+} (n=2)	1.56 \pm 0.25	163 \pm 0.0	1.9 \pm 1.6	75 \pm 7.1
<i>Tg-hCBS Cbs</i> ^{-/-} (n=4)	10.8 \pm 2.1	562 \pm 132	167 \pm 40	582 \pm 326
<i>Tg-hCBS Cbs</i> ^{+/-} (n=4)	2.6 \pm 0.8	165 \pm 24	6.1 \pm 2.4	153 \pm 21

or wild-type *Mthfr*^{+/+} mice (Table 4). Significantly more plasma N-Hcy-protein was present in heterozygous *Mthfr*^{+/-} than in wild-type *Mthfr*^{+/+} mice. Liver N-Hcy-protein levels were similar in homozygous *Mthfr*^{-/-} and in wild-type *Mthfr*^{+/+} animals (Table 4).

To determine whether *Mthfr* gene inactivation affects N-Hcy-protein accumulation in other tissues, protein extracts from mouse hearts were prepared and assayed. We found no differences in heart N-Hcy-protein between homozygous *Mthfr*^{-/-} (28.3 \pm 11.9 pmol/mg of heart protein, n=4) and wild-type *Mthfr*^{+/+} mice (26.9 \pm 7.3 pmol/mg of heart protein, n=4).

Plasma tHcy in homozygous *Mthfr*^{-/-} mice was mildly elevated relative to that in heterozygous *Mthfr*^{+/-} and wild-type *Mthfr*^{+/+} animals (Table 4), as previously reported (28). However, liver tHcy levels were similar in *Mthfr*^{-/-} and *Mthfr*^{+/+} mice (Table 4). There was a linear relationship between N-Hcy-protein and tHcy in the plasma; however, liver N-Hcy-protein essentially did not correlate with liver tHcy in these mice (Fig. 2).

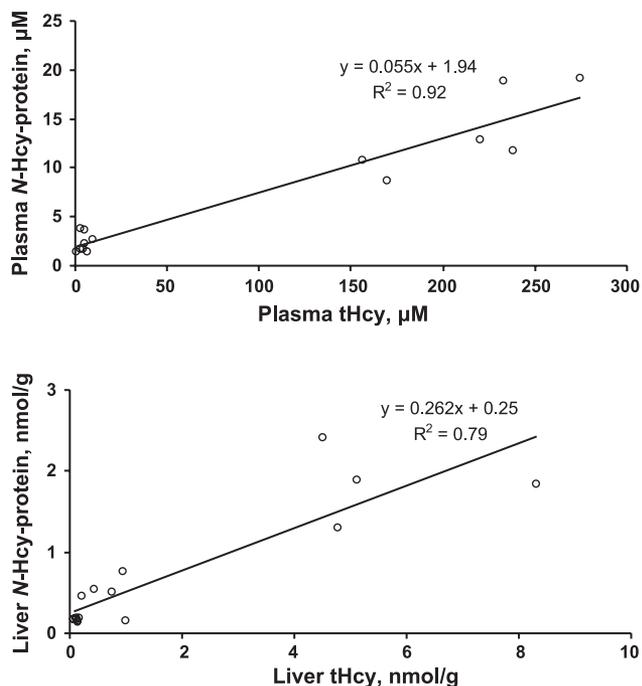


Figure 1. Relations between N-Hcy-protein and tHcy in plasma (top panel) and livers (bottom panel) of *Tg-hCBS* and *Tg-I278T* mice.

Plasma and liver N-Hcy-protein levels are elevated in *Pcft*-deficient mice

Plasma N-Hcy-protein levels were \sim 30-fold higher in homozygous *Pcft*^{-/-} mice compared with those in heterozygous *Pcft*^{+/-} and wild-type *Pcft*^{+/+} animals (Table 5). Plasma tHcy was \sim 65-fold higher in *Pcft*^{-/-} mice compared with that in *Pcft*^{+/-} and *Pcft*^{+/+} animals (Table 5). Liver N-Hcy-protein and tHcy levels were 3.6- and 5.1-fold, respectively, higher in homozygous *Pcft*^{-/-} mice than in wild type *Pcft*^{+/+} animals (Table 5).

DISCUSSION

Genetic and nutritional models have been generated in mice to approximate human disorders of Hcy or folate metabolism. Among these models, transgene treatments allowed generation of *Cbs*-deficient mice in which the presence of liver pathological changes depends on the extent of hyperhomocysteinemia (31). In the present work, we have used these models to address the question of the pathophysiological significance of N-Hcy-protein. We found that 1) N-Hcy-protein is present in the mouse, 2) wild-type mice fed a high-Met diet have significantly elevated plasma N-Hcy-protein levels, 3) mice with genetic deficiencies in folate metabolism (*Mthfr* or *Pcft* deficiency) or Hcy metabolism (*Cbs* deficiency) have significantly elevated plasma or serum N-Hcy-protein levels, and 4) although N-Hcy-protein is elevated in the plasma of mildly hyperhomocysteinemic mice (having plasma tHcy of 37.5 μM), these animals have a capacity to prevent the elevation of N-Hcy-protein in their organs: N-Hcy-protein is not affected in the liver or heart of mildly hyperhomocysteinemic mice (compared with animals with normal plasma tHcy of 4.4 μM) but is significantly elevated in severely or extremely hyperhomocysteinemic animals (serum tHcy of 167 or 272 μM , respectively). These results provide evidence that N-Hcy-protein is an important Hcy metabolite associated with mouse pathological conditions.

We showed previously that N-Hcy-protein is a significant metabolite in humans (23, 33). The findings of the present work extend these observations to mice. Similar to humans (32), in wild-type mice plasma N-Hcy-protein occurs at micromolar concentrations and increases 10-fold in *Cbs*- or *Mthfr*-deficient animals.

TABLE 4. Plasma and liver N-Hcy-protein and tHcy levels in *Mthfr*-deficient mice

Genotype	N-Hcy-protein		tHcy	
	Plasma (μM)	Liver (pmol/mg protein)	Plasma (μM)	Liver (pmol/mg protein)
<i>Mthfr</i> ^{-/-} (n=7)	7.48 \pm 2.55	49.8 \pm 38.2	37.5 \pm 48.6	184 \pm 71
<i>Mthfr</i> ^{+/-} (n=6)	2.03 \pm 0.44		9.15 \pm 3.33	
<i>Mthfr</i> ^{+/+} (n=7)	1.18 \pm 0.39	34.7 \pm 12.7	4.42 \pm 0.46	170 \pm 43

Mouse plasma N-Hcy-protein levels are increased 25-fold by a deficiency in folate transport (in *Pcft*^{-/-} animals) and 10-fold by a high-Met diet in wild-type animals. Hcy-thiolactone, a metabolic precursor of N-Hcy-protein, is also present in mice (Table 1) (7) and humans (7, 21, 22).

Diets supplemented with high Met are used to induce experimental hyperhomocysteinemia and vascular pathological changes in animal models (12, 34, 35). Our present findings that a high-Met, proatherogenic diet elevates plasma levels of N-Hcy-protein, in addition to those of Hcy-thiolactone and tHcy (Table 1), suggest that the pathway involving methionyl-tRNA synthetase-catalyzed conversion of Hcy to Hcy-thiolactone followed by protein N-homocysteinylation by Hcy-thiolactone, deduced from the *in vitro* and *ex vivo* human and rodent tissue culture studies (2, 6, 9, 19), is active in the mouse. This conclusion is strengthened by our findings that hyperhomocysteinemia caused by the inactivation of genes involved in Hcy (*Cbs*) or folate metabolism (*Mthfr* and *Pcft*) results in greatly increased accumulation of N-Hcy-protein in mice (Tables 2–5). The lowering of N-Hcy-protein levels to close to normal values and normalization of Hcy-thiolactone and tHcy levels after return to a standard chow diet (Table 1) indicate that N-Hcy-protein is metabolized in the mouse, albeit

somewhat slower than Hcy-thiolactone and tHcy. These findings also demonstrate that the plasma level of N-Hcy-protein, in addition to those of Hcy-thiolactone and tHcy, can be reversibly modified by the diet.

Although the biological significance of N-Hcy-proteins is not fully understood, accumulating evidence suggests that protein N-homocysteinylation plays a role in pathophysiology. For example, N-Hcy-proteins are autoimmunogenic (18), and titers of anti N-Hcy-protein autoantibodies are elevated in human stroke (36) and coronary artery disease patients (37, 38). N-Hcy-protein levels are elevated in coronary heart disease (24) and uremia (39, 40) in humans. Furthermore, prothrombotic N-Hcy-fibrinogen accumulates in human CBS deficiency (23), which could contribute to the increased atherothrombosis observed in these patients (1, 13). Because nutritionally (34, 35) or genetically (27, 28, 31, 41, 42) modified mice exhibit pathological changes in their vasculature, brain, or livers, the findings of the present work are consistent with a concept that N-Hcy-protein plays a role in these pathological changes.

Hereditary folate malabsorption is a rare autosomal recessive disorder caused by loss-of-function mutations in the human *PCFT* gene, which impair intestinal folate absorption and transport into the central nervous system (29). Affected infants develop anemia, immunoglobulin deficiency, seizures, and neurodevelopmental defects. Our present findings that N-Hcy-protein is elevated in plasma and livers of *Pcft*^{-/-} mice suggest that N-Hcy-protein contributes to the pathological changes of hereditary folate malabsorption syndrome.

Our findings that N-Hcy-protein accumulates in livers of transgenic *Cbs*-deficient mice provide a possible mechanism for the induction of endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in hyperhomocysteinemic animals. N-Hcy-proteins have a propensity to form aggregates (10, 19), which can induce the ER stress and UPR. The finding that N-Hcy-low-density lipoprotein, similar to other N-Hcy-proteins, has the propensity to aggregate (10, 19) and induce cell death in cultured human endothelial cells (43) is consistent with this concept. The ER stress response is observed in the livers of extremely hyperhomocysteinemic *Tg-I278T Cbs*^{-/-} mice (serum tHcy of 296 μM) (31), which accumulate 1857 \pm 452 pmol of N-Hcy-protein/mg of liver protein (Table 3), but not in the livers of severely hyperhomocysteinemic *Tg-hCBS Cbs*^{-/-} mice (serum tHcy of 169 μM) (31), which accumulate 3.3 times less N-Hcy-protein in the liver

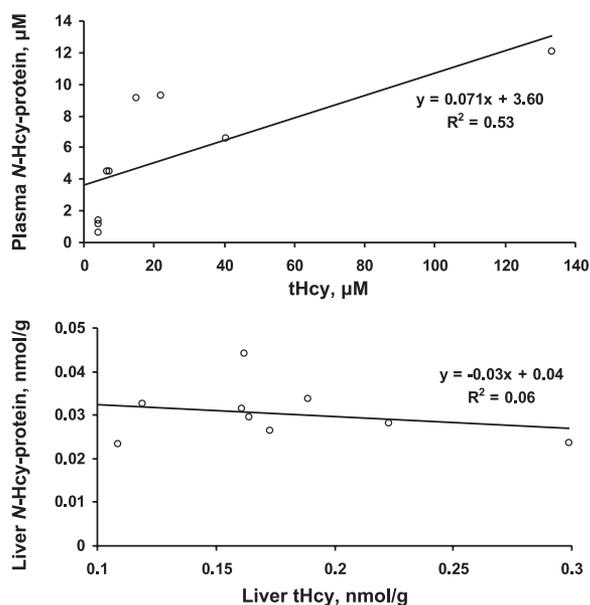


Figure 2. Relations between N-Hcy-protein and tHcy in plasma (top panel) and livers (bottom panel) of *Mthfr*^{-/-} and *Mthfr*^{+/+} mice.

TABLE 5. Plasma and liver N-Hcy-protein and tHcy levels in *Pcft*-deficient mice

Genotype	N-Hcy-protein		tHcy	
	Plasma (μ M)	Liver (pmol/mg protein)	Plasma (μ M)	Liver (pmol/mg protein)
<i>Pcft</i> ^{-/-} (n=4)	56.6 \pm 13.2	318 \pm 98	134.8 \pm 28.3	1529 \pm 390
<i>Pcft</i> ^{+/-} (n=4)	1.79 \pm 0.30		2.44 \pm 0.71	
<i>Pcft</i> ^{+/+} (n=4)	2.28 \pm 0.58	87 \pm 32	1.79 \pm 0.56	299 \pm 92

(Table 3). The involvement of N-Hcy-protein in the ER stress is also supported by our previous findings showing that immunohistochemical staining for N-Hcy-protein is increased in aortic lesions from *ApoE*^{-/-} mice fed a high-Met diet (26). The staining for N-Hcy-protein is similar to the staining for markers of the ER stress in these mice (26, 34). It is possible that Hcy, which accumulates in livers of *Tg-I278T Cbs*^{-/-} mice (Table 3), might contribute to ER stress and UPR by forming S-homocysteinylated proteins (12, 31). However, this possibility is unlikely because, in contrast to N-Hcy-protein, S-Hcy-protein is not known to aggregate or be toxic to cells.

Our finding that plasma N-Hcy-protein levels are increased in *Mthfr*^{-/-} mice compared with those in wild-type animals (Table 4) is consistent with a concept that N-Hcy-protein plays a role in brain and vascular pathological changes observed in these animals (28). The findings that N-Hcy-protein does not increase in the livers and hearts of *Mthfr*^{-/-} mice, relative to *Mthfr*^{+/+} animals, suggest that the mildly hyperhomocysteinemic *Mthfr*^{-/-} animals have a capacity to prevent N-Hcy-protein accumulation in these organs. This suggestion is supported by our findings showing that N-Hcy-protein levels increase to a significantly lower extent in the liver than in the plasma of *Pcft*-deficient mice (3.6-fold vs. 30-fold, respectively) (Table 5). Our data also suggest that this capacity can be overwhelmed and that the extremely elevated N-Hcy-protein levels (Table 3) could lead to liver pathological changes that are observed in *Tg-I278T CBS*^{-/-} mice (31). Furthermore, the data shown in Table 3 are consistent with a threshold effect of liver N-Hcy-protein accumulation on liver damage and the ER stress phenotype, which are observed in *Tg-I278T Cbs*^{-/-} mice but not in *Tg-hCBS Cbs*^{-/-} animals (31), which have 3.3-fold less liver N-Hcy-protein. Similar liver damage that is observed in 8- to 32-week-old *Cbs*^{-/-} mice fed a choline-supplemented diet (42) could be linked to the accumulation of N-Hcy-protein as well; however, N-Hcy-protein has not yet been studied in that model.

In conclusion, our findings provide evidence that hyperhomocysteinemia due to genetic deficiencies in Hcy or folate metabolism or induced by a diet leads to significant increases in the proathrogenic N-Hcy-protein in the mouse. F

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