Amino Acids (2014) 46:429–439
DOI 10.1007/s00726-013-1636-4

ORIGINAL ARTICLE

The control of hyperhomocysteinemia through thiol exchange mechanisms by mesna

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Received: 10 October 2013 / Accepted: 3 December 2013 / Published online: 14 December 2013

Abstract In hyperhomocysteinemic patients, after reaction with homocysteine-albumin mixed disulfides (HSS-ALB), mesna (MSH) forms the mixed disulfide with Hcy (HSSM) which can be removed by renal clearance, thus reducing the plasma concentration of total homocysteine (tHcy). In order to assess the HSS-ALB dethiolation via thiol exchange reactions, the distribution of redox species of cysteine, cysteinylglycine, homocysteine and glutathione was investigated in the plasma of healthy subjects: (i) in vitro, after addition of 35 μM reduced homocysteine (HSH) to plasma for 72 h, followed by MSH addition (at the concentration range 10–600 μM) for 25 min; (ii) in vivo, after oral treatment with methionine (methionine, 200 mg/kg body weight, observation time 2–6 h). In both experiments the distribution of redox species, but not the total amount of each thiol, was modified by thiol exchange reactions of albumin and cystine, with changes thermodynamically related to the pKa values of thiols in the corresponding mixed disulfides. MSH provoked a dose–response reversal of the redox state of aged plasma, and the thiol action was confirmed by in vivo experiments. Since it was observed that the dimesna production could be detrimental for the in vivo optimization of HSSM formation, we assume that the best plasma tHcy lowering can be obtained at MSH doses producing the minimum dimesna concentration in each individual.

D. Di Giuseppe and R. Priora equal experimental contribution.

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Keywords Homocysteine · Mesna · Thiol exchange reactions · Plasma thiols · p*K*a of thiols

Abbreviations

Cys	Cysteine
CysGly	Cysteinylglycine
CSH	Reduced Cys
CSSC	Cystine
CSS-ALB	Cysteine-albumin mixed disulfide
CGSH	Reduced CysGly
CGSS	CysGly disulfide
CGSS-ALB	Cysteinylglycine-albumin mixed disulfide
DTNB	5,5′-Dithiobis(2-nitrobenzoic acid)
CSSH	Cysteine-homocysteine mixed disulfide
Hcy	Homocysteine
HSH	Reduced Hcy
HSSH	Homocystine
HSS-ALB	Homocysteine-albumin mixed disulfide
GSH	Reduced glutathione
GSSG	Glutathione disulfide
GSS-ALB	Glutathione-albumin mixed disulfide
MLT	Methionine loading test
MSH	Mesna
MSSM	Dimesna
MSS-ALB	Mesna-albumin mixed disulfide
NEM	*N*-ethylmaleimide
PSH	Protein SH groups
TSH	Total plasma thiols, sum of PSH and XSH
XSS-ALB	Thiol-albumin mixed disulfides
tCSH, tGGSH, tHSH, tGSH	Sum of concentrations of redox species expressed as reduced equivalents of XSH, namely XSSX = 2XSH

XSH and XSSX	Thiols and disulfides of low molecular weight

Introduction

Despite the scarce understanding of homocysteine (Hcy) toxicity, hyperhomocysteinemia is considered a risk of pathological progression of important diseases, such as cardiovascular diseases, stroke, venous thrombo-embolism, dementia and multiple sclerosis (Loscalzo 2006; Mansoor et al. 1995; Eikelboom et al. 1999; Quéré et al. 2005; Andersson et al. 2000; Di Giuseppe et al. 2003; Mattson and Shea 2003; Perla-Kajan et al. 2007; Galimberti et al. 2008; Vrethem et al. 2003; Di Giuseppe et al. 2010).

The plasma increases in total Hcy (tHcy), sum of reduced Hcy (HSH), homocystine (HSSH) and homocysteine-albumin mixed disulfide (HSS-ALB), are prevented by different strategies, for example through appropriate diets or supplementation of class B vitamins, as well as through the administration of antioxidant thiol drugs, such as mesna (MSH) or *N*-acetylcysteine (NAC).

MSH and NAC control the tHcy growth by decreasing HSS-ALB, the Hcy most concentrated redox species. HSH, formed by methionine through cellular transmethylation processes, is recycled back to methionine or metabolized to reduced cysteine (CSH) through transulfuration pathways. However, possible HSH excesses, exported into plasma, react with disulfides by thiol exchange reactions. In particular HSH exchanges with cysteine-albumin mixed disulfides (CSS-ALB) and cystine (CSSC) forming CSH, cysteine-homocysteine mixed disulfide (CSSH), and HSSH as follows:

$$\mathrm{HSH} + \mathrm{CSS\text{-}ALB} \leftrightarrow \mathrm{HSSC} + \mathrm{HS\text{-}ALB} \quad (1)$$

$$\mathrm{CSSC} + \mathrm{HSH} \leftrightarrow \mathrm{CSSH} + \mathrm{CSH} \quad (2)$$

$$\mathrm{CSSC} + \mathrm{HSH} \leftrightarrow \mathrm{CSSH} + \mathrm{CSH}. \quad (3)$$

HSSH is in turn involved in other reactions and, for example, is prone to react with reduced albumin (HS-ALB), much better than other disulfides (XSSX) do, to form *S*-thiolated albumin (HSS-ALB):

$$\mathrm{HSSH} + \mathrm{HS\text{-}ALB} \leftrightarrow \mathrm{HSS\text{-}ALB} + \mathrm{HSH}. \quad (4)$$

Due to these equilibria, reduced thiol (XSH) of low molecular weight is rapidly metabolized to disulfide (XSSX) and mixed disulfide with albumin (XSS-ALB). In addition to albumin other proteins, such as gamma-globulin, can form mixed disulfides with endogenous thiols (Jakubowski 2002; Hortin et al. 2006) and in the case of Hcy the possibility to generate *N*-omocysteinylated proteins with albumin and other plasma proteins has well been documented (Jakubowski 2002). However, the contribution given by albumin (and cystine) for a rapid remodeling of redox species of plasma thiols by thiol exchange reactions seems to be predominant with respect to other proteins. The consequence of the albumin action is that endogenous plasma thiols exhibit a subdivision in redox species, by which disulfides (XSSX and XSS-ALB) prevail over XSH and that each compound has characteristic percentages of redox species that are rather constant over age (Di Giuseppe et al. 2004). For example, tHcy is represented for more than 95 % by HSS-ALB, by HSSH for about 4–5 %, and by HSH for <1 %, whereas the redox species percentages of Cys are greatly different, being CSS-ALB about 60 %, CSSC 30 % and CSH 8–10 % of tCys.

Thiol exchange reactions respond to thermodynamic criteria regarding the p*K*a values of thiols of mixed disulfides, and the p*K*a value is an important parameter that influences the redox species subdivision. The leaving groups of exchange reactions are thiols that have the lowest p*K*a value in the mixed disulfides (Summa et al. 2007). Thus albumin (HS-ALB) of CSS-ALB, see reaction (1) is a prone leaving group since the only free reactive cysteinyl residue (Cys34) has p*K*a value of about 5–7 that is much lower than other plasma thiols (Summa et al. 2007). CSH, whose p*K*a is about 8.0, behaves like albumin and is a leaving group when CSH is bound with thiols of higher p*K*a values in mixed disulfides, such as, for example, GSH and HSH, whose p*K*a are equal or higher than 9.0.

MSH is an antioxidant that prevents collateral damages from anti-blastic chemotherapies or from free radicals. Its clinical utilization provoked an interesting effect regarding the lowering of plasma levels of tCys and tHcy (Jakupec et al. 2004; Klastersky 2003; Lauterburg et al. 1994; Pendyala et al. 2000; Smith et al. 2003; Stofer-Vogel et al. 2003), and the consequence of this observation was the positive MSH utilization in hyperhomocysteinemic patients with end-stage renal disease (Urquhart et al. 2006, 2007). By the fact, MSH exhibited a plasma tHcy reduction mediated by the excretion of homocysteine-mesna mixed disulfide (HSSM):

$$\mathrm{MSH} + \mathrm{HSS\text{-}ALB} \leftrightarrow \mathrm{HSSM} + \mathrm{ALB\text{-}SH}. \quad (5)$$

This clinical efficacy, however, was not successively confirmed (Cutler et al. 2009), and the molecular explanation of the MSH failure was not provided.

In the present study, assuming that undesired activations of thiol exchange reactions may have a role in a possible reduction of MSH effects, we performed in vitro and in vivo experiments to verify the veracity of this assumption and to understand to what extent the plasma metabolism of redox forms of Cys, cysteinylglycine (CysGly), Hcy and glutathione was influenced by reduced thiols. In particular we investigated: (i) how changes in XSH (HSH

and MSH) could influence the redox species subdivision of plasma thiols; and (ii) whether increasing plasma concentrations of MSH could elicit mechanisms that, in contrast with reaction (5), would cause a minor HSSM production.

The redox species of endogenous thiols and mesna were investigated over time after in vitro treatment of plasma of healthy subjects with HSH (35 μM, final concentration) and a period of plasma aging of 72 h, followed by a successive treatment with increasing MSH concentrations (concentration range: 10–600 μM) for 25 min. This investigation was repeated after oral treatment with methionine of healthy volunteers, using the methionine loading test (MLT) and observation times at 2–6 h.

Our results suggest that the dimesna (MSSM) formation during the MSH treatment could be the cause of decreased production of HSSM. A possible advantage for enhancing the beneficial effects of reduction of plasma tHcy in hyperhomocysteinemic patients could be to administer the minimum dose of MSH which produces the minimum concentration of plasma MSSM.

Materials and methods

Chemicals

N-ethylmaleimide (NEM), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), mesna (sodium 2-mercaptoethanesulfonate) and all other reagents of analytical grade were purchased from Sigma Chemical Co (St. Louis, MO, USA). HPLC grade reagents were from Merck (Darmstadt, Germany). Monobromobimane (mBrB) was purchased from Calbiochem (La Jolla, CA, USA).

Study design

The study was carried out in accordance with the principles of the Declaration of Helsinki. Before entering the study, each subject gave informed consent. The design of the study conformed to current standards in Italy.

Different subjects were enrolled in the in vitro study and in the in vivo MLT study and each participant declared not to have taken any drug in the last 30 days.

In vitro study

In vitro treatment of plasma with HSH: subjects, sampling and blood processing

Eight healthy subjects (four females and four males, mean age 36 ± 10) were included in the present study. Blood was collected into plastic tubes containing ethylenediamine tetraacetic acid tripotassium salt (K_3EDTA) by venipuncture and immediately centrifuged (1,200*g* for 8 min at 4 °C). Plasma samples were collected and divided into four parts for the: (1) measurement of protein SH groups; (2) measurement of reduced thiols; (3) measurement of disulfides and protein-thiol mixed disulfides; (4) treatment with homocysteine (35 μM, final concentration) to induce an in vitro hyperhomocysteinemia condition and stored at 4 °C for 72 h. The thiol redox forms of the last samples (in vitro hyperhomocysteinemia condition) were assayed at different times and at the end the plasma was treated with mesna and assayed as subsequently described.

In vitro treatment of plasma with mesna

Increasing mesna concentrations (diluted into 5 mM K_3EDTA, pH 7) were added to hyperhomocysteinemic plasma at the final concentration of 0, 10, 150 and 600 μM and incubated at 37 °C for 0, 5, 10 and 25 min. The plasma was processed and the redox species (reduced, oxidized and bound to proteins as mixed disulfides) were assayed.

In vivo study

The methionine loading test (MLT)

Nine healthy subjects (mean age 38.6 ± 12.1, five males, and four females, mean weight 69.9 ± 10.4 kg) were enrolled in the study. All subjects were weighed in light clothing and L-methionine (100 mg/kg body weight dissolved in 250 ml of orange juice) was administered orally after overnight fasting. At different time points (2, 4 and 6 h) after methionine overload, blood samples were collected by venipuncture into plastic tubes containing K_3EDTA and immediately centrifuged (1,200*g* for 8 min at 4 °C) to obtain plasma and thiols redox species (reduced, oxidized and bound to proteins as mixed disulfides) were measured.

Analysis of protein SH groups, thiols, disulfides and thiol-protein mixed disulfides

Total plasma thiols (TSH)

Plasma samples from in vitro and in vivo study were maintained at 0 °C until they were used to assay the total thiol (TSH) concentration according to the Ellman's method (Ellman 1959) using the DTNB reagent. In plasma, TSH represents the sum of PSH and thiols of low molecular weight. In its turn, PSH is essentially represented by HS-ALB since the concentrations of other PSH are negligible (see Tables 3, 4). The TSH assay by DTNB was used to calculate the PSH concentrations (namely HS-ALB) after the subtraction from TSH of the sum of low molecular

weight reduced thiols (namely, CSH, CGSH, HSH, and GSH) assayed by HPLC.

50 μL of plasma was put into cuvette containing 0.950 μL of phosphate buffer 0.1 M (pH 7.4, containing 1 mM K_3EDTA). The reaction was started by adding 0.020 μL of DTNB and the reaction was stopped after 15 min. All TSH measurements were made at 37 °C, using a Jasco (Tokyo, Japan) UV-VS V550 spectrophotometer.

Thiols, disulfides and thiol-protein mixed disulfides

Thiols, disulfides and protein-thiol mixed disulfides were assayed according to Mansoor et al. (1992) with little modifications as reported in Di Giuseppe et al. (2010) and analyzed through high performance liquid chromatography (HPLC).

Reduced thiols

150 μL of plasma was immediately deproteinized with 6 % (w:v) trichloroacetic acid (TCA, final concentration) and centrifuged at 10,000×*g,* for 2 min at 25 °C. After neutralization of the supernatant with saturating solid $NaHCO_3$, the sample was incubated with 1 mM mBrB (final concentration) in the dark at room temperature for 15 min. After centrifugation (10,000×*g*, 2 min, 25 °C), 90 μL of the supernatant was acidified with 10 μL 37 % HCl (pH 3–4) and injected into the HPLC column (20 μL).

Disulfides

Plasma samples (100 μL) were mixed with 2 mM *N*-ethylmaleimide (NEM) (final concentration) for 3 min at room temperature and then deproteinized and centrifuged as above. The NEM excess in the supernatant was removed by extraction with dichloromethane (0.2 mL sample plus 2.5 mL dichloromethane) and 100 μL of sample was brought to alkaline pH by addition of solid NaHCO3 and treated with 1 mM DTT (dithiothreitol, final concentration) at room temperature for 20 min. After centrifugation (10,000×*g*, 2 min, 25 °C), the supernatant was treated with an excess of mBrB (3 mM, final concentration) and processed as described above.

Thiol-protein mixed disulfides

The protein pellet from NEM-treated plasma samples (see above disulfides) was washed three times with 1 mL of 1.5 % (w:v) TCA, to remove traces of NEM and other compounds. The pellet was resuspended in 0.4 mL of 1 mM K_3EDTA and saturated with solid $NaHCO_3$. Then, 18 μL of 50 mM DTT was added to the resuspension and the sample was maintained under continuous agitation at room temperature for 20 min.

After centrifugation (10,000×*g*, 2 min, 25 °C), 100 μL supernatant was deproteinized with 40 μL 60 % (w/v) TCA and the supernatant was diluted in water (1:3), neutralized with saturating solid $NaHCO_3$, reacted with 3 mM mBrB (final concentration) and processed as described above.

Chromatographic conditions

An HPLC apparatus (Hewlett-Packard 1100 Series) equipped with fluorescence detection and an OmniSpher C-18 reversed-phase column (Varian, Lake Forest, CA) was used.

Mobile phases were 0.25 % (v:v) acetic acid, adjusted to pH 3.09 with 1N of NaOH (A) and methanol (B). Elution profile was as follows: 0–8 min, 20 % B; 8–15 min, 20–40 % B; 15–25 min, 40–100 % B (1.0 mL/min flow rate; fluorimetric detector: excitation, 380 nm; emission, 480 nm). Retention times for CSH, CGSH, HSH, GSH and MSH were 6.0, 6.7, 10.1, 12.2 and 18.2 min, respectively.

The calibration curves of thiols of low molecular weight were linear from 0.3 to 100 μM (3.2 pmol/injection to 1,073 pmol/injection). The detection limit was 2 pmol/injection (injection volume 20 μL).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using the SPSS statistical package (SPSS). Statistical comparisons were carried out using the unpaired Student's *t* test. The designed level of significance was $P < 0.05$.

Results

In vitro study: the redox species subdivision in plasma by HSH exposure and aging

Figure 1a, b, c reports the redox species concentrations of XSH, XSSX and XSS-ALB at $t = 0$ and $t = 72$ h at 4 C° after the plasma treatment with 35 μM HSH.

Except for HSSH, at $t = 0$ from HSH addition (Fig. 1b), no significant changes in XSH, XSSX and XSS-ALB concentrations with respect to control (CTR, fresh plasma) were observed. HSSH was increased to about sixfold, and the change was more plausibly due to activations of thiol exchange reactions, by reactions (2) and (3) and interaction with HSS-ALB, via the reverse of reaction (4), than to simple HSH oxidation.

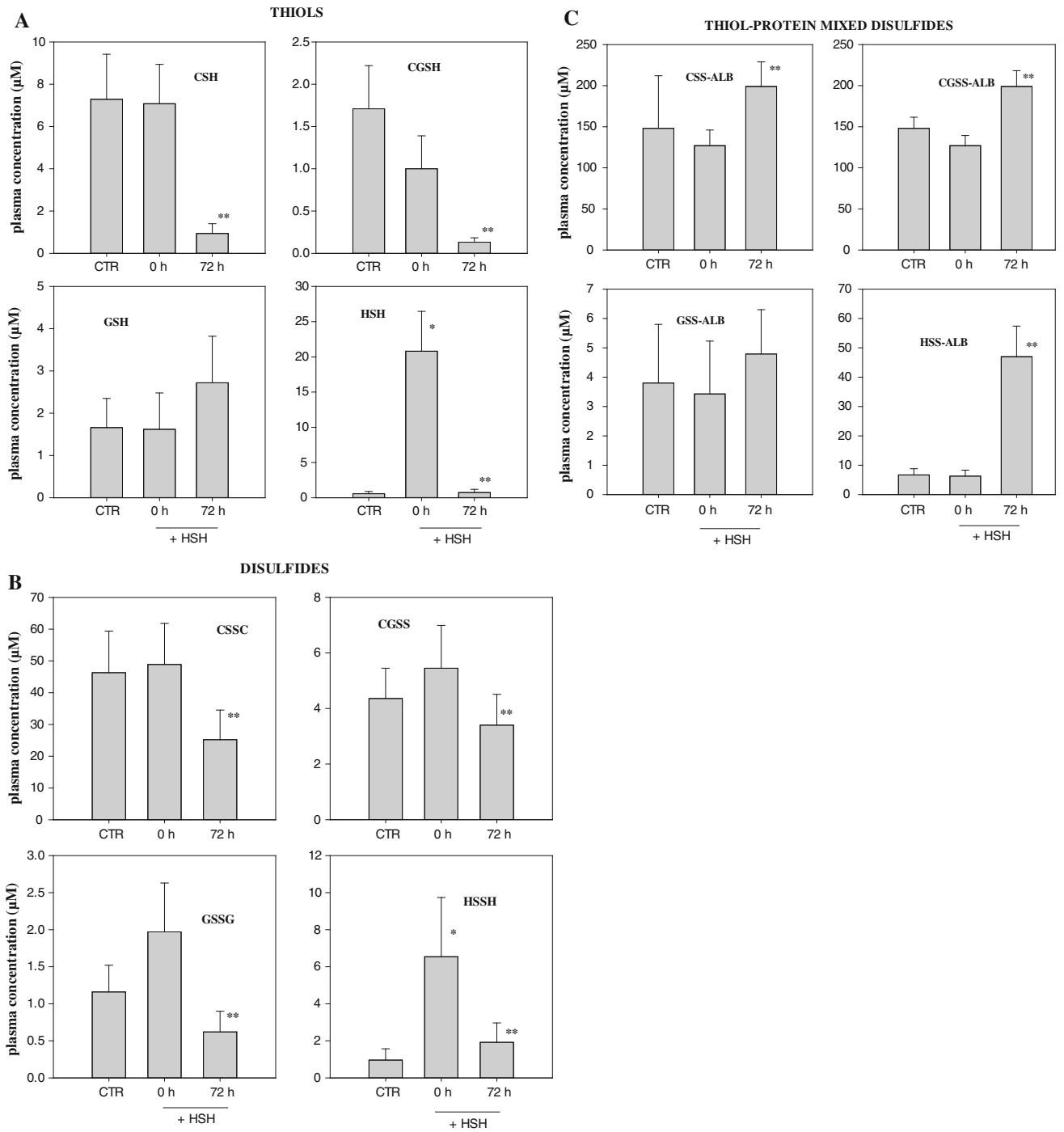

Fig. 1 Time-courses of concentrations of thiol redox species in HSH-treated plasma. XSH, XSSX and XSS-ALB were measured after blood withdrawing in plasma control (CTR). The remaining plasma, treated with 35 µM HSH and maintained at 4 °C, was assayed within the time interval of 0–72 h. **a** XSH, **b** XSSX, **c** XSS-ALB. Mean ± SD of $N = 8$. *$P < 0.05$ with respect to CTR; **$P < 0.05$ with respect to 0 h

With the exception of glutathione, the concentrations of redox species of other thiols gradually changed over time (data not shown), and resulted strongly modified at 72 h with respect to the initial values (0 h and CTR). At 72 h GSH and glutathione-albumin mixed disulfide (GSS-ALB) remained unchanged, whereas GSSG was inexplicably reduced. The GSH and GSSG interactions with albumin, namely with XSS-ALB and HS-ALB, respectively, are disfavored for mass and charge reasons with respect to the other thiols (Summa et al. 2007). Conversely, GSH may easily exchange

with CSSC by reactions (2) and (3) forming GSSG, as previously observed in in vivo and in vitro studies (Priora et al. 2010; Di Giuseppe et al. 2004).

Therefore, the state of moderate hyperhomocysteinemia, carried out by HSH addition to plasma, remodels the concentrations of redox species of thiols with Hcy and glutathione as the most and less susceptible, respectively. As far as Hcy is concerned, the following redox changes are plausible, in agreement with thermodynamic criteria regarding the thiols of mixed disulfides:

$$\text{HSH} + \text{HSS-ALB} \leftrightarrow \text{HSSH} + \text{HS-ALB} \quad (6)$$

$$\text{HSH} + \text{CSSC} \leftrightarrow \text{CSSH} + \text{CSH} \quad (7)$$

$$\text{HSH} + \text{CSS-ALB} \leftrightarrow \text{CSSH} + \text{HS-ALB} \quad (8)$$

$$\text{CSSH} + \text{HSH} \leftrightarrow \text{HSSH} + \text{CSH}, \quad (9)$$

where reactions (7) and (9) are equal to reactions (2) and (3).

In turn, CSH produced by these reactions can dethiolate CSS-ALB, as similarly described by reaction (1).

No oxidation grades higher than disulfides were observed over time because the total concentration of each thiol of low molecular weight at 0 and 72 was substantially unchanged (Table 1). Although thiols and other reductants are usually subjected to plasma oxidations (Gryzunov et al. 2003), in our experiments the potential chemical transformations of thiols to oxidation grades higher than disulfides, namely to sulfinic (XS2OH) or sulfonic acids (XSO3H), would have been negligible or absent, because tXSH of Cys, CysGly, Hcy and glutathione was unchanged over time (Table 1).

The reverse of thiol exchange reactions in aged plasma: The effect of MSH addition

We next investigated how a reducing stimulus of thiols (MSH) was able to alter the redox species distribution of aged plasma. Increasing MSH concentrations were added to 72 h aged HSH-treated plasma within observation times of 25 min. Figure 2a, b, c reports the redox species changes at $t = 0$ after MSH addition, whereas Fig. 3 shows the time-courses of the most susceptible agents, Cys and Hcy.

Table 1 Total concentrations of CSH, CGSH, HSH and GSH at 0 and 72 h after plasma treatment with HSH

HSH exposure	tCSH	tCGSH	tHSH	tGSH
0 h	232	24.2	40.2	8.99
72 h	250	26.1	51.6	8.75

The concentrations, expressed as μM, are the sum of mean values, reported in Fig. 1 of XSH, XSSX and XSS-ALB, where XSSX is calculated as reduced equivalent, e.g. XSSX = 2XSH

Dose-dependent mechanisms of thiol exchange reactions of albumin and CSSC were rapidly activated by MSH. CSH and CGSH concentrations increased at t = 0 with respect to aged plasma (CTR) (Fig. 2a), whereas no significant changes in disulfides (XSSX and XSS-ALB) were observed (Fig. 2b, c).

CSH, HSH and CGSH (not shown) continued to increase over time at increasing MSH addition and at the end of the observation period (25 min) and at the maximum MSH dose, CSH, HSH, CGSH exhibited maximum values (Fig. 3) of about 7–10 fold higher than CTR (aged plasma) and fresh plasma (Fig. 1).

At the same time, XSSX was increased, whereas XSS-ALB was decreased, above and below the levels of aged or fresh plasma, respectively.

In Table 2, the tCSH and tHSH amounts, measured over time at 600 μM MSH, are reported with the corresponding percentages of XSH and XSS-ALB. Whereas tCSH and tHSH remained unchanged over time, XSH and XSS-ALB percentages showed opposite changes (being XSH increasing, and XSS-ALB decreasing). The changes in redox states distribution of MSH were also measured (Table 3) and they suggest the following exchange mechanisms:

$$\text{MSH} + \text{CSS-ALB} \leftrightarrow \text{HS-ALB} + \text{MSSC} \quad (10)$$

$$\text{MSH} + \text{MSSC} \leftrightarrow \text{MSSM} + \text{CSH} \quad (11)$$

$$\text{CSH} + \text{CSS-ALB} \leftrightarrow \text{CSSC} + \text{HS-ALB}. \quad (12)$$

The above (10), (11), (12) reactions justify the regeneration of CSH, HSH, CSSC and HSSH at the expenses of CSS-ALB and HSS-ALB observed in Fig. 3. They also explain the paradox by which a reduced thiol (MSH), added to an oxidant environment, the plasma, at relatively high concentrations, is able to rapidly produce reducing agents (e.g., CSH and HSH).

The dimesna production is a potential sign of reduced MSH effect

Dimesna (MSSM) concentrations increased in a dose-dependent manner at increasing MSH doses (Table 3). In particular, MSSM levels were higher than the mesna-albumin mixed disulfides (MSS-ALB). These data induce to infer the following coupled reactions:

$$\text{MSH} + \text{CSSC} \leftrightarrow \text{MSSC} + \text{CSH} \quad (13)$$

$$\text{MSSC} + \text{MSH} \leftrightarrow \text{MSSM} + \text{CSH}, \quad (14)$$

in agreement with the notion that CSH of MSSC would be a good leaving group [reaction (14)], because the CSH p*K*a value is lower than that of MSH (MSH, p*K*a = 9.0) (Urquhart et al. 2006).

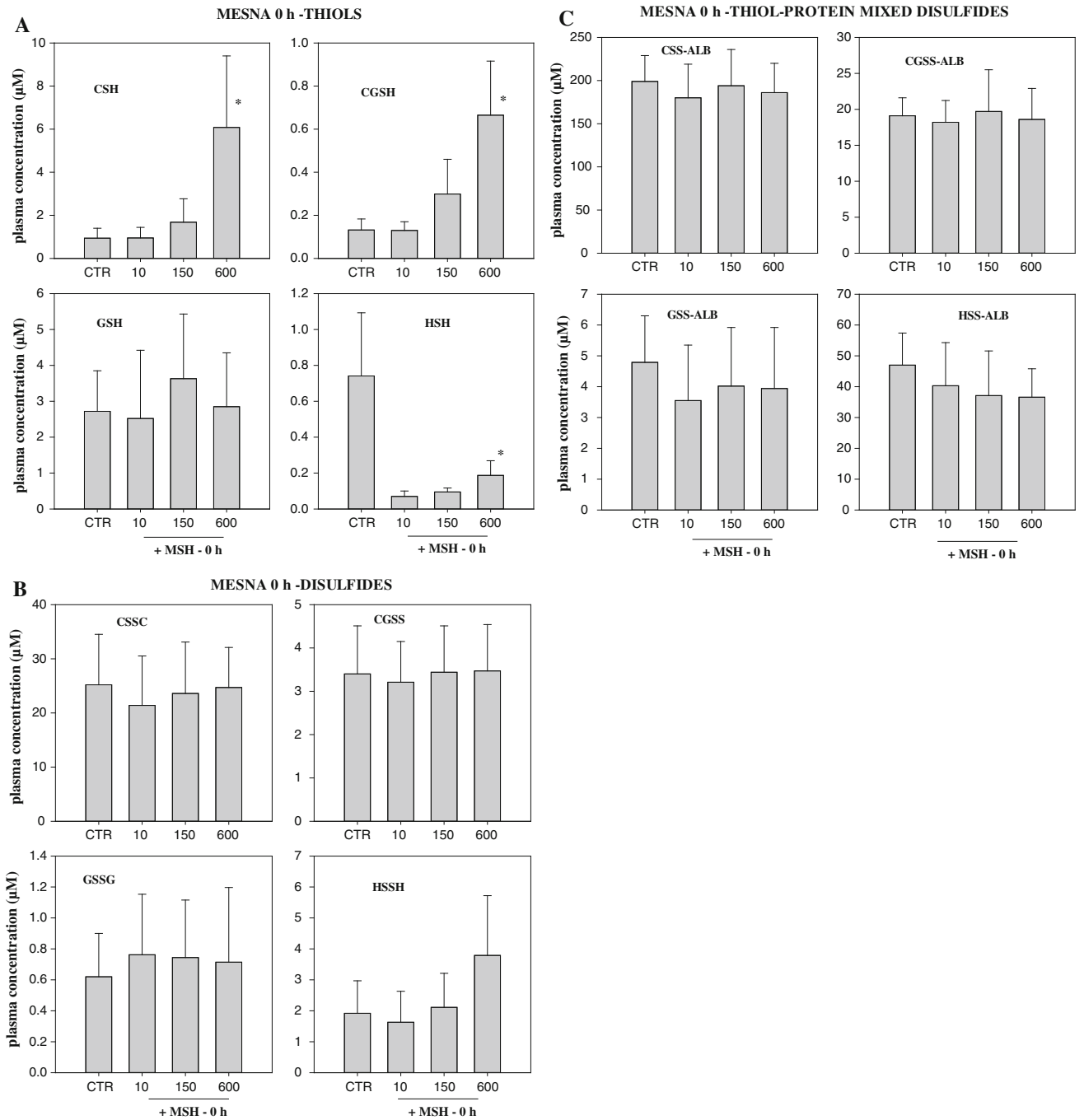

Fig. 2 Concentrations of thiol redox species at 0 min after treatment with MSH of aged plasma for 72 h from the plasma addition of 35 μM HSH. Human plasma treated with 35 μM HSH for 72 h was the control (CTR). Aliquots of aged plasma were taken and treated with increasing MSH concentrations (MSH 10, 150, 600 μM) at room temperature. **a** XSH, **b** XSSX, **c** XSS-ALB. Mean ± SD, $N = 8$. *$P < 0.05$ with respect to CTR

In vivo study: time-course of plasma distribution of thiol redox species after the methionine loading test

The methionine loading test (MLT) is clinically used to detect the onset of hyperhomocysteinemia in subjects with a moderate risk of hyperhomocysteinemia. The plasma HSH increase caused by the methionine metabolism is able to activate complex thiol exchange reactions. The plasma concentrations of redox species of Cys, Hcy, CysGly, glutathione and albumin were measured over time and data of Table 4 report the results of the most susceptible thiols (Cys, Hcy, and albumin HS-ALB).

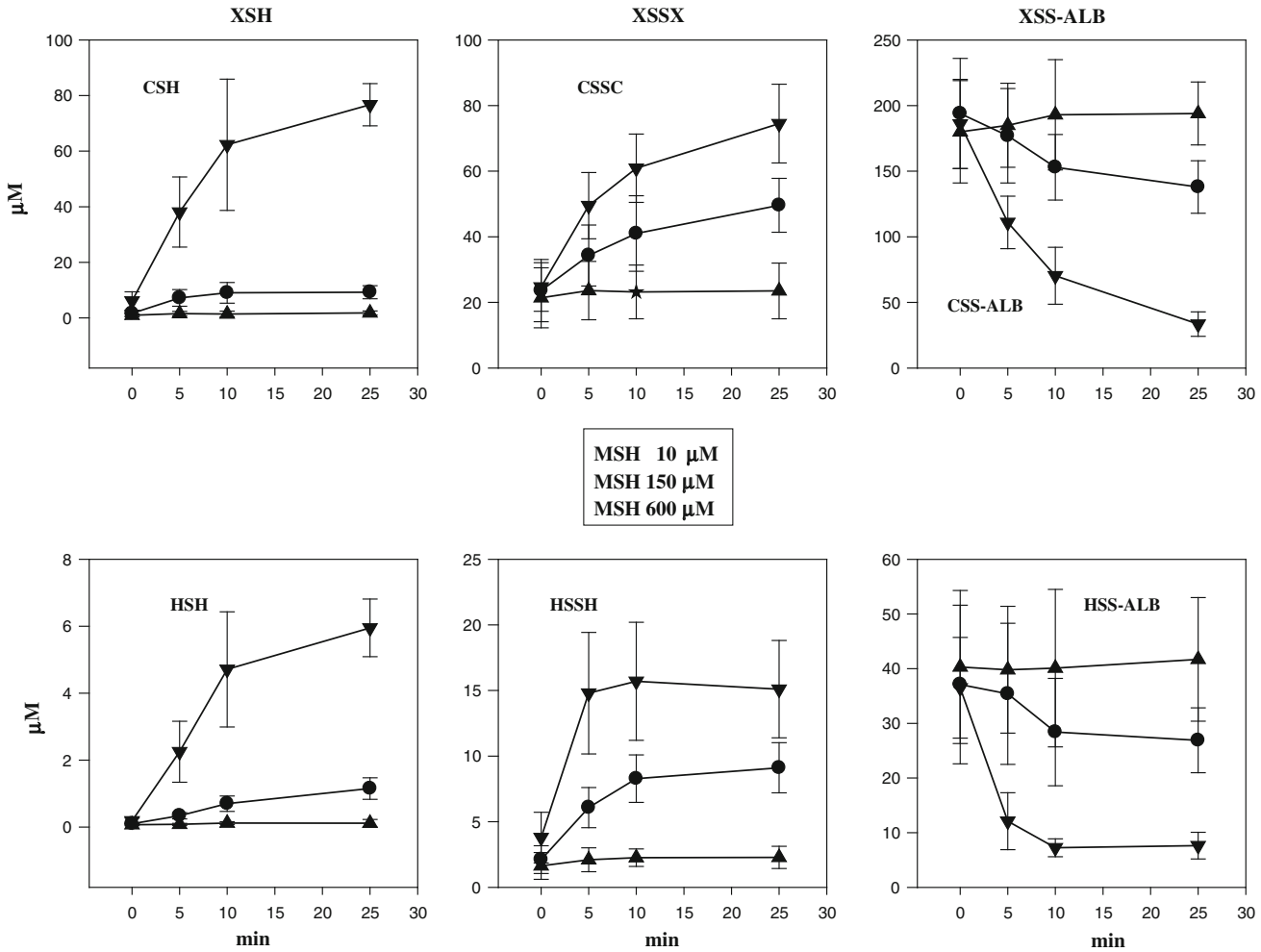

Fig. 3 Time-course of redox species of Cys and Hcy in human plasma, aged for 72 h after HSH addition and treated with increasing mesna concentrations. Mean ± SD, $N = 8$; the symbols of statistical significance were omitted for clarity of representation

Table 2 Time-courses of percentages of XSH and XSS-ALB with respect of total concentrations of Cys and Hcy concentrations, calculated at each time after 600 µM MSH addition to aged plasma

Time (min)	tCSH	tHSH	%CSH	%HSH	%CSS-ALB	%HSS-ALB
0	241	44.3	2.52	0.422	77.1	82.9
5	248	43.9	15.4	5.12	44.5	27.6
10	254	43.3	24.5	10.9	27.7	16.7
25	259	43.8	29.6	13.6	12.9	17.4

tXSH are the sum of XSH, XSSX and XSS-ALB, where XSSX is calculated as reduced equivalents, e.g., XSSX = 2XSH. Each value at each time, expressed as µM, is the mean of tXSH of eight different subjects whose SD was omitted for clarity

HSH concentrations showed a significant increase at 2 h and then they remained constant. A similar significant trend was observed for HSSH, whereas HSS-ALB concentrations increased significantly and continuously up to 6 h. These variation trends are in agreement with previous MLT experiments (Di Giuseppe et al. 2010) and with in vitro experiments here presented, in which HSSH increases had been attributed to exchanges of HSH with CSSC (via reactions (2) and (3) and HSS-ALB (via the reverse of reaction (4). Therefore, the in vivo kinetic profiles of HS-ALB and CSS-ALB, respectively, characterized by an increase and a drop (with a CSS-ALB rebound, at 4 and 6 h) are the consequence of thiol exchange reactions evoked by plasma HSH increases and described by the previous reactions (6), (7), (8), (9).

Discussion

The pharmacological treatment with MSH is used to reduce the collateral effects associated with cis-platinum therapy. In fact, MSH reduces the platinum toxicity chelating the metal excesses (Hausheer et al. 2010) and blocks the toxic

Table 3 Changes of mesna redox forms at 0 time and 25 min from the plasma treatment of aged plasma with increasing mesna

MSH 10 μM			MSH 150 μM			MSH 600 μM		
MSH	MSSM	MSS-ALB	MSH	MSSM	MSS-ALB	MSH	MSSM	MSS-ALB
0 min								
11.5	1.32	0.361	131	5.70	1.25	616	24.1	4.36
±3.6	±0.61	±0.072	±38	±3.12	±0.52	±117	±13.7	±3.22
25 min								
0.343	5.43	4.21	9.2	58.2	29.8	159	275	61.5
±0.162	±1.03	±1.02	±2.35	±12.5	±5.5	±30	±53	±12.3

Values are mean ± SD of eight different subjects

Table 4 Time-course of redox forms of plasma thiols after methionine administration

	CSH	HSH	CSSC	HSSH	CSS-ALB	HSS-ALB	HS-ALB
Basal	18.2 ± 7.9	0.237 ± 0.090	72.3 ± 8.9	1.30 ± 0.33	151 ± 18	6.86 ± 2.54	420 ± 81
2 h	22.9 ± 10.7	0.967 ± 0.395*	78.9 ± 11.6	5.03 ± 1.88*	132 ± 10	20.9 ± 5.9*	475 ± 58
4 h	22.3 ± 9.01	0.829 ± 0.349*	76.5 ± 8.7	5.26 ± .14*	135 ± 13	25.1 ± 5.4*	507 ± 55*
6 h	24.1 ± 11.5	0.937 ± 0.382*	79.5 ± 10.5	5.86 ± 1.61*	150 ± 20	28.2 ± 6.6*,#	486 ± 50*

Mean ± SD; $n = 9$

* $P < 0.05$ with respect to control (basal)

# $P < 0.05$ with respect to 2 h

metabolite of oxazaphosphorine, acrolein (Ormstad et al. 1983; Manz et al. 1985). Moreover, MSSM which is formed through MSH oxidation (Burkert 1983) is reduced back to MSH in the liver and renal tubular epithelium, thus exerting uroprotection (Ormstad and Uehara 1982; Ormstad and Ohno 1984).

In addition to these clinical applications, MSH has been successful in reducing moderate hyperhomocysteinemia in end-stage renal disease patients, who were not responding to the vitamin/cobalamin administration. MSH, through activation of thiol exchange reactions, is able to produce HSSM, which is eliminated through urine (Urquhart et al. 2007), and to reduce total Hcy. However, this effect was not equally effective when MSH was administered through a different route (Cutler et al. 2009). Since the molecular reason for this drawback was not previously investigated, we verified to what extent this could be related to activations of competing thiol exchange mechanisms.

The results of the present study regarding the metabolism of plasma thiols by thiol exchange reactions indicate that: (i) these reactions are rapidly activated by XSH (HSH and MSH); (ii) the perturbation of redox species is further influenced by plasma aging; (iii) XSS-ALB and CSSC are fundamental actors of reversible processes performed in harmony with thermodynamic rules regarding the p*K*a values of thiols of mixed disulfides (see "Introduction"). For example, the addition of HSH to plasma determines a rapid HSSH increase due to exchanges of HSH with CSSC and XSS-ALB. Successively, during plasma aging, other similar reactions overlap with a further subdivision of redox species by which XSS-ALB increases, and XSH and XSSX decrease with respect to their original levels of fresh plasma.

The reversibility of these processes is mainly linked to structural characteristics of albumin which as XSS-ALB is better exposed to the milieu and more reactive than HS-ALB (Christodoulou et al. 1994). Consequently, XSH added to plasma is more permeable toward XSS-ALB than that XSSX is permeable toward HS-ALB.

In the rat, whose albumin is more reactive than the human and bovine species (Spiga et al. 2011), the GSH efflux from the liver is rapidly buffered in plasma (Priora et al. 2010) to GSSG by the concurrent action of CSSC, via reactions (2) and (3), and XSS-ALB, via reactions similar to those above described by (6), (7), (8), (9) reactions.

For charge and mass reasons, the exchanges of XSSX with HS-ALB are per se more impeded than those of XSH with XSS-ALB (mostly represented by CSS-ALB), and, in addition to p*K*a differences, different charges and masses contribute to create specific plasma distribution of redox species for each compound. In the case of glutathione and Hcy, the p*K*a values are less important than their masses and charges. In fact, GSSG and GSH, having greater mass and being more negatively charged than HSSH and HSH,

are disfavored in reactions with HS-ALB or XSSALB, respectively, as compared with HSSH and HSH (Summa et al. 2007). These facts explain why glutathione is more resistant than Cys and Hcy to change the redox species distribution when HSH and MSH are added to plasma (Figs. 1, 2).

The comparison at $t = 0$ between the distribution of redox species of mesna (MSSM) (Table 3) with that of endogenous thiols (HSSH) (Fig. 1) emphasizes the importance of the electric charges, more than the mass, in determining specific thiol redox states. The MSSM percentages of tMSH, calculated from the data of Table 3, are much lower than those of the endogenous XSSX (Fig. 1b). For example, at $t = 0$ h, MSSM was 18.2 % (experiment at 10 μM MSH) and 7.21 % of total mesna (experiment at 600 μM MSH), whereas the HSSH percentages of tHcy calculated at 0 h from the experiment of Fig. 1 (addition to plasma of 35 μM HSH) were about 32 %. Having Hcy and mesna similar masses, similar p*K*a values, but different electric charges (mesna is more negatively charged than Hcy), it means that MSH and MSSM are more subjected to electric repulsion in exchange reactions with albumin (respectively with XSS-ALB and HS-ALB) with respect to HSH and HSSH, with a minor percentage of disulfide (MSSM) formation.

An interesting biological question concerns the origin of plasma GSS-ALB because GSS-ALB increases could be related to enhanced production of inflammatory states. GSS-ALB, as any other mixed disulfide of proteins, is usually supposed to be formed through thiol exchange mechanisms of XSSX with PSH (specifically with HS-ALB). However, the rate of GSSG exchange with human albumin is very slow:

$$\mathrm{GSSG} + \mathrm{HS\text{-}ALB} \leftrightarrow \mathrm{GSS\text{-}ALB} + \mathrm{GSH}, \quad (15)$$

and this fact induces to suggest that GSS-ALB might be formed by other plausible mechanisms, involving the formation of sulfenic acid of albumin (HOS-ALB) by hydrogen peroxide (Carballal et al. 2003; Turell et al. 2008), as described by the following reactions:

$$\mathrm{HS\text{-}ALB} + \mathrm{H2O2} \leftrightarrow \mathrm{HOS\text{-}ALB} + \mathrm{H2O} \quad (16)$$

$$\mathrm{HOS\text{-}ALB} + \mathrm{GSH} \leftrightarrow \mathrm{GSS\text{-}ALB} + \mathrm{H2O}. \quad (17)$$

However, on the basis of the data here presented, the H2O2 production, and consequently that of HOS-ALB, in aged plasma seems to be relatively scarce because the prolonged exposure to air oxygen did not evidence signs of GSH decrease, (Fig. 1a), or GSS-ALB increases (Fig. 1c). Therefore, the GSS-ALB origin in the plasma of healthy or unhealthy subjects, via reactive oxygen species (ROS) or reactive nitrogen species (RNS) remains an open and interesting question to be resolved.

The other goal of the present study was to verify whether the ineffectiveness of MSH in lowering plasma tHcy concentration could depend on a minor HSSM production due to undesired activations of thiol exchanges reactions. In this connection we have supposed that, at relatively high levels of plasma MSH the increasing MSSM production could occur at expenses of HSSM formation. This hypothesis has been confirmed by the data presented in Table 3. In this case, MSSM would be produced by the following reactions:

$$\mathrm{MSH} + \mathrm{HSSM} \leftrightarrow \mathrm{MSSM} + \mathrm{HSH} \quad (18)$$

$$\mathrm{MSH} + \mathrm{CSSC} \leftrightarrow \mathrm{MSSC} + \mathrm{CSH} \quad (19)$$

$$\mathrm{MSSC} + \mathrm{MSH} \leftrightarrow \mathrm{MSSM} + \mathrm{CSH}, \quad (20)$$

and the MSSM in vivo formation has already been demonstrated (Burkert 1983; Ormstad and Uehara 1982). Therefore, excessive MSH concentrations, obtained unintentionally under different administration routes, endovenous (negative) (Cutler et al. 2009) and oral (Urquhart et al. 2007) (positive) would explain the possible therapeutic failure of MSH in end-stage renal disease patients (Cutler et al. 2009).

In our opinion, a further confirmation of the above-mentioned hypothesis must be obtained by monitoring the concentrations of redox species of mesna, Cys and Hcy in MSH-treated subjects; in this way it will be possible to get the best MSH dose associated with the minor MSSM production and the best HSSM elimination.

In conclusion, the results here obtained call for future studies to verify the best appropriate dose of MSH capable at reducing the levels of tHcy in subjects at risk of pathological progression and whether GSS-ALB can be used as an inflammatory index of radical diseases.

Informed consent The authors have given their consent to be included in the present study.

Conflict of interest The authors declare that they have no conflict of interest.

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