Cystathionine β-synthase deficiency increases collagen N-homocysteinylation in mice

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Summary

Clinical manifestations of severe hyperhomocysteinemia due to cystathionine β-synthase (CBS) deficiency include connective tissue abnormalities affecting skin, bone, lung, eye and vascular (Mudd et al, 1985). Similar abnormalities are observed in Cbs−/− mice (Hamelet et al., 2009), but molecular mechanisms underlying these abnormalities remain obscure.

Key words: Homocysteine metabolism, protein N-homocysteinylation, collagen, cystathionine β-synthase deficiency

Introduction

Homocysteine (Hcy) is a sulphur amino acid, which comes from the dietary methionine. Hcy can exist in serum in three chemical forms: free reduced homocysteine, as an oxidized disulfide with itself or with cysteine (S-Hcy), or it can be bound to cysteine in serum proteins such as albumin (N-Hcy) (Jakubowski, 2006).

Homocysteine is metabolized, mostly in the liver and the kidney, by two classical pathways, transsulfuration to cysteine and remethylation to methionine, which is shown in Fig. 1. These reactions require the enzymes cystathionine β-synthase (CBS), 5-methyltetrahydrofolate-reductase (MTHFR), methionine synthase (MS) and vitamins B6 and B12, folates as cofactors. Hcy is also metabolized to Hcy-thiolactone by methionyl-tRNA synthetase (MetRS). Genetic or nutritional deficiencies in Hcy metabolism lead to the
accumulation of Hcy and increase the flow through the Hcy-thiolactone pathway (Fowler, 1997; Jakubowski, 1997).

![Figure 1. Schematic representation of the metabolism of homocysteine (Hcy) CBS – cystathionine β-synthase, MS - methionine synthase, MTHFR - methylenetetrahydrofolate reductase, SAH - S-adenosylhomocysteine, SAM - S-adenosylmethionine (Fowler, 1997; Jakubowski, 1997).](image)

**Hyperhomocysteinemia**

When normal metabolism is disturbed, due to deficiency of cystathionine β-synthase (CBS) (Fig. 2), Hcy accumulates in the blood and in the urine. CBS is a multidomain enzyme (EC 4.2.1.22), which catalyses the first step of the transsulfuration pathway, from homocysteine to cystathionine.

![Figure 2. Structure of cystathionine β-synthase (CBS)](image)
CBS deficiency is the most common cause of classical homocystinuria (HCU), an autosomal recessive metabolic disease, which causes skeletal abnormalities, osteoporosis, dislocated optic lenses, mental retardation, and a dramatically increased incidence of vascular disorders. Elevated level of Hcy is a risk factor for cardiovascular diseases (Cavalca et al., 2001), stroke (Yoo and Lee, 2001), venous thrombosis (den Heijer et al., 1996), the development of pregnancy complications (Nelen et al., 1997) and neurodegenerative diseases (Seshadri et al., 2002). How Hcy accumulation causes those abnormalities is unclear.

Clinical manifestations of severe hyperhomocysteinemia due to cystathionine β-synthase (CBS) deficiency include connective tissue abnormalities affecting skin, bone, lung, eye, and vasculature (Mudd et al., 1985) and characterized by an accumulation of homocysteine in the serum and an increased excretion of homocysteine in the urine.

**Tg-I278T Cbs-/- mouse model**

Similar abnormalities are observed in Cbs−/− mice (Fig. 3) (Hamelet et al., 2007). Characteristic include: lower weight, thin tail, facial alopecia, extreme hyperhomocysteinemia, low bone density, which lead to osteoporosis, endoplasmic reticulum (ER) stress in the liver and kidney and 20% reduction in mean survival time (Gupta et al., 2009).

![Figure 3. Comparison of Tg-I278T Cbs−/− mouse model (left) and Tg-I278T Cbs+/+ mouse (right)](image)

This model mouse was developed by utilized a mutant human CBS transgene (Tg-I278T), which expressed human CBS containing the I278T mutation, the most common allele found in CBS-deficient patients. In transgenic mouse, the human CBS cDNA is under control of the zinc inducible metallothionein promoter (Gupta et al., 2009).
**Thiolactone homocysteine (HTL)**

HTL is a cyclic tioester of Hcy, which is chemically reactive, even in low concentrations around 10 nM, and easily acylates free amino groups of proteins under physiological conditions. It reacts preferentially with the ε-amino groups of protein lysine residues, forming isopeptide bonds, –εNH-αCO-. This adduct can form with any available lysine amino residue. The process is called N-homocysteinylation (Fig. 4).

![Hcy-thiolactone-mediated incorporation of Hcy into protein occurs at lysine residues](image)

**Figure 4. N-homocysteinylation of protein amino groups**

**Mechanism of homocysteine toxicity**

Protein N-homocysteinylation is a major reaction of Hcy-thiolactone, generates modified proteins and results in a loss of their enzymatic activities. It can lead to protein functional damage of an important enzymes, such as lysyl oxidase, which is required for normal collagen biogenesis (Jakubowski, 2004; Jakubowski, 2011).

N-homocysteinylated proteins are prone to multimerization and further structural changes, which lead to their denaturation. Protein N-homocysteinylation effects on protein structure and function, causes cellular toxicity, elicits autoimmune response, leading to the synthesis of antibodies against N-Hcy-proteins and aggregation of N-Hcy-fibrinogen in thrombosis, inflammation and tightening of blood vessels (Jakubowski, 2004, 2011).

**Collagen**

Collagen is the major protein of connective tissues in animals and the most abundant protein in mammals, making up about 25% of the total protein content (Fig. 5, 6). Collagen is the main component of skin, tendons, ligaments, fascia, cartilage, bone and teeth. So far there are identified 27 types of collagen. Collagen is composed of a triple helix, which generally consists 3 α chains (2 α-1 and 1 α-2), each chain consists of a constant amino acid sequence of Gly-X-Y (where X and Y are any amino acid residues). About 20% of the amino acids in the collagen structure are proline and hydroxyproline. They play important roles in the stabilization of the tropocollagen (Shoulders and Raines, 2009).
Collagen fibers have a great tensile strength due to crosslinks between specific lysine residues in triple helical structures of collagen molecules. The side-by-side interactions of collagen helices are stabilized by an aldol cross-link between two lysine (or hydroxylysine) side chains. The extracellular enzyme, lysyl oxidase catalyzes formation of the aldehyde groups and participate in cross-linking collagen fibers (Fig. 7).
Hcy and connective tissue

Hcy is known to regulate extracellular matrix turnover and to inhibit lysyl oxidase activity and expression. Hcy interferes with the formation of collagen cross-linking, prevents the insolubilization of fibrils, and may delay the synthesis of more complex cross-links in collagen. Indeed, a reduced number of cross-links was observed in patients with homocystinuria. Collagen N-homocysteinylation would block the ε-NH2 groups of lysine residues, which would impairs cross-linking of extracellular matrix fibers (Liu et al., 1997; Coral et al., 2009; Anagnostis et al., 2009). Additionally, in vitro exposure of smooth muscle cells to homocysteine results in a proliferative response and enhanced collagen production and accumulation (Mohammad et al., 2011).

Because lysine residues participate in cross-linking collagen fibers, we predict that N-homocysteinylation of these lysine residues will prevent the formation of essential collagen cross-links and cause connective tissue abnormalities. Because connective tissues are complex supramolecular assemblies of collagen monomers, even low levels of N-Hcy-lysine could result in a structural defect.

So we set out to determine whether hyperhomocysteinemia is associated with elevated N-homocysteinylation in skin collagen of CBS-deficient mice.
Aim

Because lysine residues participate in cross-linking collagen fibers, we predict that $N$-homocysteinylation of these lysine residues will prevent the formation of essential collagen cross-links and cause connective tissue abnormalities. Because connective tissues are complex supramolecular assemblies of collagen monomers, even low levels of $N$-Hcy-lysine could result in a structural defects.

Materials and method

We studied Tg-I278T Cbs$^{-/-}$ and Tg-I278T Cbs$^{+/+}$ mice kindly provided by Warren Kruger, Fox Chase Cancer Center, Philadelphia, PA. Tg-I278T Cbs$^{-/-}$ mice have severe hyperhomocysteinemia (serum tHcy 272±50 μM vs. 1.9±1.6 μM for Tg-I278T Cbs$^{+/+}$) and elevated serum $N$-Hcy protein levels (16.6±4.1 μM vs. 1.6±0.3 μM for Tg-I278T Cbs$^{+/+}$) (Jakubowski et al., 2009).

We prepared collagen from skin of these mice using the acetic acid extraction method, confirmed its purity by SDS-PAGE, and analyzed tHcy and $N$-Hcy collagen content in the purified collagen using HPLC.

Results

First of all, our mice were weighted. Cbs$^{-/-}$ mice are smaller around 40% than wild type, but weight of skin is around 11% of total body weight of both transgene mice and weight of tail is around 2% of total body weight (Table 1).

<table>
<thead>
<tr>
<th>Weight of mice [g]</th>
<th>Cbs$^{-/-}$</th>
<th>Cbs$^{+/+}$</th>
</tr>
</thead>
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<tr>
<td>60 days</td>
<td>16.02 ± 1.62</td>
<td>25.2 ± 1.92</td>
</tr>
</tbody>
</table>

Table 1. Weight of Tg-I278T Cbs$^{-/-}$ and Tg- I278T Cbs$^{+/+}$ mice

Actually we should say that Cbs$^{-/-}$ are heavier than wild type mice, because collagen is main component of skin and tail.

In next step, we quantified tHcy and $N$-Hcy in serum. As shown in Table 2, tHcy protein level was 272±50 μM for Tg-I278T Cbs$^{-/-}$ vs. 1.9±1.6 μM for Tg-I278T Cbs$^{+/+}$) and $N$-Hcy protein level was 16.6±4.1 μM for Tg-I278T Cbs$^{-/-}$ vs. 1.6±0.3 μM for Tg-I278T Cbs$^{+/+}$).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>tHcy protein [μM]</th>
<th>$N$-Hcy protein [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg-I278T Cbs$^{-/-}$</td>
<td>272.0 ± 50.0</td>
<td>16.6 ± 4.1</td>
</tr>
<tr>
<td>Tg-I278T Cbs$^{+/+}$</td>
<td>1.9 ± 1.6</td>
<td>1.6 ± 0.3</td>
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Table 2. tHcy and $N$-Hcy protein content in collagen
We used acetic acid extraction method to collagen isolation. In first step, 0.5 M sodium acetate was used, in second one – 0.075 M sodium citrate and third one – 0.5 M acetic acid. In the first and second steps we received insoluble collagen and in third one – soluble collagen. Amount of collagen, based on hydroxyproline (OHPro) concentration, is almost the same in both mice genotype (Fig. 8). It means that amount of collagen does not depend on genotype.

![Figure 8. Amount of soluble and insoluble collagen](image)

Collagen itself is an insoluble protein because of extensive cross-linking. As cross-linking of collagen increases it becomes less soluble in a variety of solvents, such as salt and acid solutions. As shown in Table 9, N-Hcy collagen level was higher in insoluble collagen for Tg-I278T Cbs<sup>+</sup>/ vs. Tg-I278T Cbs<sup>+/+</sup>.

![Figure 9. Amount of tHcy and N-linked Hcy in insoluble and soluble collagen](image)
After the acetic acid extraction method, we confirmed purity of isolated collagen from tissue by SDS-PAGE (Fig. 10), then we analyzed thcy and N-Hcy collagen content in the purified collagen using HPLC.

![Figure 10. SDS-PAGE](image_url)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>thcy [pmol/mg tail]</th>
<th>Skin collagen [pmol/mg skin]</th>
<th>N-Hcy Tail collagen [pmol/mg tail]</th>
<th>Skin collagen [pmol/mg skin]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg-I278T Cbs&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>58.2 ± 35.9</td>
<td>125.0 ± 81.3</td>
<td>15.7 ± 4.4</td>
<td>89.9 ± 25.1</td>
</tr>
<tr>
<td>Tg-I278T Cbs&lt;sup&gt;++/+&lt;/sup&gt;</td>
<td>1.3 ± 0.9</td>
<td>6.2 ± 0.8</td>
<td>0.7 ± 0.9</td>
<td>5.0 ± 2.4</td>
</tr>
</tbody>
</table>

Table 3. thcy and N-Hcy-collagen level in Tg-I278T Cbs<sup>-/-</sup> and Tg-I278T Cbs<sup>++/+</sup> mice in skin and tail.
Conclusions

These findings demonstrate that collagen is a target for $N$-homocysteinyltion in vivo in mice and can account for connective tissues deficiencies observed in severe hyperhomocysteinemia. Hyperhomocysteinemia affects the connective tissue of skin in CBS-deficient mice:

- hyperhomocysteinemia is associated with elevated $N$-homocysteinyltion in skin and tail collagen of CBS-deficient mice
- Cbs$^{−/−}$ mice are smaller around 40% than wild type, but weight of skin is around 11% of total body weight of both transgene mice and weight of tail is around 2% of total body weight. Actually we should say that Cbs$^{−/−}$ are heavier than wild type mice, because collagen is main component of skin and tail
- increase amount of collagen (OHPRO was elevated Tg-I278T Cbs$^{−/−}$ mice, compared with Tg-I278T Cbs$^{+/+}$ animals)
- increased $N$-homocysteinyltion of skin and tail collagen ($N$-Hcy-collagen was elevated 18-fold in Tg-I278T Cbs$^{−/−}$ mice, compared with Tg-I278T Cbs$^{+/+}$ animals (89.9±25.1 vs. 5.0±2.4 pmol/mg skin) and was elevated 24-fold in Tg-I278T Cbs$^{−/−}$ mice, compared with Tg-I278T Cbs$^{+/+}$ animals (15.7±4.4 vs. 0.7±0.9 pmol/mg tail))

Hyperhomocysteinemia may interfere with connective tissue quality, primarily by altering the properties of collagen crosslinks. We are only beginning to understand pathophysiological consequences of $N$-Hcy-protein accumulation in tissues.

Reference

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