Sex affects N-homocysteinylation at lysine residue 212 of albumin in mice

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The modification of protein lysine residues by the thioester homocysteine (Hcy)-thiolactone has been implicated in cardiovascular and neurodegenerative diseases. However, only a handful of proteins carrying Hcy on specific lysine residues have been identified and quantified in humans or animals. In the present work, we developed a liquid chromatography/mass spectrometry targeted assay, based on multiple reaction monitoring, for quantification of \( N\)-Hcy-Lys212 (K212Hcy) and \( N\)-Hcy-Lys525 (K525Hcy) sites in serum albumin in mice. Using this assay, we found that female (n = 20) and male (n = 13) \( \text{Cbs}^{-/-} \) mice had significantly elevated levels of K212Hcy and K525Hcy modifications in serum albumin relative to their female (n = 19) and male (n = 17) \( \text{Cbs}^{+/-} \) littermates. There was significantly more K212Hcy modification in \( \text{Cbs}^{-/-} \) males than in \( \text{Cbs}^{-/-} \) females (5.78 ± 4.21 vs. 3.15 ± 1.38 units, \( P = 0.023 \)). Higher K212Hcy levels in males than in females were observed also in \( \text{Cbs}^{+/-} \) mice (2.72 ± 0.81 vs. 1.89 ± 1.07 units, \( P = 0.008 \)). In contrast, levels of the K525Hcy albumin modification were similar between males and females, both in \( \text{Cbs}^{-/-} \) and \( \text{Cbs}^{+/-} \) mice. These findings suggest that the sex-specific K212Hcy modification in albumin might have an important biological function in mice that is not affected by the \( \text{Cbs} \) genotype.

The sulfur-containing amino acid homocysteine (Hcy) is an intermediate in the metabolic pathways of two canonical amino acids that participate in the genetic code: methionine (Met) and cysteine (Cys). Hcy levels are regulated by re-methylation to Met, catalyzed by Met synthase (with methyltetrahydrofolate cofactor provided by the MTHFR enzyme) and betaine-Hcy methyltransferase, as well as by transsulfuration to cysteine catalyzed by cystathionine \( \beta \)-synthase (CBS) and cystathionine \( \gamma \)-lyase1. Although Hcy, in contrast to Met and Cys, is a non-coded amino acid that cannot participate in canonical protein biosynthesis, it can be incorporated into proteins via distinct mechanisms2–5. In one mechanism Hcy is first erroneously selected in place of Met by methionyl-tRNA synthetase and metabolized to Hcy-thiolactone3,5. Like other biological thioesters (e.g., acetyl-coenzyme A6), Hcy-thiolactone is chemically reactive and modifies protein lysine residues generating KHcy-proteins in a process called \( N\)-homocysteinylation7. \( N\)-homocysteinylation alters protein’s structure/function and contributes to a variety of pathologies associated with genetic or dietary hyperhomocysteinemia (HHcy)1,3,5.

The major cause of genetic HHcy in humans is CBS deficiency with world-wide incidence of 1:344,0001 that in some countries can be as high as 1:65,000 (Ireland)8, 1:1,800 (Qatar)9, or even 1:240 (an Austronesian Taiwanese Tao tribe)10. CBS deficiency is associated with mental retardation, ectopia lentis, osteoporosis, and vascular complications (thromboembolism), which are the major cause of morbidity and mortality1. Hcy-thiolactone and \( N\)-Hcy-protein levels are elevated in CBS-deficiency, both in humans and mice11–14. In CBS-deficient patients, \( N\)-Hcy-protein accumulation has been linked to an autoimmune response and atherothrombosis3,5.

We have previously identified K525Hcy15, K212Hcy, and K137Hcy in human serum albumin16,17, as well as \( \alpha\text{K562Hcy}, \beta\text{K344Hcy}, \) and \( \gamma\text{K385Hcy} \) in human fibrinogen18 from CBS-deficient patients. Although protein \( N\)-homocysteinylation is increased in mouse models of HHcy, individual mouse \( N\)-Hcy-proteins and their sites of Hcy modification have not yet been identified in vivo.

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The objective of the present study was to identify and quantify KHcy residues in mouse serum albumin and to study how sex, age, total Hcy (tHcy), and Cbs genotype affect mouse albumin KHcy modification in vivo using Tg-I287T Cbs−/− and Tg-I287T Cbs+/− mice.

**Results**

**Identification of KHcy sites in mouse albumin modified with Hcy-thiolactone in vitro.** We modified mouse serum albumin in vitro with increasing concentrations of Hcy-thiolactone and analysed the changes in molecular weight of albumin using electrospray ionization mass spectrometry (ESI MS). There was a linear increase in the molecular weight, from 66,565 Da for unmodified albumin to 68,695 Da for the modified KHcy-albumin (Fig. 1). The 2,140 Da increase in molecular weight indicates incorporation of ca. $[2,140/119.2] = 18$ moles of Hcy per mol of albumin for the highest Hcy-thiolactone concentration used (Fig. 1). This suggests that at least 18 out of 51 lysine residues (35.3%) in mouse serum albumin were modified under these conditions. Similar relationships were observed for the KHcy modification of human serum albumin (Fig. 1).

Using liquid chromatography with tandem mass spectrometry (LC/MS-MS), we identified twenty eight KHcy residues in mouse serum albumin modified in vitro with Hcy-thiolactone (Table 1). Two of those modifications, K212Hcy and K525Hcy, present in $^{212}\text{K}^{\text{Hcy}}\text{QTAELVK}^{334}$ (m/z 637.8) and $^{210}\text{AFKHcyAWVAR}^{234}$ (m/z 597.3) peptides, were the most abundant.

Quantification of K212Hcy and K525Hcy modifications in the in vitro-modified human and mouse albumins showed that there was a linear relationship between Hcy-thiolactone concentration and the magnitude of these modifications (Fig. 2). Notably, the K212 and K525 residues were more susceptible to modification in the mouse than in human albumin, as indicated by the greater slopes of the 'Intensity vs. Hcy-thiolactone' plots for the mouse K212Hcy (4.4-fold) and K525Hcy (2.9-fold) residues in Fig. 2.

**Identification/quantification of albumin KHcy modifications in mouse plasma in vivo.** Having established the masses of KHcy-peptides from tryptic digests of the in vitro-prepared mouse KHcy-albumin, we quantified these modifications directly in tryptic digests of mouse plasma. Examples of LC/MS-MS MRM analyses and extracted ion chromatograms for KHcy albumin modifications identified in mouse plasma in vivo and in in vitro-prepared mouse KHcy-albumin are shown in Fig. 3. We found that albumin K212Hcy and K525Hcy

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**Figure 1.** Relationships between Hcy-thiolactone concentration and molecular weight of (A) and number of KHcy residues (B) in mouse (MSA) and human (HSA) albumin.
modifications, present in 210AFKHcyAWAVAR.218 (m/z 597.3) and 525KHcyQTALAEVK.334 (m/z 637.8) peptides, respectively, were detectable in each mouse plasma sample. Other KHcy modifications were detectable in some samples, most likely because of their low abundance.

To examine determinants of K212Hcy and K525Hcy modifications we quantified 210AFKHcyAWAVAR.218 (597.3 m/z) and 525KHcyQTALAEVK.334 (637.8 m/z) peptides in tryptic digests of plasma from HHcy Cbs−/− mice (median plasma tHcy 200.8 µM, range 47.4 to 346 µM, n = 23), which also have elevated levels of KHcy-protein (16.6 ± 4.1 µM)12 and from control Cbs+/− mice (median plasma tHcy 6.4 µM, range 1.2 to 11.2 µM, n = 12), which have low levels of KHcy-protein (2.62 ± 1.73 µM)12.

**Table 1.** KHcy-peptides identified in tryptic digests in vitro-modified mouse KHcy-albumin.

| Sequence | Area | m/z [Da] | Range | Modification |
|----------|------|----------|-------|--------------|
| 1 R.AFKAWAVAR.L | 4.04E11 | 597.32 | 210–218 | K212Hcy |
| 2 K.QLTAEVLK.H | 3.44E11 | 637.36 | 525–534 | K525Hcy |
| 3 K.QLTCDKPLLK.K | 3.11E11 | 517.26 | 275–286 | K281Hcy |
| 4 R.YTQAKPQVYPTLVEAR.N | 2.53E11 | 1086.56 | 411–428 | K414Hcy |
| 5 K.QLTCDKPLLK.K.A | 1.59E11 | 559.95 | 275–286 | K281/285Hcy |
| 6 K.TYPESHTKCCSGSILLER.R | 1.57E11 | 1110.51 | 467–484 | K475Hcy |
| 7 R.RPCSLALTVDYETYPKEFK.A | 1.46E11 | 821.07 | 484–503 | K500Hcy |
| 8 R.VGTCKCCTPLPEDQR.L | 1.24E11 | 869.39 | 433–445 | K436Hcy |
| 9 K.EKALVSVYR.Q | 1.09E11 | 581.82 | 187–195 | K188Hcy |
| 10 K.NLCTCDLKVYK.L | 1.06E11 | 835.90 | 386–397 | K389Hcy |
| 11 R.VYCLHKEPTPVSEHVTK.C | 1.01E11 | 684.35 | 460–475 | K460Hcy |
| 12 R.HPKATAEQKL.T | 8.59E10 | 712.89 | 235–245 | K236/238Hcy |
| 13 K.KYATTEK.C | 8.57E10 | 578.29 | 352–359 | K352Hcy |
| 14 K.AADKDFCSFSTGEPLVLTR.C | 8.27E10 | 1078.49 | 561–578 | K564Hcy |
| 15 K.AEITFTPHSDFICTLPEKEK.Q | 8.24E10 | 776.36 | 504–521 | K519Hcy |
| 16 K.LATDLITKVYN.E | 6.94E10 | 638.85 | 234–243 | K240Hcy |
| 17 K.EPKAETFTHFSDICTLPEK.Q | 6.09E10 | 1237.57 | 501–519 | K503Hcy |
| 18 K.LQEVQTDFAKTCDVASEDAANCDS.K | 5.58E10 | 915.74 | 42–64 | K51Hcy |
| 19 K.LDGVEKALVSVYR.Q | 5.55E10 | 924.99 | 182–195 | K186Hcy, K188Hcy |
| 20 K.QIKKQTALAEV.A.H | 5.14E10 | 909.50 | 522–534 | K524Hcy, K525Hcy |
| 21 R.ENYGLADCCDKQPEQR.N | 4.36E10 | 1136.97 | 82–98 | K93Hcy |
| 22 K.CSSQMGKFER.A | 4.07E10 | 702.30 | 200–209 | K206Hcy |
| 23 K.QLTCDKPLLK.K.A | 3.67E10 | 926.45 | 275–286 | K281Hcy, K285Hcy |
| 24 K.TCVADESANCDKSHTLFGDK.L | 2.89E10 | 654.04 | 52–73 | K64Hcy |
| 25 K.VNKECHGDILLEADATA.R | 2.01E10 | 1132.96 | 241–257 | K243Hcy |
| 26 K.CSYDHEAKLVQVETFDAK.T | 1.98E10 | 772.02 | 34–51 | K41Hcy |
| 27 K.HPKATAEQKL.T | 1.93E10 | 533.31 | 235–245 | K236Hcy, K238Hcy |
| 28 K.SLHITLFGDNLCAIPNL.R.E | 1.78E10 | 1065.05 | 65–81 | K73Hcy |

Figure 2. Relationships between Hcy-thiolactone concentration and the magnitude of K212Hcy and K525Hcy modifications in mouse (MSA) and human (HSA) serum albumins.
Mean levels of K212Hcy and K525Hcy modifications were significantly higher (~2-fold) in Tg-I287T Cbs−/− mice compared with Tg-I287T Cbs+/− littermates, both in females and in males (Table 2). Notably, the levels of K212Hcy modification were significantly higher in male than in female mice. In contrast, the levels of K525Hcy modification were similar in females and males.

Total Hcy explained 7.5% (Fig. 4A) and 3.8% (Fig. 4B) of the variance in K212Hcy and K525Hcy, respectively. Age explained 4.6% (Fig. 5A) and 10.08% (Fig. 5B) of the variance in K212Hcy in Tg-I287T Cbs−/− mice and their Tg-I287T Cbs+/− littermates, respectively. In contrast, only 1.76% (Fig. 5C) and 0.16% (Fig. 5D) of the K525Hcy variance in these mice was explained by their age.

**Discussion**

Since the discovery of KHcy-protein in human plasma19, the list of KHy-proteins identified in vivo has grown to a few dozen5. For some of these proteins the in vivo sites of KHcy modifications have been identified. These include human serum albumin15–17, fibrinogen18, histones20 and DNA damage repair proteins21, rat dynein22, actin and E-cadherin23, and mouse collagen13. The present findings add mouse serum albumin to this list.

An unexpected finding of the present work is that the K212Hcy modification in albumin is sex-specific and is significantly higher in male than in female mice, in contrast to the K525Hcy modification, which was not affected by sex. Interestingly, the sex dependence of the K212Hcy modification was independent of the Cbs genotype. These findings suggest that the sex-specific K212Hcy modification in albumin is likely to play an important biological function in mice, which remains to be elucidated.

In humans, factors that affect KHcy-protein levels include the PON1 gene variants and HHcy caused by the CBS or MTHFR gene mutations. In mice, the determinants of KHcy-protein levels include the status of genes involved in the metabolism of Hcy (Cbs), Hcy-thiolactone (Pon1, Blmh), or folate (Mthfr, Pcf1), as well as a high methionine diet3. In general, KHcy-protein levels increase in HHcy and in Hcy-thiolactonase deficiencies. For instance, plasma N-Hcy-protein levels increase 31.4-fold in CBS-deficient patients13 and 8.1-fold in Cbs−/− mice, relative to unaffected individuals3. Elevated KHcy-protein levels are associated with low Hcy-thiolactonase activity of PON1 in humans and Pon1 or Blmh in mice6.
The present study identifies Cbs genotype as a determinant of albumin K212Hcy and K525Hcy modifications in mice. The mass spectrometry MRM assay shows about 2-fold higher albumin K212Hcy and K525Hcy modifications in plasma of Cbs−/− mice than in their Cbs+/− littermates. A chemical assay used in previous studies shows 8.1-fold higher KHcy-protein levels in plasma of Cbs−/− mice than in their Cbs+/− littermates. This suggests that

Figure 4. Relationships between albumin K212Hcy (A) and K525Hcy (B) modifications and tHcy in Cbs+/− and Cbs−/− mice.

Figure 5. Relationships between albumin K212Hcy (A,B) and K525Hcy (C,D) modifications and age in Cbs−/− (A,C) and Cbs+/− (B,D) mice.
the total KHcy modifications of all other plasma proteins exceed KHcy modifications of albumin in Cbs$^{-/-}$ mice. As shown in the present work, age and tHcy levels explain at best up to 10% of the variation in albumin K212Hcy and K525Hcy modifications (Figs 4 and 5). Notably, the K212Hcy modification exhibits greater variation with age than the K525Hcy modification (Fig. 5), again suggesting that the K212Hcy modification in albumin is likely to play an important biological function in mice.

Quantification of N-homocysteinylatation at K212 and K525, lysine residues most susceptible to the modification in mouse and human albums in vitro, revealed a linear increase in the magnitude of these modifications with the increasing concentration of Hcy-thiolactone (Fig. 2). While total N-homocysteinylatation (at all sites) was similar for mouse and human albums (Fig. 1), the site-specific N-homocysteinylatation at K212 and K525 was greater in mouse than in human albumin (Fig. 2). This suggests that K212 and K525 residues are more reactive with Hcy-thiolactone in mouse albumin than in human albumin.

The KHcy modification is conserved in serum albums from a variety of species, from human, pig, sheep, rabbit, rat and mouse to chicken. More KHcy is present in rodent albums (0.5% to 0.9% in mice and rats) than in human albumin (0.3%)24. The present findings, showing that K212 and K525 have greater reactivity towards Hcy-thiolactone in mouse albumin than in human albumin (Fig. 2), provide a possible explanation for these differences.

Identification of K212Hcy and K525Hcy residues in mouse serum albumin both in vitro and in vivo strongly suggests that these modifications are formed in vivo as products reactions of Hcy-thiolactone with the protein lysine residues. Analogous albumin modifications occur in vivo in humans15,18, indicating the conservation of the KHcy albumin modifications between rodents and humans.

In conclusion, to the best of our knowledge, the present findings represent the first identification and quantification of KHcy modifications at specific lysine residues of albumin in mice. We identified the sex-specific K212Hcy modification in albumin that is not affected by the Cbs genotype. These findings suggest an important biological function for the K212Hcy modification in mice and underscore the need to identify other determinants of the KHcy modifications and elucidate their roles in health and disease.

**Methods**

**Mice.** Tg-I278T Cbs$^{-/-}$ mice on the C57BL/ByJ genetic background were kindly provided by Warren Krueger25. These mice express human CBS I278T transgene under control of the zinc-inducible metallothionein promoter, which allows one to rescue the neonatal lethality phenotype of Cbs$^{-/-}$ mice by supplementing the drinking water of pregnant dams with 25 mM zinc chloride. Zinc-water is replaced by plain water after weaning at 4 weeks. Mice are fed a standard rodent diet (TD.04352, Harlan Teklad, Madison, WI). We examined 1 to 9-months-old Tg-I278T Cbs$^{-/-}$ mice with severely elevated tHcy and their Tg-I278T Cbs$^{+/+}$ siblings with normal tHcy levels as controls12. Animal procedures were approved by the Institutional Animal Care and Use Committee at the Rutgers-New Jersey Medical School. All experiments were performed in accordance with relevant guidelines and regulations.

**Preparation of mouse KHcy-albumin.** KHcy-albumin was prepared by incubation of mouse serum albumin (150 μg; MilliPoreSigma) with 0.01–10 μM L-Hcy-thiolactone·HCl (MilliPoreSigma), 0.1 M sodium phosphate buffer (pH 7.4), 0.1 mM ethylenediaminetetraacetic acid (EDTA) (overnight, 37°C).

**Trypsin digestion of KHcy-albumin and mouse plasma.** KHcy-albumin was reduced with 5.5 mM dithiothreitol (5 min, 95°C), free thiols were blocked with 11 mM iodoacetate (20 min, darkness), and digested with sequencing-grade trypsin in 50 mM NH₄HCO₃ (trypsin-protein ratio 1:50, overnight, 37°C). To identify sites of KHcy modifications in mouse albumin in vivo, plasma from Tg-I287T Cbs$^{-/-}$ and Tg-I287T Cbs$^{+/+}$ mice was diluted 60-fold in 50 mM NH₄HCO₃, processed and trypsinized as above.

**Mass spectrometry and data analysis.** In vitro assays of KHcy-albumin. Tryptic peptides from in vitro-prepared KHcy-albumin were analyzed using Dionex UltiMate 3000 RSLC nanoLC System connected to Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). The peptides were separated on Acclaim PepMap RSLC nanoViper C18 column (75 μm × 25 cm, 2 μm granulation) eluted with acetonitrile (4–60% gradient in human albumin (0.3%)24. The present findings, showing that K212 and K525 have greater reactivity towards Hcy-thiolactone in mouse albumin than in human albumin (Fig. 2), provide a possible explanation for these differences.

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In vivo analysis of albumin K212Hcy and K525Hcy modifications in mouse plasma. For multiple reaction monitoring (MRM) analysis on Ion trap MS the transitions for main KHcy peptides were 597.3 → 673.4 m/z (K212Hcy) and 637.8 m/z → 672.4 m/z (K525Hcy). Analyzes were carried out using an ESI-IonTrap (Amazon SL, Bruker Daltonics) mass spectrometer coupled with a UPLC system (nanoAQUITY, Waters). The effluent from the nanoLC column (15 cm, 75-μm-i.d. C18 column fitted with a C18 pre-column (nanoAQUITY)), was directly introduced into the Ion Trap in positive ESI mode. The column was eluted with acetonitrile a (4 to 60% gradient in 0.1% formic acid, flow rate 300 nL/min, 140 min, 30°C). Ion trap charge control was used to control ion accumulation in the trap. For precursor ion isolation, a 3-Da window was set up, and the precursor fragmentation
amplitude was set to 1.0. Acquisitions were run under the control of Trap Control 7.1 software (Bruker Daltonics).

All MRM data were processed using Data Analysis 4.0 software (Bruker Daltonics). The relative amounts of each target peptide were calculated as the average ratios of peak areas corresponding to the analyzed peptides. All data were manually inspected to ensure correct peak detection and accurate integration.

All analyses of plasma samples were repeated twice and standard deviations of peptides containing K525Hcy and K212Hcy, were ~20%. The interassay accuracy, determined from duplicate assays on 2 different analyses, was ≤10%.

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Author Contributions
M.S., I.M. carried out mass spectrometry analyses; J.P. performed Hcy quantification; H.J. designed the study, bred the Tg-I278T Chs−/− and Tg-I278T Chs+/− mice and collected plasma samples, analysed the data, wrote the paper with contributions from M.S., and had primary responsibility for the final content; all authors read and approved the final manuscript.
