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S-Homocysteinylation of transthyretin is detected in plasma and serum of humans with different types of hyperhomocysteinemia

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Abstract

While the association of homocystinuria with disease is known for more than four decades, mild hyperhomocysteinemia has been detected more recently as a risk factor for a number of diseases. However, the mechanism which apparently renders (even mild) hyperhomocystenemia harmful is not known. Following reports on N-homocysteinylation of proteins by the homocysteine derivative homocysteine thiolactone, it has been suggested that homocysteinylation of proteins may contribute to the induction of biological effects by homocysteine. This has prompted us to study by electrospray ionization mass spectrometry homocysteinylation of transthyretin (TTR) in plasma and serum of humans with different types of hyperhomocysteinemia. We did not detect any N-homocysteinylation, but found pronounced S-homocysteinylation of TTR, if the concentration of total homocysteine was high. Our findings support a possible role of S-homocysteinylation of proteins in the mediation of detrimental effects of hyperhomocysteinemia. Careful study of posttranslational modifications of individual proteins may contribute to a better understanding of diseases associated with hyperhomocysteinemia.

Keywords: Homocysteine; Homocysteine thiolactone; Homocysteinylation; Lysine; Vitamin B_{12}; Cobalamin; Folate; Transthyretin; High pressure liquid chromatography–electrospray ionization mass spectrometry

Several inherited diseases, including cystathionine β-synthase deficiency, 5,10-methylenetetrahydrofolate reductase (MTHFR) deficiency, disorders in cobalamin metabolism locus C or D (cbl C/D disease), and nutritional deficiencies can result in elevated levels of the sulfur-containing amino acid homocysteine (Hcy) [1–4]. While the association of homocystinuria with disease is known for more than 40 years, mild hyperhomocysteinemia has been recognized more recently as a risk factor, e.g., for atherosclerotic cardiovascular diseases, stroke, peripheral arterial occlusive disease, and venous thrombosis. However, the mechanism which apparently renders (even mild) hyperhomocysteinemia harmful is not known.

In 1997, Jakubowski [5] had proposed the conversion of Hcy to its cyclic thioester Hcy thiolactone and the acylation of proteins by this chemically reactive compound. Such a reaction may contribute to the induction of biological effects by homocysteine, especially via N-homocysteinylation of the ε-amino group of lysine residues, which may yield highly immunogenic products [6]. A number of subsequent papers have followed this path [5,7–17], however, mostly not in patients' samples and without a specific focus on a particular protein within biological samples.

This has prompted us to study the influence of hyperhomocysteinemia of different causes on the formation of covalent adducts with transthyretin (TTR). The thyroid-hormone binding plasma protein TTR is a tetramer with four identical subunits, each consisting of 127 amino acids [18]. The established clinical relevance of TTR analysis is the diagnosis of familial amyloidotic

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polyneuropathy (FAP) by the detection of TTR variants, as we have performed by high pressure liquid chromatography–electrospray ionization mass spectrometry (LC-ESI-MS) [19]. Pioneering work on TTR mutations in FAP was by Saraiva et al. [20,21], and the first TTR analysis by mass spectrometry by Wada et al. [22] and Suzuki et al. [23].

More recently, S-sulfonation of plasma or serum TTR has been recognized as a promising marker for a disorder of sulfur metabolism [24,25], thus rendering more diagnostic applications of TTR analyses feasible.

Here, we describe for the first time S-homocysteinylation of TTR in plasma and serum of patients with hyperhomocysteinemia of different causes.

Materials and methods

Specimen. Plasma and sera were obtained for determination of amino acids concentrations or transthyretin isoforms as part of selective screening for inborn errors of metabolism or for monitoring of already diagnosed patients. Hyperhomocysteinemia samples included two samples of two patients with cystathionine β-synthase deficiency, three of one patient with methylene tetrahydrofolate reductase deficiency, and 12 samples obtained from five patients with cbl C/D deficiency or nutritional vitamin B_{12} deficiency.

Sample preparation. Serum or plasma TTR was prepared by immunoprecipitation as previously reported [25]. In brief, 25 μl of anti-human TTR antiserum (Sigma, St. Louis, MO, USA) was added to 50 μl of Protein G/A agarose (Cat. No. IP05, Oncogene Research Products, Boston, MA, USA), which had been washed before three times with 0.15 M sodium chloride solution. After shaking for 30 min at ambient temperature, the resulting agarose–antibody complex was washed four times with saline. Subsequently, 10 μl of sample serum or plasma was added to the complex and the mixture was incubated for one hour at room temperature. The generated precipitates were washed seven times with 0.5 ml of 0.15 M sodium chloride solution. Each washing step consisted of turning the tube over several times, centrifuging for 5 s, and discarding the supernatant.

Finally, the precipitate was suspended in 20 μl of 2% acetic acid and the agarose was removed by filtration, using an ultrafree-MC centrifugal 0.45 mm filter unit (Millipore, Bedford, MA). The filtration was repeated twice and the pooled filtrate was applied to high pressure liquid chromatography–electrospray ionization mass spectrometry (LC-ESI-MS), with a PLRP-S reversed-phase column (1 × 50 mm, 1000 Å, 8 μm; Michrom Bioresources, Pleasanton, CA, USA), as described earlier [25]. An ESI mass spectrometer, TSQ7000 (Thermo Quest, San Jose, CA, USA) was utilized. The scanning range was m/z 500–2000 in 3 s. Calibration was performed using the peptide Met–Arg–Phe–Ala (Thermo Quest) and multiple charged ions of horse apo-myoglobin (Sigma) according to the manufacturer's instructions. All other chemicals were purchased from Nacalai tesque (Kyoto, Japan).

To confirm the disulfide linkage of adducts, 300 mM dithiothreitol (DTT) solution in water was added to the filtrate at the volume ratio of 1:9 (DTT solution:TTR filtrate). The mixture was incubated for 2 h at room temperature and was subsequently submitted to LC-ESI mass spectrometry. For further confirmation of the identity of the additional signal with a mass increment of 134, which we expected to represent *S*-homocysteinyl-TTR, homocysteine solution (1 mg/ml in water) was added to commercially obtained TTR solution (3 mg/ml) in 20 mM sodium acetate buffer (pH 6.0), at a volume ratio of 10:1 (Hcy solution:TTR solution). TTR was purchased from Biogenesis (Poole, England, UK). The mixture was incubated for 2 days at room temperature before analysis by LC-ESI-MS. After the in vitro generation of the additional signal with a mass increment of 134 (which is compatible with *S*-homocysteinyl-TTR), an aliquot of the reaction mixture was reduced at room temperature by adding one part of 300 mM DTT to nine parts of the sample solution and incubation for 2 h.

The ratio of Hcy-TTR/Cys-TTR was calculated from the peak heights obtained from transformed spectra. Concentrations of total homocysteine (tHcy) in serum and plasma were determined with a commercially available enzyme immunoassay (Axis Shield AS, Oslo, Norway) with the limit of quantification given as 1.0 μmol/L.

Results and discussion

In samples of patients with different causes of hyperhomocysteinemia, e.g., cystathionine β-synthase deficiency, MTHFR deficiency or cbl C/D deficiency as well as nutritional vitamin B_{12} deficiency, deconvoluted ESI-MS spectra of TTR revealed an additional signal with a mass increment of 134 which is compatible with TTR S-homocysteinylated at the Cys-10 residue.

Fig. 1 displays deconvoluted ESI-MS spectra of serum TTR prepared from three patients. The spectrum of a sample with a normal serum level of homocysteine (Fig. 1I) shows a normal profile, well in agreement with our earlier reports which have provided the peak assignments given in the legend of Fig. 1 [24,25]. The spectra shown in Figs. 1II and III are from two patients with elevated concentrations of tHcy in serum/plasma. In contrast to the first spectrum (Fig. 1I), they both present with an extra peak (labeled with an arrow head) just behind peak E. We assumed that the extra signal is due to S-homocysteinylation of TTR because the mass is 14 units higher than that of peak E (corresponding to cysteinyl-TTR) and because the peak was detected only in patients with elevated serum/plasma levels of tHcy. Fig. 2 shows deconvoluted ESI-MS spectra of serum TTR prepared from a patient with a high level of tHcy. The signal with a mass increment of 134 (see arrow head) in Fig. 2I decreased after reduction with dithiothreitol and the signal of free TTR increased, as shown in Fig. 2II. This observation confirms that the adduct is due to disulfide linkage.

Fig. 3 demonstrates that the adduct formed via the disulfide linkage is indeed a reaction product of homocysteine and TTR. Commercially obtained TTR contained mainly TTR + 120, which corresponds to cysteinyl-TTR, as shown by the high peak E in Fig. 3I. By reaction with homocysteine, cysteinyl-TTR disappeared and the signal with a mass increment of 134 (if compared to free TTR) increased (Fig. 3II). In addition, the signals of other TTR adducts with disulfide linkage (peaks D, E, F, and G) decreased, while that of free TTR (peak B) and those of the peaks A and C increased.

After reduction with DTT (Fig. 3III), both the signal with a mass increment of 134 and that of cysteinyl-TTR (peak E) disappeared, while free TTR (peak B) increased

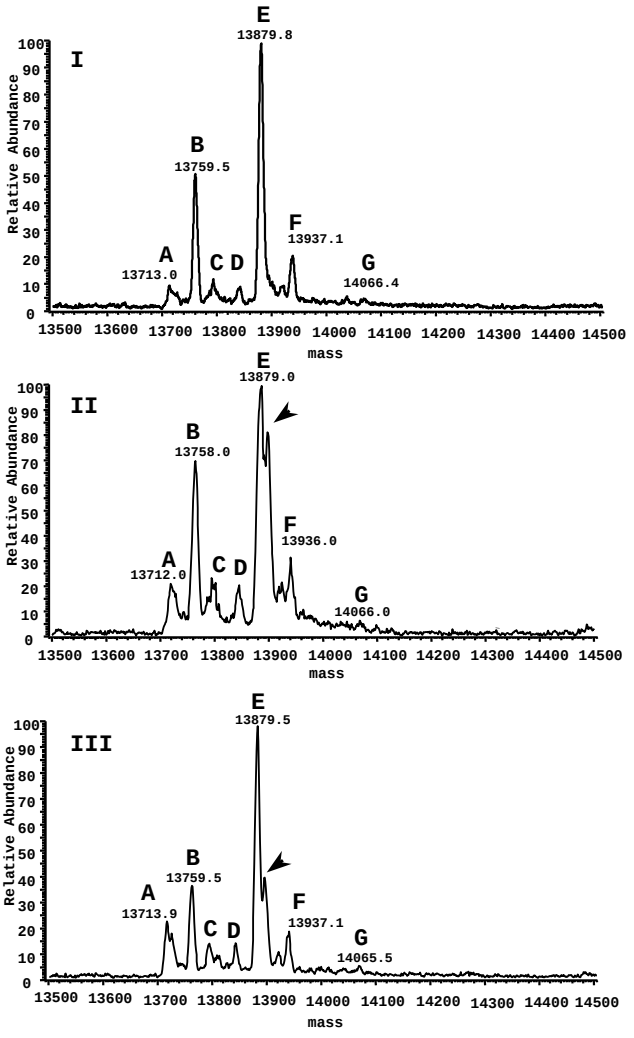

Fig. 1. Deconvoluted ESI-MS spectra of TTR from a control sample (normal total homocysteine [tHcy] of 12.2 μmol/L) (I), a patient with MTHFR deficiency ([tHcy] of 105 μmol/L) (II) and a patient with cystathionine β-synthase deficiency ([tHcy] of 120 μmol/L) (III). The arrow indicates an additional signal with a mass increment of 134 which is compatible with TTR S-homocysteinylated at the Cys-10 residue (Hcy-TTR). (A) TTR modified at position 10, cysteine to glycine, Gly10TTR [19]; (B) free TTR; (C) oxidized TTR; (D) sulfonated TTR; (E) cysteinyl-TTR; (F) cysteinylglycinyl-TTR; and (G) glutathionyl-TTR.

and peaks A and C remained prominent, thus indicating that—in contrast to the signal with a mass increment of 134—peaks A and C are not due to disulfide linkage. Our experiments document that the peak with a mass increment of 134 indeed represents *S*-homocysteinyl-TTR. For TTR with a mass increment of 120, we have previously confirmed the identity in a similar way and reported it as cysteinyl-TTR [19].

The signal of Hcy-TTR disappeared, when the concentration of tHcy had normalized in serum (Fig. 4). There was a significant linear correlation between the concentrations of tHcy in serum or plasma and the ratios of Hcy-TTR/Cys-TTR (Fig. 5).

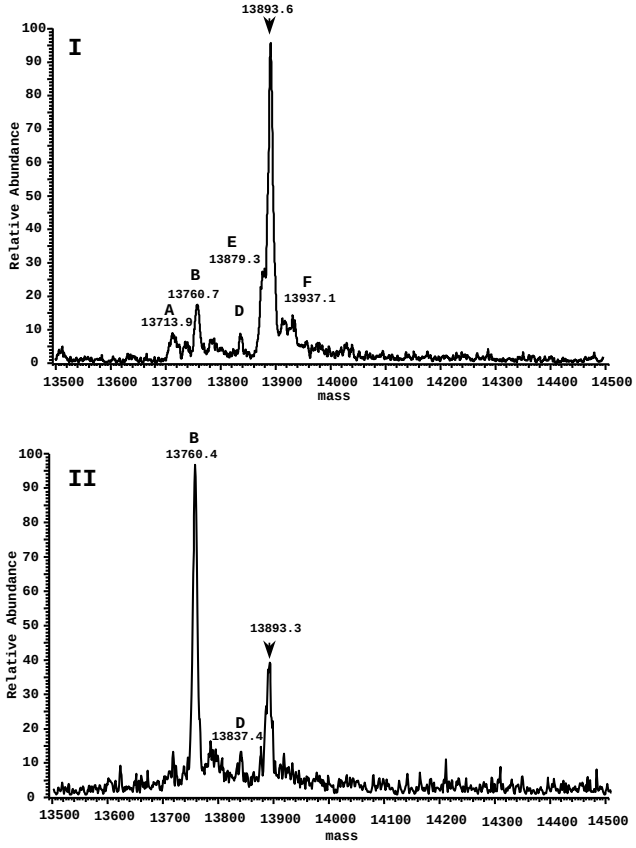

Fig. 2. Deconvoluted ESI-MS spectra of plasma TTR prepared from a patient with high level of homocysteine, before (I) and after (II) reduction with dithiothreitol. Peak assignments are the same as in Fig. 1.

Hcy-TTR was not detected if the concentration of tHcy was $\leqslant 30$ μmol/L. In such samples the ratio of the signals of Hcy-TTR and Cys-TTR was clearly below 0.1.

We normalized Hcy-TTR signals to Cys-TTR in order to minimize effects of variations in preanalytical conditions on mixed disulfide formation at the Cys-10 residue.

We were unable to detect any adducts resulting from reactions of Hcy thiolactone with the ϵ-amino group of lysine residues, with the N-terminal amino group or with the C-terminal carboxy group of TTR, i.e., did not observe additional signals with a mass increment of 117 or 250, as would be expected based on recently reported mechanistic studies [17]. For the latter adduct, which represents a *N*-homocysteinyl-cysteine derivative of TTR, the detection limit was about 2% of Cys-TTR and less than 1% of total TTR. Resolution of the signal compatible with a mass increment of 117 is impaired by the signal representing Cys-TTR and therefore more limited. For a high precision quantification by mass spectrometry, a different approach with stable isotope-labeled internal standards would be required [26].

Several explanations are possible for the lack of detectable N-homocysteinylation. For instance, formation of covalent adducts at lysine residues may be sterically

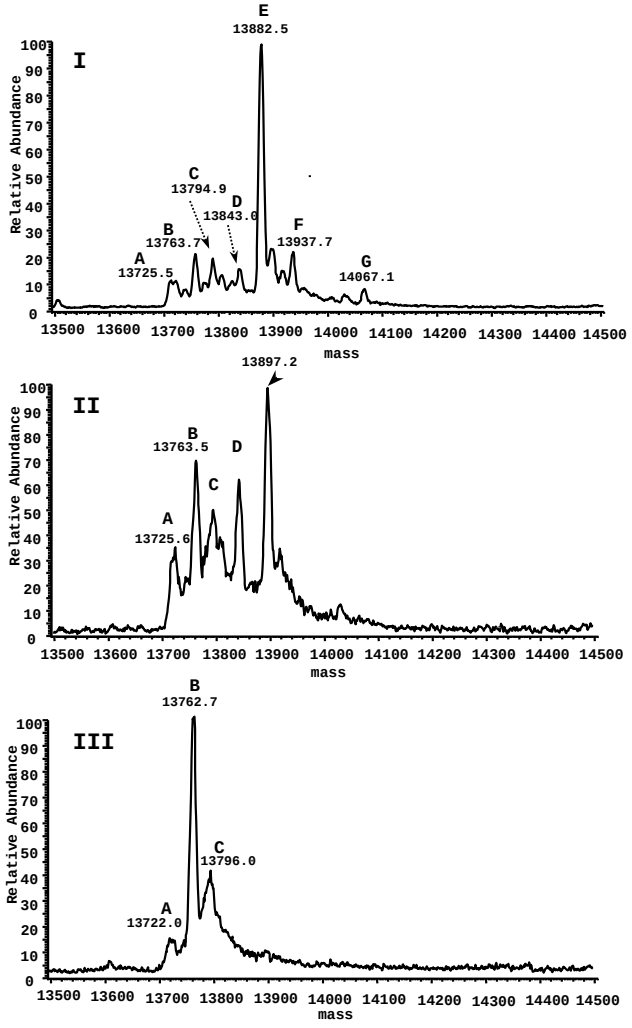

Fig. 3. An experiment for in vitro generation of transthyretin with a mass increment of 134 by reaction of commercially available purified TTR (I) with homocysteine (II) and subsequent reduction with dithiothreitol (III). Peak assignments are the same as in Fig. 1.

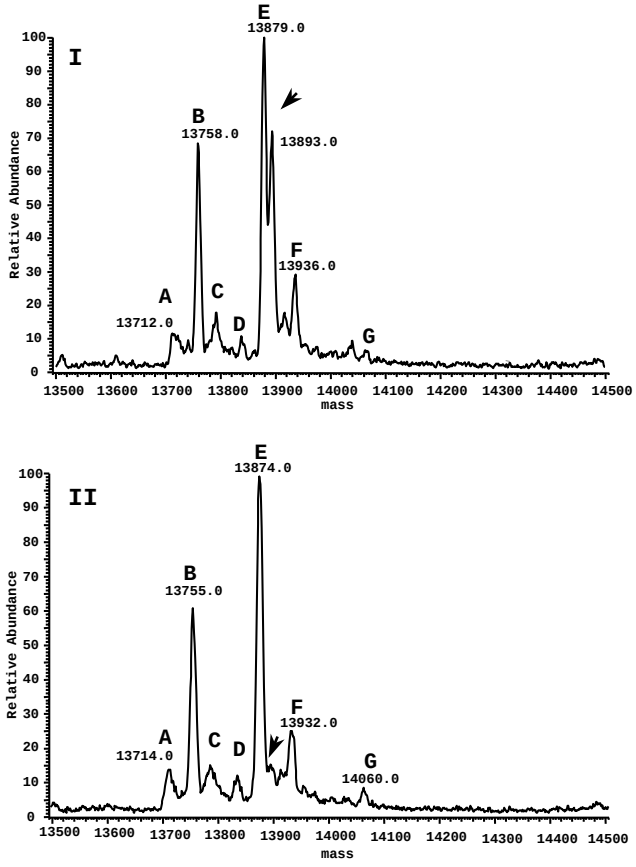

Fig. 4. Deconvoluted ESI-MS spectra of TTR from serum of a patient with vitamin B_{12} deficiency both before (I) and after (II) normalization of the total homocysteine level from 109 to 7.0 μmol/L two weeks later, following administration of vitamin B_{12}. Peak assignments are the same as in Fig. 1.

hindered. However, there are seven lysine residues in different parts of the TTR molecule, one of them even at position 9, just next to Cys-10, which makes such an assumption not very likely. Possibly, Hcy thiolactone, the key molecule in N-homocysteinylation, reacts preferably with other proteins than TTR, regardless of S-homocysteinylation of TTR due to the binding of Hcy to Cys-10. Jakubowski [16] has found variable levels of Hcy-*N*-protein when studying commercially obtained blood proteins. Furthermore, in theory high levels of Hcy-thiolactonase/paraoxonase in our samples might account for low levels of N-homocysteinylation of TTR. Hcy-thiolactonase/paraoxonase catalyzes the enzymatic hydrolysis of Hcy-thiolactone to Hcy, an alternative pathway to N-homocysteinylation by Hcy-thiolactone [12,13]. We could not study the activity of this enzyme in our samples, but there is no evidence indicating that pronounced hyperhomocysteinemia can affect thiolac-

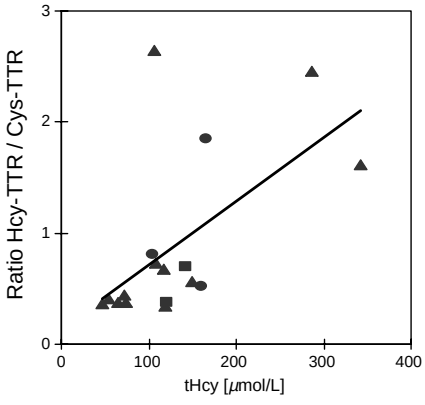

Fig. 5. Linear regression analysis: ratio of the peak heights of Hcy-TTR/Cys-TTR versus concentration of total plasma or serum homocysteine (tHcy). $y = 0.0057x + 0.144$; correlation coefficient (Pearson) $r = 0.5921$ ($p = 0.0123$). Triangle, hyperhomocysteinemia due to vitamin B_{12} deficiency or vitamin B_{12} related disease (cbl C/D defect); circle, MTHFR deficiency; and square, cystathionine β-synthase deficiency.

tonase/paraoxonase activity in vivo. To our knowledge, such a (possibly protective) mechanism has not been studied in hyperhomocysteinemia so far.

Our findings support a possible role of S-homocysteinylation of proteins in the mediation of detrimental effects of hyperhomocysteinemia, the latter, e.g., known to be associated with vascular disease. Our results are compatible with recent in vitro data by Majors et al. [27] on Hcy binding to human plasma fibronectin. The literature published on N-homocysteinylation has mostly focused on in vitro studies [5,7–17]. Even if cells of humans with cystathionine β-synthase deficiency or plasma samples "from homocystinuric patients" were studied, homocysteine, its thiolactone and overall Hcy-*N*-protein were examined, but not the homocysteinylation of individual proteins in affected patients, as we have investigated here.

For a better understanding of processes associated with hyperhomocysteinemia, further work is necessary which should combine the various attempts and focus them on individuals with hyperhomocysteinemia and proteins with a long half-life which may possibly reflect a variety of posttranslational modifications during the course of the disease.

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