Hydrolysis of homocysteine thiolactone results in the formation of Protein-Cys-S-S-homocysteinylation

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An increased level of homocysteine, a reactive thiol amino acid, is associated with several complex disorders and is an independent risk factor for cardiovascular disease. A majority (>80%) of circulating homocysteine is protein bound. Homocysteine exclusively binds to protein cysteine residues via thiol disulfide exchange reaction, the mechanism of which has been reported. In contrast, homocysteine thiolactone, the cyclic thioester of homocysteine, is believed to exclusively bind to the primary amine group of lysine residue leading to N-homocysteinylation of proteins and hence studies on binding of homocysteine thiolactone to proteins thus far have only focused on N-homocysteinylation. Although it is known that homocysteine thiolactone can hydrolyze to homocysteine at physiological pH, surprisingly the extent of S-homocysteinylation during the exposure of homocysteine thiolactone with proteins has never been looked into. In this study, we clearly show that the hydrolysis of homocysteine thiolactone is pH dependent and at physiological pH, 1mM homocysteine thiolactone is hydrolysed to ~ 0.71 mM homocysteine within 24 hours. Using albumin, we also show that

incubation of HTL with albumin leads to a greater proportion of S-homocysteinylation (0.41 mol/mol of albumin) than N-homocysteinylation (0.14 mol/mol of albumin). S-homocysteinylation at Cys³⁴ of HSA on treatment with homocysteine thiolactone was confirmed using LC-MS. Further, contrary to earlier reports, our results indicate that there is no cross talk between the cysteine attached to Cys³⁴ of albumin and homocysteine attached to lysine residues.

Introduction

The thiol amino acid, homocysteine (Hcy), is formed in the ubiquitous methionine cycle. It is a key branch point intermediate of the folate pathway and the remethylation and transulfuration pathways in the methionine cycle. An elevated level of Hcy is considered to be an independent risk factor for cardiovascular disease (1) and several epidemiological studies have shown the association of homocysteine with a wide spectrum of disease conditions like neural tube defects (2), schizophrenia (3), stroke (4), placental abnormalities (5), diabetes (6), Alzheimer's disease (7) etc. In circulation, free reduced homocysteine accounts for only a small fraction (~2%) of the total homocysteine, while about 10-20% exists as low molecular weight oxidized disulfide such as homocystine and homocysteine cysteine mixed disulfides. A majority of homocysteine (~70-80%) exists in the protein bound form through disulfide (-S-S-) linkage. Protein bound homocysteine is also a predominant form of homocysteine in homocystinuria (8-12) and various other conditions such as end stage renal disease (13-15) peripheral vascular disease (16), stroke

(17), cobalamin deficiency (18), coronary artery disease (19) etc. In addition, it is generally believed that a small fraction of homocysteine is metabolically converted to homocysteine thiolactone (HTL), a cyclic form of homocysteine, by methionine t-RNA synthetase (20). Homocysteine thiolactone is believed to bind solely to the ϵ -amino group of lysine and reports suggest that this form of homocysteine (homocysteinamide) may be present in circulation *albeit* accounting for a small percentage of the total plasma homocysteine levels (21).

Since, a majority of the circulating homocysteine is bound to protein, one of the mechanistic hypothesis put forth to explain the cytotoxicity of homocysteine is the “molecular targeting hypothesis”. Molecular targeting of proteins by homocysteine leads to the formation of stable disulfide bonds with protein cysteine residue, known as S-homocysteinylation while formation of stable amide bonds with activated carbonyl group of HTL and primary amino group of protein lysine residue (reaction 1) is referred to as N-homocysteinylation (22, 23). The detailed mechanisms of both S- and N-Homocysteinylation have been proposed using human serum albumin (23-25). Albumin is the most abundant human plasma protein with concentration ranges from 3.5 – 5 (g/dl) and constitutes about 50% of the total plasma protein (26). Albumin is a single polypeptide chain folded in three different domains which consist of 35 cysteine residues of which 34 are involved in the formation of 17 intra-chain disulfide bonds. Interestingly, albumin has a single cysteine residue at position 34 (Cys³⁴) which is not involved in the intra-chain disulfide bond formation and this cysteine accounts for the bulk of free thiol in plasma (27). A majority of this cysteine (Cys³⁴) exists in the free reduced form (albumin-Cys³⁴-SH,

>65%) while one-third of albumin-Cys³⁴ is bound to cysteine (albumin-Cys³⁴-S-S-Cys) and about 2% is bound to homocysteine (albumin-Cys³⁴-S-S-Hcy) in circulation. It has been established that homocysteine binds to albumin-Cys³⁴ through a two-step reaction (24). In the first step, homocysteine binds to the cysteine attached to Cys³⁴ forming albumin thiolate anion and homocysteine-cysteine mixed disulfide. In the second step, albumin thiolate anion, thus formed, binds to homocysteine-cysteine mixed disulfide to primarily form Albumin bound homocysteine (Alb-Cys³⁴-S-S-Hcy).

The mechanism of N-homocysteinylation has also been proposed and it has been shown that HTL binds to the ϵ -amino group of lysine residues exclusively forming N-homocysteinylation albumin, Albumin-Lys-Hcy-SH (23). Further, it has also been reported that there is a cross talk between cysteine attached to Cys³⁴ of albumin and homocysteine bound to albumin lysine residue, resulting in the formation of Albumin-Lys-Hcy-S-S-Cys (23). Interestingly, there are reports showing that HTL readily hydrolyses *in vitro* and in circulation undergoes enzymatic hydrolysis by paraoxonase (PON1) to form homocysteine (22, 28, 29). If this is indeed the case, then the reaction of HTL with albumin should lead to both protein-S-homocysteinylation along with N-homocysteinylation since, if HTL hydrolyses at physiological pH to homocysteine, then the homocysteine formed could potentially bind to Cys³⁴-SH. However, till date, surprisingly, there are no reports showing that incubation of HTL with proteins could lead to protein-S-homocysteinylation.

In this study, we not only show that S-homocysteinylation is the predominant product formed during the incubation of HTL with albumin, but also show that there is no cross talk between cysteine attached to Cys³⁴ and homocysteine attached to lysine (N-homocysteinylation).

Materials and methods

Reagents

DL-homocysteine (Hcy), DL-Homocysteine-thiolactone (HTL), Dithiothreitol (DTT), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent), Ethylenediamine tetra-acetic acid (EDTA), Formic Acid, Glutathione, Iodoacetamide, L-homocystine, L-cysteine, Trizma® (Tris base), Monobromobimane, Sodium borohydride, Sodium acetate and human serum albumin were purchased from Sigma (St. Louis, MO, U.S.A.). Perchloric acid, HPLC grade acetonitrile and methanol were purchased from Fisher (Fair Lawn, NJ, U.S.A.). HPLC grade acetic acid was purchased from HIMEDIA (Mumbai, India). Isoamyl alcohol was purchased from BDH laboratory (Poole, UK) and Trypsin from Promega (Madison, U.S.A). All other chemicals used in this study were of analytical grade.

Determination of albumin bound homocysteine and cysteine during HTL binding to albumin

To determine the amount of homocysteine bound to albumin by Hcy or HTL, 0.75 mM HSA in 1 ml of 0.1 M sodium phosphate buffer (pH-7.4) was incubated with 1 mM of either HTL or homocysteine. The reaction mixture was incubated at 37°C in shaker with continuous shaking.

From this, 0.05 ml aliquots were withdrawn at various time points and transferred to tubes containing 0.1 ml of 1.5M perchloric acid to stop the reaction by precipitating albumin. The tubes were vortexed and incubated in ice for 10 minutes. After centrifugation at 12,000 rpm for 10 minutes, the protein pellet was washed three times with 0.1 ml of 1.5 M perchloric acid. The washed pellet was dissolved in 0.1 ml of 0.5 M Tris (pH 8.5) and stored at -20°C . Albumin bound homocysteine and cysteine was measured using high performance liquid chromatography (HPLC) equipped with fluorescence detector (30). Briefly, 0.1 ml of the dissolved pellet was treated with 0.035 ml of freshly prepared 1.43 M sodium borohydride in 0.10 M sodium hydroxide (to reduce oxidized thiols) followed by short and quick vortexing with further addition and mixing of 0.035 ml of 1.0 M HCl. To this 0.05 ml of 7 mM monobromobimane in 5 mM sodium EDTA (pH-7.0) was added to conjugate the reduced thiols and the solution was incubated at 42°C for 12 min. Albumin was then precipitated by the addition of 0.05 ml of 1.5 M perchloric acid followed by centrifugation at 12,000 rpm for 10 minutes. The supernatant (0.10 ml) was then transferred to injector vials for automated HPLC analysis. 0.06 ml of cold 2.0 M Trizma was added to the vials to neutralize the acidic pH. HPLC measurements were carried out using reverse phase C18 column (5 μM particle size, 4.6 x 150 mm, Phenomenex, USA). Standard curves were generated with known amounts of cysteine and homocysteine to calculate the concentrations of these two thiols in the reaction mixture.

Binding capacity of HTL to Plasma protein

Blood collection and Plasma separation Blood samples were collected from healthy individuals (n=5) in vacutainer tubes (Becton-Dickinson) containing EDTA. All the individuals provided their written consent on the day of collection by filling a questionnaire on the same day when blood samples were collected from the individuals. The study was approved by institutional ethical committee following the Helsinki Declaration. Whole blood sample was collected from these individuals (after overnight fasting) and immediately put on ice for 30-45 minutes and centrifuged at 12000 g for 20 minutes at 40°C. Plasma was separated and stored at -80°C until further use.

Plasma protein bound homocysteine and cysteine

The amount of S-homocysteinylated plasma protein was determined during HTL binding to human plasma. Plasma protein bound homocysteine and cysteine was determined as described by Togawa *et al.* (31). Briefly, human plasma (1 ml) was diluted with 1 ml of 0.1 M sodium phosphate buffer (pH-7.4) and pre-incubated at 37°C for 10 minutes. To this DL- HTL (final concentration, 1 mM), was added and the reaction mixture was incubated at 37°C in a shaker. From this, 0.05 ml aliquots were withdrawn at various time points and transferred to tubes containing 0.02 ml of 1.5 M perchloric acid. Following this, the method as described above for HSA was used. Plasma protein bound homocysteine and cysteine were measured using reverse-phase high performance liquid chromatography equipped with fluorescence detector as mentioned above.

Determination of homocysteine-bound lysine during HTL binding to albumin

To determine the amount of N-homocysteinylation albumin formed during HTL binding to albumin, the free Cys³⁴ thiol group of albumin was treated with DTT to form albumin thiolate anion and subsequently alkylated using iodoacetamide (IAA) as described below.

Preparation of albumin thiolate anion (free reduced albumin Cys³⁴) and albumin Cys³⁴-S-IAA

Albumin thiolate anion (mercaptalbumin) was prepared as described by Sogami *et al.*, (1984)(32) with slight modification. Briefly, 50 mg of HSA was dissolved in 1 ml of 0.1 M sodium phosphate buffer with 10 mM EDTA at physiological pH and treated with 1 mM dithiothreitol (DTT) at 37°C for 45 min to prepare albumin thiolate anion. Albumin Cys³⁴-S-IAA was prepared by completely blocking the thiol group (-SH) of free reduced albumin-Cys³⁴ (albumin-Cys³⁴-SH) using IAA. For this, albumin thiolate anion (50 mg/ml) was treated with 55 mM IAA at 37°C for 30 minutes in dark. Aliquots (50 µl) were withdrawn at various time points (0, 15, 30, 45, 60, 90, 120, 180, 300, 480 minutes) and transferred to tubes containing 0.1 ml of 1.5 M perchloric acid to stop the reaction by precipitating the protein. The tubes were then vortexed, incubated for 10 minutes on ice and centrifuged at 12000 rpm for 10 minutes. The pellet was washed three times with 1.5 M perchloric acid to remove excess DTT and IAA, which

is then dissolved in 0.1 ml of 0.5 M Tris (pH-8.5). The amount of free reduced thiol and addition of IAA to albumin, at the time points mentioned above, were determined using Ellman's reagent (33).

Preparation of N-homocysteinylated albumin

N-homocysteinylated albumin was prepared by incubating HTL with IAA treated albumin (albumin-Cys³⁴-S-IAA). After solubilizing the IAA treated albumin as mentioned above, buffer exchange was carried out with 0.1 M sodium phosphate (pH 7.4) by ultra-filtration through 10 kDa cut-off membrane centricon (Millipore) at 4⁰C. 1 mM of HTL was added to the solution and the reaction mixture was incubated at 37⁰C in a thermo-mixture. Aliquots (0.05 ml) were withdrawn at various time points and precipitated with 0.1 ml of 1.5 M perchloric acid as mentioned above, under this condition only lysine bound HTL, i.e. IAA-S-Cys³⁴-Albumin-Lys-N-Hcy-SH could be formed. However, HTL in the solution could potentially hydrolyse to homocysteine which in turn could bind to the free thiol of homocysteine attached to the lysine residue of albumin forming IAA-S-Cys³⁴-Albumin-Lys-N-Hcy-S-S-Hcy. Thus, to determine the actual amount of homocysteine bound directly to albumin lysine residue, we first solubilized the perchloric acid precipitate and added 0.035 ml sodium borohydride to reduce any homocysteine that may be bound to albumin-lysine bound homocysteine and the total amount of lysine bound

homocysteine [albumin-Lys-N-Hcy-SH-(albumin-Cys³⁴-S-IAA)] (product 1 and 2 in the reaction 2) was determined by using Ellman's reagent (33).

Mass spectrometric analysis of S/N-homocysteinylation albumin tryptic peptides

S-homocysteinylation Albumin peptide

To confirm S-homocysteinylation during HTL binding to albumin, high resolution electrospray ionization (ESI) mass spectrometry (MS) (QTOF 5600, AB Sciex) was used. For this 750 μ M native albumin, and HTL treated albumin-Cys³⁴-SH was digested using trypsin (enzyme:substrate, 1:25) overnight at 37°C. Then, 10 μ l of tryptic peptides were loaded onto an Eksigent C18 nanoLC Trap column (350 μ m $\times$ 0.5 mm) and desalted at a flow rate of 3 μ l/min for 40 mins. The peptides were then resolved on an analytical column (Eksigent C18 nanoLC column 0.075 $\times$ 150 mm) with a multistep linear gradient of mobile phase A (100% water, 0.1% formic acid) and mobile phase B (100% ACN, 0.1% formic acid) at a flow rate of 300 nL/min. The gradient started at 5% buffer B for 0.1 min, with linear increase up to 30% B at 40 min, 50% B at 50 min and 90% B at 51 min. The gradient was maintained at 90% B for 8 min, before re-equilibration to 5% B for 2 min. QTOF 5600 (AB Sciex) was operated in information dependent acquisition (IDA) mode with a TOF/MS survey scan (m/z 350–1250) and an accumulation time of 200 millisecond (ms). The MS settings were as follows: Ionspray voltage floating: 2400 V, curtain gas: 25, ion source gas 1: 25, interface heater temperature: 130, and declustering potential: 80 V. Eighteen precursor ions per cycle were selected for fragmentation, and each

MS/MS spectrum was accumulated for 100 ms (m/z 100–1800) with a total cycle time of approximately 2.05 seconds.

Data Processing and Data Analysis

Data in the constituted MS and MS/MS spectra scan was obtained from QTOF 5600 in the form of .wiff files. These .wiff files from each experiment were submitted to ProteinPilot™ software (v.4.5 AB Sciex, Foster City, CA) to identify peptides of human serum albumin. The search parameters were as follows: trypsin as the digestion enzyme with two missed cleavages. The theoretically predicted peptide's intact mass from the protein pilot was matched to the mass of the experimentally identified peptide. LC-ESI-MS data, MS/MS spectral data (.wiff files) were imported in Peak View (v. 2.1 AB Sciex, Foster City, CA) for identification of S-homocysteinylation manually. In case of S-homocysteinylation, mass of homocysteine ($C_4H_9NO_2S$, 135 Da) was added, i.e., $\Delta M = +133$ Da to protein, for identification of homocysteine in the tryptic peptide.

The MS/MS data of HTL modified peptides were analyzed manually based on the theoretically calculated m/z values of peptide fragment ions obtained from the standalone software, Peptide Fragment Ion Analyzer (34). Mass of homocysteine (+ 133 Da) was added to the b and y ions of the unmodified tryptic peptide(s) and such theoretically calculated modified m/z values of b and y ions were then matched with the experimentally detected ESI MS/MS spectra of the tryptic peptide(s).

RESULTS AND DISCUSSION

HTL hydrolysis leads to formation of Albumin bound homocysteine through disulfide (-S-S-) linkage (S-homocysteinylation)

Homocysteinylation of proteins is gaining attention, since it is increasingly being recognized that the binding of homocysteine to proteins could potentially alter the structure and/or the function of the proteins. We had earlier shown that the binding of homocysteine to fibronectin potentially alters its ability to bind fibrin (35). Similarly there are other reports that clearly points to the modulation of protein function on homocysteinylation (36). Using molecular dynamic simulation, we have recently shown that homocysteine could also potentially alter the structure of proteins granzyme B and junctional adhesion molecule 1(36, 37). Binding of homocysteine to proteins via thiol disulfide exchange reaction has been documented (24). However, with the discovery that homocysteine could exist in its thiolactone form led to the hypothesis of protein-lysine-N-homocysteinylation which was later elegantly demonstrated by Jakubowski and his colleagues. The concentration of HTL in plasma was found to be about 1.4% of plasma total homocysteine (28). It was also reported that incubation of radiolabeled HTL with human serum resulted in the disappearance of thiolactone, which was 25 fold faster than the rate of non-enzymatic hydrolysis of thiolactone (28). Further, it was shown that incubation of HTL with

serum resulted in at least 50% of binding to be resistant to DTT indicating they were not linked to protein via disulfide linkage.

It has been more than a decade that the mechanisms of protein N-homocysteinylation have been proposed. Homocysteine thiolactone binds to ϵ -amino group of protein-lysine residues forming protein-Lys-N-homocysteine. It has also been proposed that during albumin-N-homocysteinylation, cysteine attached to Cys³⁴ of albumin binds to homocysteine attached to the lysine residue thereby forming a mixed disulfide at this lysine residue. However, the concentration of protein N-linked homocysteine is still a matter of debate, since the concentration of HTL is very low compared to homocysteine. Further, it is known that HTL is relatively unstable and can be hydrolysed to homocysteine. It is therefore surprising to note that most of the studies done to estimate the concentration of Protein N-linked homocysteine formed by HTL, did not assess the extent of S-homocysteinylation, although HTL could be hydrolyzed to homocysteine (22, 23, 28, 38-41). This led us to revisit the mechanism of binding of HTL to albumin.

It is known that commercially available HSA contains thiols bound to Cys³⁴ and hence, we initially estimated the amounts of cysteine and homocysteine that is bound to Cys³⁴ of albumin. The albumin, used in this study contained 0.32 mol of bound cysteine (albumin-Cys³⁴-S-S-Cys)/mol of protein and ~0.019 of bound homocysteine (albumin-Cys³⁴-S-S-Hcy)/mol of protein and is in agreement with previous reports (25). To ascertain, if hydrolysis of HTL results in the formation of albumin-Cys³⁴ bound homocysteine (S-homocysteinylation) in addition to albumin-

Lysine-N-homocysteinylation, we incubated 1 mM HTL with 0.75 mM HSA in 0.1 M phosphate buffer (pH 7.4). Incubation of HTL with albumin resulted in the formation of albumin-Cys³⁴S-S-homocysteine (S-homocysteinylation). The formation of albumin-Cys³⁴S-S-homocysteine increased with time and after 24 hours of reaction 0.41 mol of albumin-Cys³⁴S-S-homocysteine per mol of albumin was formed (Figure 1A). This was lower than albumin-Cys³⁴S-S-homocysteine formed during the incubation of 1 mM homocysteine with 0.75 mM albumin (Figure 1A). After 3 h of reaction, approximately 0.34 mol of albumin-Cys³⁴S-S-homocysteine (mol/mol albumin) was formed, when 1 mM of HTL was used, compared to 0.14 mol homocysteine/mol albumin formed during incubation of 1 mM homocysteine with 0.75 mM albumin. This is expected, since the concentration of homocysteine in solution available for S-homocysteinylation will be lower when 1 mM HTL is used. As mentioned earlier, in the first step of S-homocysteinylation, homocysteine binds to the cysteine attached to Cys³⁴ forming homocysteine-cysteine mixed disulfide and albumin thiolate anion leading to a time dependent decrease in the concentration of cysteine bound to albumin-Cys³⁴. Thus, if albumin-Cys³⁴S-S-homocysteine is formed during the reaction of HTL with albumin, we would expect similar decrease in the cysteine concentration which was indeed observed (Figure 2B). Addition of 1 mM HTL to 0.75 mM albumin, decreased the concentration of albumin bound cysteine (albumin-Cys³⁴-S-S-Cys) from 0.33 mol cysteine/mol albumin to 0.21 mol cysteine/mol albumin, after 2 h of reaction (Figure 1B).

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Apart from albumin, there are several other proteins in plasma that could potentially bind to homocysteine and/or HTL. To ascertain, if binding of HTL with plasma also leads to S-homocysteinylation, we incubated 1 mM HTL with plasma and determined the extent of protein-S-homocysteinylation. The formation of protein-S- homocysteine in plasma increased from 0.04 mol/mol of protein to 0.13 mol/mol of protein after an hour of reaction and equilibrium binding was achieved after 10 h of incubation (Figure 2A). At the same time, the amount of plasma protein bound cysteine decreased. Addition of 1 mM HTL reduced the protein bound cysteine concentration from 0.35 mol/mol of protein to 0.05 mol/mol of protein after an hour of incubation. After this time point, the concentration of bound cysteine began to increase slightly until it reached equilibrium at 16 h, where the final concentration of protein bound cysteine was 1.4 mol/mol of protein (Figure 2B). HTL treated with human plasma protein revealed that the reaction was biphasic in nature; corroborating with the earlier study of homocysteine binding to human plasma protein (31). At the start of the reaction, there was a slow formation of protein-Cys³⁴-S-S-Hcy and after 1 h the formation of protein-Cys-S-S-Hcy increased rapidly till 16 h, where it reached its equilibrium binding and then saturated.

All these results indicate that, homocysteine formed due to the hydrolysis of HTL, binds to the cysteine attached to Cys³⁴ in the first step forming albumin thiolate anion and homocysteine-cysteine mixed disulfide. Albumin thiolate anion thus formed in the first step then reacts with

homocysteine-cysteine mixed disulfide and preferentially binds to homocysteine as has been shown earlier (25).

To further confirm that homocysteine binds to Cys³⁴ of albumin, during the reaction of HTL with albumin, we performed mass spectral analysis. For this, native albumin or HTL treated albumin was digested with trypsin in 10 mM ammonium bicarbonate buffer (enzyme substrate ratio, 1:25). The albumin bound fraction (16 or 24 h HTL treatment) was collected as mentioned above (see Materials and methods) and was digested with trypsin without reduction with DTT. In native albumin, the tryptic peptide [21-41] “ALVLIAFAQYLQQCPFEDHVK” (Uniprot KB ID: P02768, with a calculated monoisotopic mass: 2432.25 Da) contained unmodified Cys³⁴. Binding of cysteine or homocysteine to Cys³⁴ of this peptide would increase its mass to 2551.26 Da or 2565.276 Da, respectively (Table 1).

In mass spectrum of this tryptic peptide obtained from native albumin, we observed peaks at m/z 609.07 ($z = +4$) and 811.7 ($z = +3$) eluted at 48.167 min corresponding to native albumin (figure 3 A & table 1, MS/MS spectra is shown in figure 3B and 3C) and m/z 638.82 ($z = +4$), which corresponds to modification of Cys³⁴ by cysteine eluted at 47.582 min (figure 4A & table 1, MS/MS spectra is shown in figure 4B). The change in retention time is observed for native peak and cysteine modified peak, which may be due to modification. However, homocysteine modified peptide [21-41] was not observed in the native albumin. Incubation of albumin with

HTL for 16 hours resulted in the appearance of peaks at m/z 642.33 ($z = +4$) that corresponds to homocysteine modified tryptic peptide, indicating that incubation of albumin with HTL results in the formation of S-homocysteinylated albumin. This mass spectrum also contained the peak at m/z 638.82, which is due to albumin-Cys³⁴-S-S-Cys (Figure 4A and C & Table 1). The site-specific modification of the tryptic peptide “ALVLIAFAQYLQQCPFEDHVK”, was confirmed from its LC-ESI- MS/MS spectrum of at RT: 47.61 min., which was acquired from QTOF 5600 (Figure 5). Theoretically calculated m/z values of b and y ions from homocysteine modified tryptic peptide were compared with experimentally detected fragment ions’ m/z values and accordingly peaks were assigned (Figure 5A, MS/MS spectra is show in figure 5B-C). Thus, S-homocysteinylation of albumin-Cys³⁴ can be confirmed, which is evident particularly from the peaks of y - ion series: y_1 - y_{13} , whose m/z values clearly reveal the mass increment due to homocysteine modification of Cys³⁴.

Cys³⁴-SH and N-homocysteinylation of albumin

It has been reported earlier that HTL binds to albumin at the ϵ -amino group of lysine leading to the formation of albumin-Lys-N-Hcy-SH. Further, it was reported that there is a cross talk between cysteine attached to Cys³⁴ of albumin and the homocysteine bound to the primary amine of lysine residues (23). This resulted in the formation of Albumin-Lys-N-Hcy-S-S-Cys, where the cysteine bound to homocysteine was believed to be the one attached to Cys³⁴. However, from our results, it is clear that a large proportion of HTL is hydrolyzed to homocysteine, which

causes S-homocysteinylation, whereby homocysteine binds to cysteine attached to Cys³⁴ in the first step forming albumin thiolate anion and homocysteine-cysteine mixed disulfide. In the second step, albumin thiolate anion so formed preferentially binds to homocysteine of the mixed disulfide forming albumin-Cys³⁴-S-S-Hcy and cysteine thiolate anion. We hypothesize that this free cysteine thiolate anion binds to homocysteine attached to ϵ -amino group of lysine and there is no cross talk between Cys³⁴ and lysine residues.

To test this and to determine the amount of N-homocysteinylation of albumin in the presence of HTL, albumin thiolate anion (albumin-Cys³⁴-S⁻), formed after reduction of the thiols attached to Cys³⁴ using DTT, was blocked by IAA. Under these circumstances, if there is a cross talk between Cys³⁴ and lysine residues as reported earlier, then, it is expected that homocysteine attached to lysine would remain in reduced form (-SH). Addition of 1 mM DTT to 1 mM albumin for 1 h. completely reduced all the thiols attached to Cys³⁴ as determined by Ellman's reaction (Figure 6A). The albumin thiolate anion (free reduced thiol) was completely blocked using 55 mM IAA within 15 minutes (Figure 6B; see Methods section). DTT/IAA treated albumin (albumin-Cys³⁴-S-IAA) was then treated with 1 mM HTL to determine the amount of N-homocysteinylation and to see, if there is any cross talk between Cys³⁴ and homocysteine bound to lysine (Schematic reaction 2).

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In this case, HTL can undergo only N-homocysteinylation, since albumin-Cys³⁴-S is blocked, thereby preventing S-homocysteinylation. Further, the hypothesis that cysteine bound to Cys³⁴ cross reacts with homocysteine bound to lysine cannot be operative here, as in this case Cys³⁴ is blocked and it is expected that homocysteine bound to lysine will be in the reduced form (see Scheme 2, shown above). Addition of HTL to the DTT/IAA treated albumin resulted in formation of only 13 μ M (0.017 moles) of homocysteine/mole of albumin after 16 hours of reaction as determined by Ellman's reaction (Figure 7A).

Interestingly, after 3 h of reaction with HTL no free thiol could be detected indicating that homocysteine attached to lysine was disulfide bound and no longer existed in the free reduced form. This could only be possible if thiolate anion of homocysteine (Hcy-SH), which was hydrolyzed from HTL, reacted with the lysine bound homocysteine forming albumin-Lys-N-Hcy-S-S-Hcy [(N-linked homocysteine bound S-homocysteinyl albumin moiety), (reaction 2)]. To investigate this, DTT/IAA treated albumin was incubated with 1 mM HTL and 50 μ l aliquots were added to perchloric acid at different time intervals. The precipitated albumin was washed and then solubilized as mentioned in Materials and methods. The albumin solution was then treated with sodium borohydride to reduce any homocysteine bound to lysine bound homocysteine. Sodium borohydride treated albumin was further precipitated, washed and re-dissolved and the amount of reduced homocysteine bound to lysine was determined using Ellman's reagent (DTNB). After borohydride reduction we found that about 0.15 moles of

homocysteine was bound per mole of albumin (Figure 7B) clearly indicating that homocysteine formed due to hydrolysis of HTL at physiological pH binds to the homocysteine that is bound to lysine. This clearly contradicts the hypothesis that cysteine or homocysteine bound to Cys³⁴ cross talks with homocysteine bound to lysine.

CONCLUSION

Our study, thus, clearly reveals that S-homocysteinylation is the major modification that takes places in circulation during the reaction of HTL with plasma proteins and N-homocysteinylation is only a minor component. And also our study suggests that Hcy is in a very large excess of HTL in plasma but it questions the utility of physiological relevance of HTL in terms of human diseases such as cardiovascular disease.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

YS, TB and SS conceived and developed the methodology. YS performed experiments, data curation formal analysis and writing the original draft. SV and TB performed data curation, validation and writing, review editing of the manuscript. YS, AR and SV helped in visualization

and writing, review editing of the manuscript. AZ, CC and SS conceptualized the project. SS supervised the project and editing of the manuscript.

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Tables and Figures legend:

Table 1: Theoretically calculated intact molecular mass and m/z of multiply protonated molecular ions that can be anticipated in the ESI mass spectrum corresponding to unmodified, cysteine modified and homocysteine modified peptide: ALVLIAFAQYLQQCPFEDHVK, which is [21-41] of HSA (Uniprot KB ID: P02768).

Figure 1: Binding of HTL with human serum albumin through disulfide (-S-S-) linkage. A) Formation of protein bound homocysteine (A) and decrease in bound cysteine (B) during incubation of 1 mM HTL with human serum albumin (0.75 mM) in 0.1 M sodium phosphate buffer at physiological pH (pH-7.4). All the experiments were performed in triplicates.

Figure 2: Binding of HTL with plasma protein through disulfide (-S-S-) linkage. A) Formation of protein bound homocysteine B) amount of bound cysteine during incubation of 1 mM HTL with human Plasma (1ml diluted in 1:1 in buffer). All the experiments were done in triplicates.

Figure 3: (A)LC-ESI-MS Spectra of tryptic peptide of native HSA (750 μ M) is shown . The LC-ESI-MS spectra of native albumin peaks (609.07 and 811.7 Da) is shown (B) And (C) LC-ESI-MS/MS spectrum of precursor ion 609.07 ($z=4$) and 811.7 ($z=3$) of native albumin peptide with b and y ions is shown. b and y ions that matched with the theoretically generated list of

peptides are shown in green. (D) LC-ESI-MS spectrum of bound cysteine (Albumin-Cys³⁴-S-S-Cys) (638.8 Da, z=4) precursor ion were represented with black arrow. (E) LC-ESI-MS/MS spectrum of precursor ion m/z 638.8 with b and y ions is shown. b and y ions that matched with the theoretically generated list of peptides are shown in green.

Figure 4: A) LC-ESI- MS Spectra of tryptic peptide of 16 h HTL (1 mM) treated HSA) is shown. The LC-ESI-MS spectra of bound cysteine (Albumin-Cys³⁴-S-S-Cys, 638.8 Da) and bound homocysteine (Albumin-Cys³⁴-S-S-Hcy, 642.3 Da) peptide mass were represented with black arrow respectively. B) LC-ESI-MS/MS spectrum of precursor ion m/z 638.8, which is $[M+4H]^{4+}$ of Cys modified peptide (i.e Albumin-Cys³⁴-S-S-Cys) with b and y ions is shown. b and y ions that matched with the theoretically generated list of peptides are shown in green. C) LC-ESI-MS/MS spectrum of precursor ion m/z 642.3, which is $[M+4H]^{4+}$ of Hcy modified peptide (i.e., Albumin-Cys³⁴-S-S-Hcy) peptide with b and y ions is shown. b and y ions that matched with the theoretically generated list of peptides are shown in green.

Figure 5: Amount of free reduced thiol (albumin-Cys³⁴-SH) when 0.75 mM albumin was treated with 1 mM DTT at different time point, B) Free reduced thiols were completely blocked by 55 mM IAA and No free thiols were detected after 45 mins of incubation.

Figure 6: N-homocysteinylation of albumin-Cys³⁴-S-IAA. A) Lysine bound homocysteine mixed disulfide (Albumin-NH-Hcy-S-S-Hcy), B) Lysine bound homocysteine (Albumin-NH-Hcy-SH) during incubation of 1 mM HTL with DTT/IAA treated human serum albumin.

21 ALVLIAFAQYLQQCPFEDHVK 41				
	Intact	m/z (calculated)		
	Molecular Mass (calculated)	$[M+4H]^{4+}$ z = +4	$[M+3H]^{3+}$ z = +3	$[M+2H]^{2+}$ z = +2
Unmodified	2432.256 Da	609.072	811.760	1217.136
Cysteine modified	2551.260 Da	638.823	851.428	1276.638
Homocysteine modified	2565.276 Da	642.327	856.099	1283.646

21 ALVLIAFAQYLQQCPFEDHVK 41				
	Intact	m/z (calculated)		
	Molecular Mass (calculated)	$[M+4H]^{4+}$ z = +4	$[M+3H]^{3+}$ z = +3	$[M+2H]^{2+}$ z = +2
Unmodified	2432.256 Da	609.072	811.760	1217.136
Cysteine modified	2551.260 Da	638.823	851.428	1276.638
Homocysteine modified	2565.276 Da	642.327	856.099	1283.646

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