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ORIGINAL ARTICLE

Regulation of redox forms of plasma thiols by albumin in multiple sclerosis after fasting and methionine loading test

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Abstract Increases in plasma concentrations of total homocysteine (tHcy) have recently been reported in multiple sclerosis (MS) as the alteration of the methionine cycle for the onset of autoimmune diseases. Homocysteine (Hcy) and cysteine (Cys) are generated by the methionine cycle and transsulfuration reactions. Their plasma levels are subjected to complex redox changes by oxidation and thiol/disulfide (SH/SS) exchange reactions regulated by albumin. The methionine loading test (MLT) is a useful in vivo test to assay the functionality of the methionine cycle and transsulfuration reactions. Time courses of redox species of Cys, cysteinylglycine (CGly), Hcy, and glutathione have been investigated in plasma of MS patients versus healthy subjects after an overnight fasting, and 2, 4, and 6 h after an oral MLT (100 mg/kg body weight), to detect possible dysfunctions of the methionine cycle, transsulfuration reactions and alterations in plasma distribution of redox species. After fasting, the MS group showed a significant increase in cysteine-protein mixed disulfides (bCys) and total Cys (tCys). While plasma bCys and tCys in MS group remained elevated after methionine administration when compared to control, cystine (oxCys) increased significantly with respect to control. Although increased plasma concentrations of bCys and tCys at fasting might reflect an enhance of transsulfuration reactions in MS patients, this was not confirmed by the analysis of redox changes of thiols and total thiols after MLT. This study has also demonstrated that albumin-dependent SH/SS exchange reactions are a potent regulation system of thiol redox species in plasma.

Keywords Multiple sclerosis · Methionine loading test · Plasma thiols · Homocysteine · Cysteine

Abbreviations

Ado	Adenosine
AdoHcy	*S*-Adenosyl-homocysteine
Cys	Cysteine
CGly	Cysteinylglycine
DTNB	5,5′-Dithiobis-(2-nitrobenzoic acid)
EAE	Experimental autoimmune encephalomyelitis
GSH	Reduced glutathione
bGSH	Glutathione-protein mixed disulfide
GSSG	Glutathione disulfide
Hcy	Homocysteine
tLWT	Sum of total thiols of Cys, CGly, Hcy, and glutathione, where disulfides are expressed as reduced forms, XSSX = 2XSH
NPSH	Non-protein thiols, sum of rCys, rCGly, GSH, rHcy
MLT	Methionine loading test
MS	Multiple sclerosis
MTHFR	Methylene tetrahydrofolate reductase
oxCys	Cystine
PSH	Protein SH groups

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ROS	Reactive oxygen species
RSH	XSH thiols of low molecular weight
r	Prefix of reduced thiols
ox	Prefix of disulfides
b	Prefix of protein-thiol mixed disulfides
t	Prefix of total amounts
SAM	*S*-Adenosyl-methionine
SAH	*S*-Adenosyl-homocysteine
SH	Sulfhydryl group
SH/SS	Thiol/disulfide
TSH	Total reduced thiols
tXSSX	Total disulfides, sum of oxCys, oxHcy, oxCGLy, and GSSG
tXSSP	Total thiol-protein mixed disulfides, sum of bCys, bCGly, bHcy, and bGSH
XSSX	Symmetrical disulfides
XSSP	Thiol-protein mixed disulfides
XSSR	Asymmetrical disulfides

Introduction

The redox status of biological compartments relies on appropriate mechanisms of oxidant/antioxidant balance to accomplish physiological functions and minimize damage due to reactive oxygen species (ROS) (Sies 1985). Thiol compounds such as cysteine (Cys), homocysteine (Hcy), cysteinylglycine (CGly), and glutathione have a significant role as components of the intracellular and extracellular redox buffer (Moriarty-Craige and Jones 2004), and are important scavengers of ROS and other electrophilic agents.

The main plasma thiols are present at total concentrations of reduced forms of 20–30 μM, which are lower than those of corresponding disulfides (XSSX) and thiol-protein mixed disulfides (XSSP) (70–90 and 130–150 μM, respectively). The plasma distribution of thiols as reduced and oxidized species is rather constant and well maintained over a wide age range, from birth to advanced adulthood (Di Giuseppe et al. 2004). This distribution represents the in vivo response of complex equilibria involving oxidations, SH/SS exchange reactions, plasma/tissues exchanges, and excretion.

Despite SH group presence, thiols are not always beneficial antioxidants, and the case of Hcy is paradigmatic. As a matter of fact, increased plasma levels of total Hcy have emerged as a risk factor for cardiovascular disease, stroke, venous thrombo-embolism, and other diseases (Mansoor et al. 1995; Quere et al. 2005; Eikelboom et al. 1999; Andersson et al. 2000; Di Giuseppe et al. 2003; Mattson and Shea 2003). However, it is still uncertain whether hyperhomocysteinemia is a causative factor or a marker of vascular disease, though, according to more recent studies on animal models, the cell target of Hcy toxicity seems to be in the vascular endothelium, where it causes dysfunctions at several levels (Welch and Loscalzo 1998; Dayal et al. 2001; Weiss et al. 2002; Hossain et al. 2003).

Cys and Hcy are intimately related in health and disease (Andersson et al. 2000; Di Giuseppe et al. 2003). For example, a possible increase in cell Hcy, namely rHcy, may be regulated by remethylation, regenerating methionine, as well as by transsulfuration processes, forming rCys. When these cellular pathways are disrupted, increased amounts of cellular rHcy or rCys are exported into plasma and subjected to metabolic changes such as oxidations, by virtue of heavy metals bound to proteins (albumin and ceruloplasmin) (Gryzunov et al. 2003; Sengupta et al. 2001a; Løvstad 2002; Ozawa et al. 1993), and to SH/SS exchange reactions (or thiol exchanges) by albumin (Sengupta et al. 2001b; Summa et al. 2007).

Albumin exerts buffering actions of redox species of plasma thiols (Sengupta et al. 2001b; Summa et al. 2007) by a variety of thiol exchanges reactions such as:

$$\mathrm{RSH} + \mathrm{XSSP} \rightleftharpoons \mathrm{PSH} + \mathrm{RSSX} \quad (1)$$

$$\mathrm{RSH} + \mathrm{XSSP} \rightleftharpoons \mathrm{RSSP} + \mathrm{XSH} \quad (2)$$

$$\mathrm{XSSX} + \mathrm{PSH} \rightleftharpoons \mathrm{XSSP} + \mathrm{XSH} \quad (3)$$

Albumin reactions rely on a conserved reactive cysteine residue (Cys_{34}) that has an unusually low pKa value (Lewis et al. 1980; Pedersen and Jacobsen 1980). By virtue of this characteristic, *S*-thiolated albumin (XSSP) is involved in dethiolation (reaction 1), rather than thiol substitution (reaction 2) (Summa et al. 2007), in dependence of thermodynamic principles inherent in the pKa value.

The orchestration of redox states of plasma thiols of healthy and pathological subjects by albumin is not well known, because clinical studies generally rely on the changes in total amounts of thiols rather than in specific redox species. Information regarding redox modification is important because it may serve to better understand mechanisms of development of a pathological state, especially in inflammatory diseases where thiols are inevitably involved.

Multiple sclerosis (MS) is a chronic auto-immune disease characterized by central nervous system inflammation and demyelination (Noseworthy 1999). It has been suggested that the redox system modulations are involved in the progression of MS. For example, although the contribution of free radicals to MS pathogenesis is still debated (Spector 1977; Bush 2000; Bö et al. 1994; Calabrese et al. 2000), free radicals are thought to contribute to demyelination (Lucchinetti et al. 1998).

Elevated plasma tHcy levels have been found in MS patients, but the relationship between redox forms of Hcy and other plasma thiols such as Cys, in this and other

diseases is still unknown (Ramsaransing et al. 2006; Besler and Čomoğlu 2003; Vrethem et al. 2003). It remains to be proven whether mild hyperhomocysteinemia directly causes tissue damage or reflects other mechanisms related to dietary factors or a simple adaptation to pathological disorders. In favor of the former hypothesis, there are recent studies of experimental autoimmune encephalomyelitis (EAE) which show a strong linkage between the methionine cycle/*S*-adenosyl-L-homocysteine (AdoHcy) hydrolase and T cell activation or other autoimmune/inflammatory processes (Fu et al. 2006; Lawson et al. 2007).

The methionine loading test (MLT) was originally devised to detect heterozygotes for deficiency of activity of transsulfuration reactions (cystathionine β synthase) (Fowler et al. 1971) and is known as an in vivo assay to test the efficiency of the methionine cycle and transsulfuration reactions in pathological subjects. The methionine cycle and transsulfuration reactions represent one of the sources generating plasma amino acids containing SH groups, such as Cys and Hcy (Finkelstein 2000; Brosnan and Brosnan 2006). The MLT stresses and unmasks possible defects of Hcy metabolism in normal and pathological subjects with normal Hcy concentrations at fasting (Mansoor et al. 1995; Graham et al. 1997; van der Griend et al. 1998; Mansoor et al. 1992a; van der Griend et al. 2002). In particular an abnormal response to a methionine load would primarily reflect a defect in the transsulfuration pathway. Since MLT is an important biological assay to study the regulation of plasma thiols by albumin, we sought to investigate possible changes in plasma distribution of thiol redox forms in MS patients when compared to healthy individuals.

In this study, we investigated plasma changes of thiol redox forms, particularly those of Hcy and of the main other compounds Cys, glutathione, and CGly, induced by MLT in patients with MS, to shed lights on the redox dynamics operating at plasma level. The information about the redox metabolism of thiols may be useful to better ascertain the thiol role in pathological mechanisms of redox origin such as inflammatory diseases. The main aim of this study is to understand whether possible plasma changes modulated by albumin might be related to the hypothesis of disruption of the methionine cycle and transsulfuration reactions in MS.

Materials and methods

Patients

Twelve patients with relapsing-remitting MS (5 males, mean age 44.9 ± 12.3, mean weight 73.3 ± 13.1 kg) and 11 healthy subjects (8 males, mean age 38.6 ± 12.1, mean weight 69.9 ± 10.4 kg) were included in the study. Diagnosis of MS was based on the McDonald/Polman criteria (Polman et al. 2005). No patients had ever been treated with disease-modifying therapy and they all were free from drugs and relapses since at least 2 months at the time of 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. Blood was gently withdrawn into plastic tubes containing EDTA 2, 4, and 6 h after methionine overload. The study was carried out in accordance with the principles of the Declaration of Helsinki. Before entering the study, each patient gave informed consent. The design of the study conformed to current standards in Italy.

Chemicals

All chemicals of analytical grade were purchased from Sigma Chemical Co (St. Louis, MO, USA). Methanol, water, and HPLC grade reagents were obtained from Merck (Darmstadt, Germany). Monobromobimane (mBrB) was obtained from Calbiochem (La Jolla, CA, USA).

Plasma analysis of reduced thiols, disulfides, and thiol-protein mixed disulfides in MS

After collection, blood samples were immediately centrifuged (10,000×*g*, 20 s) and the plasma was divided into two parts, one of which was used for reduced thiol analysis, and the other for disulfide and protein-thiol mixed disulfide assays. Remaining plasma samples were stored at 0°C until TSH determination by Ellman's method (Ellman 1959). Thiols, disulfides, and thiol-protein mixed disulfides were assayed by chromatographic methods according to Mansoor et al. (1992b), using mBrB as derivatizing agent of thiols, with slight modifications regarding sample preparations as reported by Di Giuseppe et al. (2004).

Plasma perturbation of thiol redox species by SH/SS exchange reactions by reduced thiols

Blood of healthy donors (2 females, 2 males) was drawn by venipuncture into vacutainer tubes containing EDTA. After centrifugation at 10,000×*g* for 20 s, plasma was transferred to tubes and treated with reduced thiols at room temperature. Plasma aliquots were taken to assay the original concentration of the redox species of various thiols. Then the original plasma was treated with 50 µl of rCys, GSH, rHcy in physiological solution (60 µM, final concentration) or equal volume of physiological solution. Redox species of Cys, glutathione (not shown), Hcy (not shown), and CGly (not shown) were assayed on plasma aliquots stopped by TCA addition (6%, final concentration) at 5, 15, 30, and 60 min by HPLC, as above indicated (Di

Giuseppe et al. 2004). Results of a typical experiment are reported in Fig. 5.

Chromatography

Thiols were measured with an HPLC apparatus (Hewlett Packard Series 1100) equipped with fluorimeter detector. Aliquots (20 µl) of derivatized samples were separated on a ChromSep C_{18} column (250 × 4 mm) (Varian, Lake Forest, CA, USA). The thiol derivatives were detected fluorimetrically with excitation at 390 nm and emission at 480 nm. Calibration curves were constructed after addition of thiols and disulfides to phosphate buffer. The detection limit was 2 pmol/injection (injection volume 0.02 ml).

Analysis of total thiols and albumin

Total plasma SH groups (TSH) is the sum of protein SH groups (PSH) and low molecular weight thiols. TSH was determined spectrophotometrically with the use of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (Ellman 1959). All spectrophotometric determinations were carried out with a JASCO V550 UV–Vis apparatus.

Albumin concentrations of plasma samples were assayed with the Synchron LX 20 Chemical System.

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was performed by a general linear model for repeated measures (ANOVA) using the SPSS package version 10.3. This analysis provided *P* values for differences between groups (*G*), differences over time (*T*), and for the interaction of groups with time (*GT*). If significant differences between groups were documented, values at each time points were compared by one-way ANOVA. Comparisons between before and 2, 4, and 6 h after MLT within each group were made by a paired Student's *t* test.

Results

Redox forms of homocysteine, cysteine, cysteinylglycine, and glutathione

The plasma redox forms of Hcy, Cys, CGly, and glutathione of MS and control groups were assayed under fasting conditions (time zero) and after MLT at 2, 4, and 6 h.

Homocysteine

Plasma concentrations of rHcy, oxHcy, and bHcy of MS patients at fasting (Fig. 1) were not significantly different

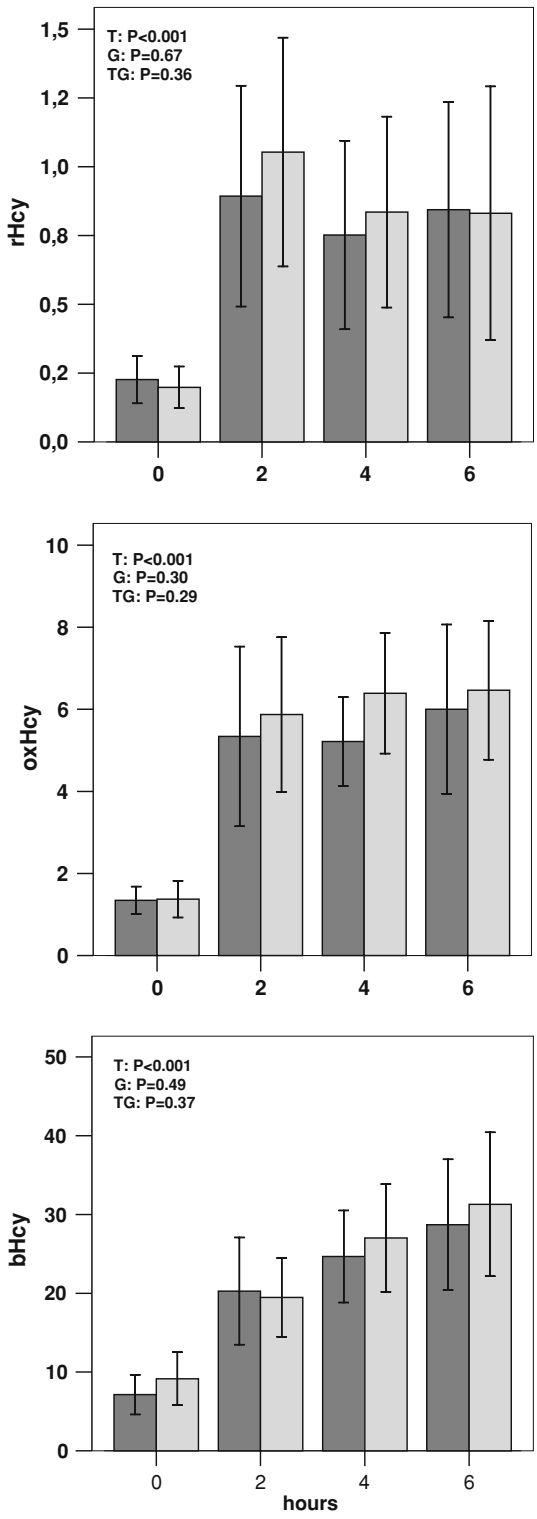

Fig. 1 Time courses of rHcy, oxHcy, and bHcy concentrations in plasma of patients with MS (*gray bars*) and controls (*black bars*) after MLT. Values are expressed as µM ± SD. *P* values were calculated using repeated-measures ANOVA and denoted as *T* differences over time, *G* differences between groups, and *TG* differences due to the interaction of groups with time. One-way ANOVA showed no significant differences between groups at each time point

when compared with control group. rHcy, oxHcy, and bHcy increased significantly over time ($P < 0.001$) after MLT, without significant differences between groups.

Cysteine

Plasma concentrations of bCys of MS patients at fasting (Fig. 2) were higher than controls ($P = 0.003$), whereas no significant differences were found when rCys and oxCys of MS group were compared with control group. After MLT, the overall repeated measures ANOVA revealed significant changes over time in rCys ($P = 0.003$) and bCys ($P < 0.001$), and significant interactions between time and groups for rCys ($P = 0.007$) and bCys ($P = 0.006$). rCys concentrations of MS group showed a peak trend, whereas bCys of both the groups showed a minimum at 2 h and then a recovery at 6 h. Plasma oxCys concentrations did not vary over time. However, significant differences between groups were found for oxCys ($P = 0.006$) and bCys ($P = 0.041$). In particular, plasma concentrations of oxCys of MS group become significantly higher than control group at all time points after MLT.

From bCys data of both the groups, we also determined the mean of the difference between values at each time point and basal bCys (referred to as ΔbCys in Table 1). At 2 and 4 h, ΔbCys was in MS significantly higher than in control group. For example, ΔbCys of MS at 2 h was −36.2 μM when compared to ΔbCys of control of −19.1 μM ($P = 0.017$).

Cysteinylglycine

No significant differences in fasting levels of rCGly, oxCGly, and bCGly were observed between groups (data not shown). MLT did not affect levels of rCGly and oxCGly over time, whereas bCGly decreased significantly over time ($P < 0.001$) without differences between groups ($P = 0.12$). As in the case of bCys, the estimate of bCGly variation (ΔbCGly) at each time point with respect to fasting levels for each subject was measured (data not shown). ΔbCGly resulted to be more pronounced in MS patients than in controls with a significant difference of the means at 2 h (−5.7 vs. −2.4, respectively; $P = 0.027$).

Glutathione

No significant differences in fasting concentrations of GSH, GSSG, and bGSH were observed between groups (data not shown). MLT had no significant effects on GSSG and bGSH over time, whereas GSH increased ($P = 0.037$), without significant differences between groups.

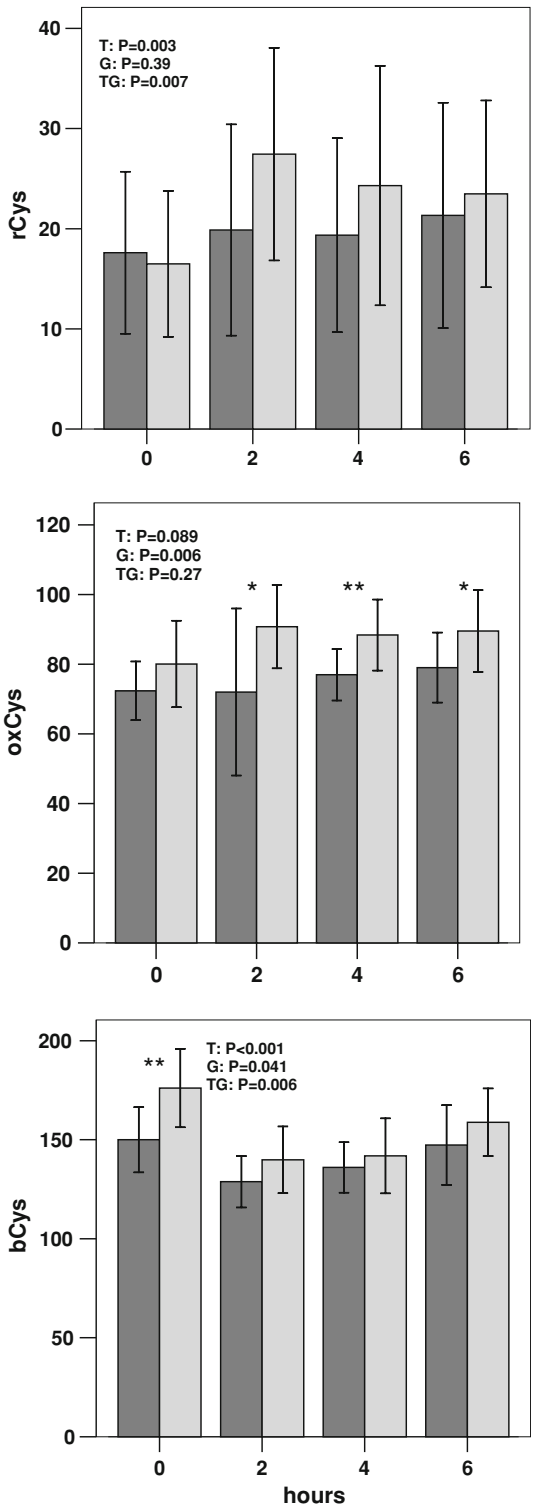

Fig. 2 Time course of rCys, oxCys, and bCys concentrations in plasma of patients with MS (*gray bars*) and controls (*black bars*) after MLT. Values are expressed as μM ± SD. *P* values were calculated using repeated-measures ANOVA and denoted as *T* differences over time, *G* differences between groups, and *TG* differences due to the interaction of groups with time. Significant differences between groups at each time points (*$P < 0.05$, **$P < 0.01$) are indicated as showed by one-way ANOVA

Table 1 Plasma variations of differences in bCys, bHcy, tXSSX, and tXSSP concentrations with respect to fasting levels in patients with MS and controls after MLT

Hours	ΔbCys	ΔbHcy	ΔtXSSX	ΔtXSSP
Control group ($n = 11$)				
2	$-19.1^{\#}$	$13.2^{\#\#\#}$	3.7	−11
4	−12.9	$17.6^{\#\#}$	8.5	0
6	−3	$21.6^{\#\#\#}$	11	16
MS group ($n = 12$)				
2	$-36.2^{*,\#\#\#}$	$10.3^{\#\#}$	14.6	$-31^{**,\#}$
4	$-34.2^{**,\#\#\#}$	$17.8^{\#\#}$	13.6	−21*
6	−18	$22.1^{\#\#}$	14.6	1

Mean values are expressed in micromolar, standard deviations were omitted for clarity. tXSSX and tXSSP are the sum of disulfides and thiol-protein mixed disulfides, respectively, of Cys, CGly, Hcy, and glutathione. Δ represents the difference between mean at each time and baseline. Significant differences between groups at each time points are indicated (* $P < 0.05$, ** $P < 0.01$) (one-way ANOVA). Significant differences from baseline within each groups are also reported (# $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$)

TSH, PSH, and NPSH

Total plasma SH groups (TSH) determined by DTNB represents the sum of protein (PSH) and non-protein thiols (NPSH). PSH (Table 2) was estimated as difference between TSH and the sum of reduced forms (rHcy, GSH, rCys, and rCGly), named NPSH. In good approximation this sum represents the total plasma NPSH population. TSH and PSH of the two groups at fasting (0 h) were not significantly different. Plasma TSH and PSH concentrations of each group changed significantly over time after MLT, without differences between groups. In particular, TSH and PSH of MS and control groups showed a significant increase at 4 and 6 h with respect to the corresponding basal levels. The PSH increase over time (Δ in Table 2) reflected the bCys decrease (Fig. 2) and was dependent on dethiolation reactions by reaction (1).

Plasma concentrations of NPSH of controls and MS patients at fasting were nearly equal (24.6 ± 9.7 and 24.0 ± 9.3 μM, respectively). MLT did not affect NPSH concentrations in control group (e.g., value at 2 h = 28.8 ± 13.4 μM), whereas NPSH in MS group showed a peak at 2 h (36.9 ± 12.8 μM), statistically significant ($P = 0.024$). The NPSH increase of MS patients was related to rHcy (Fig. 1), rCys (Fig. 2), and GSH (data not shown). No NPSH differences between groups were observed at 4 and 6 h.

Total oxidized forms

Total XSSX (tXSSX) represents the sum of disulfides (oxCys, oxCGly, oxHcy, and GSSG) and total XSSP (tXSSP), the sum of thiol-protein mixed disulfides (bCys, bHcy, bCGly, and bGSH). The increase in reduced thiols in plasma is able to provoke variations in tXSSX and tXSSP concentrations by complex thiol exchanges. Plasma concentrations of tXSSX and tXSSP were estimated and reported in Fig. 3. We also determined the concentration difference between each time point and corresponding basal levels at fasting of tXSSX and tXSSP in both the groups (referred to as ΔtXSSX and ΔtXSSP in Table 1). No significant differences in fasting levels of tXSSX between MS and control groups were observed (Fig. 3). After MLT, tXSSX showed an overall increment over time ($P = 0.003$) with a more marked increase in MS patients than in controls and a statistically significant difference at 2, 4, and 6 h.

Table 2 TSH and PSH plasma levels in patients with MS and controls after MLT

Time (h)	Control ($n = 11$)			MS ($n = 12$)		
	TSH	PSH	ΔPSH	TSH	PSH	ΔPSH
0	447 ± 83	423 ± 84	0	419 ± 57	395 ± 57	0
2	501 ± 53	472 ± 60	49	478 ± 51	441 ± 54	46
4	$535 \pm 55^{\#}$	$507 \pm 57^{\#}$	84	$499 \pm 58^{\#}$	$465 \pm 51^{\#}$	70
6	$516 \pm 54^{\#}$	487 ± 53	64	$527 \pm 74^{\#\#}$	$494 \pm 69^{\#\#}$	99

Mean values are expressed as μM ± SD. TSH represents total plasma thiols assayed by DTNB; PSH is the difference between TSH and the sum of reduced thiols (rCys, rHcy, GSH, and rCGly assayed by HPLC). Δ represents the difference between mean at each time and baseline. One-way ANOVA showed no significant differences between groups at each time point. Significant differences from baseline within each group are reported (# $P < 0.05$, ## $P < 0.01$)

tXSSP at fasting was in MS significantly higher than in control group ($P < 0.01$). After MLT, tXSSP showed an initial decrease followed by a slow recovery up to basal levels ($P < 0.001$). This trend was similar in both MS patients and controls, without significant differences between groups but with significant interactions between time and groups ($P < 0.019$).

When ΔtXSSX and ΔtXSSP of both the groups were compared after MLT (Table 1), we confirmed the tendency of an opposite trend between tXSSX and tXSSP, more evident in MS group. For example, at 2 h, ΔtXSSP was significantly more accentuated in MS than in control group (−31 vs. −11 μM). tXSSP changes of both the groups are interpreted as consequence of complex dethiolation and thiolation processes, where ΔbCys and ΔbHcy decreased and increased, respectively (Table 1). Interestingly, tXSSP decreases were to some extent related with tXSSX increases. This opposite relationship of tXSSX and tXSSP would suggest that plasma disulfide formation is more linked to protein dethiolations (as described by reaction 1) than to oxidations.

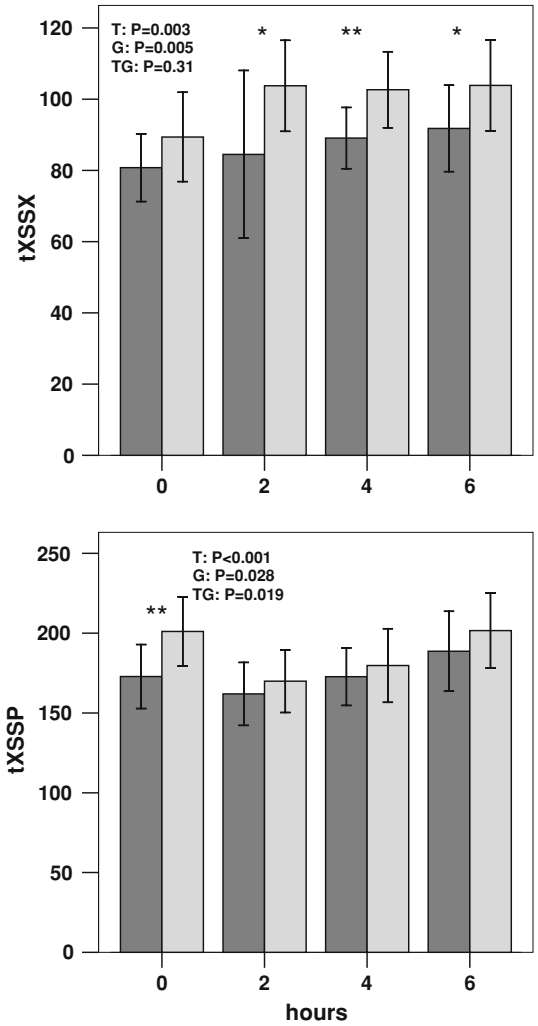

Fig. 3 Time course of tXSSX and tXSSP concentrations in plasma of patients with MS (*gray bars*) and controls (*black bars*) after MLT. Values are expressed as μM ± SD. *P* values were calculated using repeated-measures ANOVA and denoted as *T* differences over time, *G* differences between groups, and *TG* differences due to the interaction of groups with time. Significant differences between groups at each time points (*$P < 0.05$, **$P < 0.01$) are indicated as showed by one-way ANOVA

Regulation of total thiols

Figure 4 reports the total amount, expressed as reduced forms, of redox species of Hcy (tHcy) and Cys (tCys). After fasting, tHcy, tGSH (data not shown), and tCGly (data not shown) concentrations in MS patients did not differ from those of controls, whereas tCys of MS group was significantly higher than control. MLT induced a significant increase over time in tHcy ($P < 0.001$), but not in tCys (Fig. 4). tGSH and tCGly levels over time of MS and control groups did not differ from the corresponding basal values (tCGly control value at fasting: 27.9 ± 6.0 μM; tGSH control value at fasting: 8.67 ± 2.9 μM).

No significant differences between groups were observed for tHcy after MLT. tCys levels of MS group remained significantly higher than those of controls ($P < 0.01$), confirming the differences at fasting. No significant interaction between time and groups was observed for tCys (Fig. 4) when compared to the significant interaction found for bCys (Fig. 2). In other words, the increase in tCys levels in MS group was a stable parameter, not modified by MLT, even though Cys redox species had been subjected to great plasma remodeling.

Relationship between plasma tCys of MS patients and cellular Cys production by MLT

To verify whether increased tCys levels observed in MS group at fasting and after MLT (Fig. 4) were expression of increased Cys production in tissues by transsulfuration reactions or other metabolic pathways, differences (Δ) of total amounts of thiols with respect to basal levels were estimated (Table 3). ΔtHcy resulted to be nearly equally increased in control and MS groups, whereas the trend of ΔtCys (and ΔtCGly) was unexpectedly more frequently decreased than increased in both the groups. The increase in tHcy induced by MLT in all subjects resulted to be promptly counterbalanced by the decreasing trend of other total thiols. Therefore, no support was obtained by ΔtCys data that explained the increase of tCys (and bCys) levels in MS patients at fasting. After MLT, the Cys concentration seems to be promptly regulated, but the regulation site, whether plasmatic or cellular, remains unknown.

Plasma perturbation of thiol redox species by SH/SS exchange reactions by reduced thiols

The absence of plasma response of tCys after MLT could be explained by cellular responses addressed to control rCys levels. Since rCys may be transformed into cysteinesulfinate, hypotaurine, and other metabolites by cysteine dioxygenase (Stipanuk et al. 2009), it is not excluded that rCys increases caused by methionine administration are promptly metabolized. However, the observation that plasma tCys did not change over time in both the groups could also be dependent on phenomena of Cys excretion or plasma/tissue exchanges. To better clarify the plasma metabolism of Cys and the contribution from SH/SS exchange reactions, we investigated redox changes over time deriving from increased concentrations of reduced thiols in plasma. The aim of the experiment is to simulate possible redox modulations evoked by the efflux of reduced thiols into plasma, at concentrations correspondent to the maximum in vivo increase of tHcy after MLT (60 μM).

The plasma distribution of thiol redox forms changed in a complex manner when solutions of reduced thiols, freshly

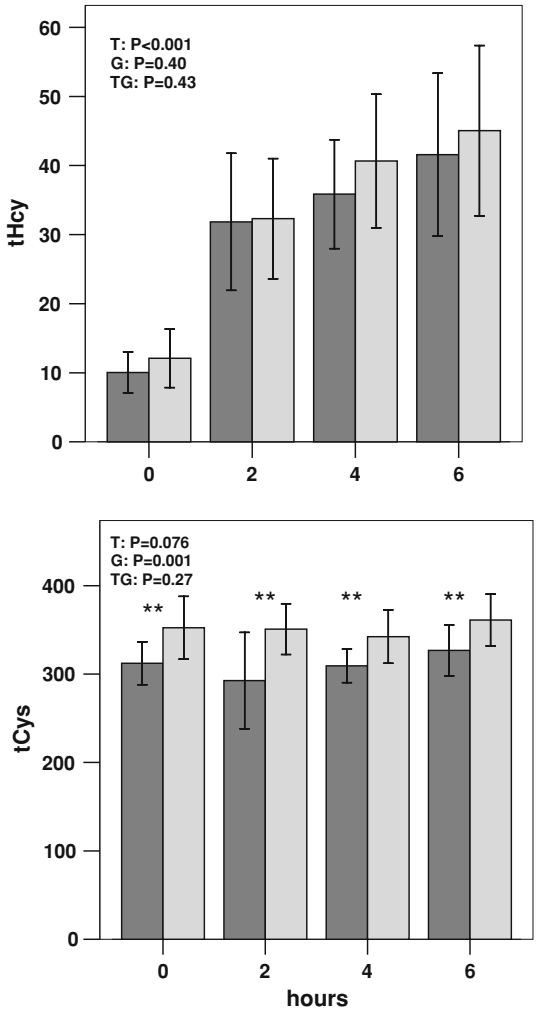

Fig. 4 Time courses of tHcy and tCys concentrations in plasma of patients with MS (*gray bars*) and controls (*black bars*) after MLT. Values are expressed as μM ± SD. *P* values were calculated using repeated-measures ANOVA and denoted as *T* differences over time, *G* differences between groups, and *TG* differences due to the interaction of groups with time. Significant differences between groups at each time points ($*P < 0.05$, $**P < 0.01$) are indicated as showed by one-way ANOVA

prepared, were added to plasma. These variations were attributed to the overlapping reactions of oxidations and thiol exchanges, where SH/SS exchange reactions seemed to prevail over oxidations. For example, when rCys was added (+rCys, Fig. 5), the observed oxCys increases were more plausibly linked to dethiolation reactions than to oxidations, by the following SH/SS exchange reaction:

$$\mathrm{rCys} + \mathrm{bCys} \rightleftharpoons \mathrm{oxCys} + \mathrm{PSH}. \quad (4)$$

Activations by reaction (4) are also supported by the comparison of bCys trends over time. In fact, when plasma was stimulated by a reduced thiol, this trend was decreasing, whereas it was opposite (increasing) for the control (plasma plus buffer, Fig. 5). Moreover, since the addition of reduced thiols to plasma (+GSH and +rHcy, Fig. 5), frequently caused rCys increases, we deduced from this that transformation effects of a reduced species (GSH or rHcy) into another reduced species (increases in rCys) are a further evidence of activation of thiol exchanges.

Discussion

Albumin is recognized to be the main determinant of plasma bHcy accumulation, through complex SH/SS exchange reactions between rHcy and bCys (Di Giuseppe et al. 2003; Summa et al. 2007; Sengupta et al. 2001b). This in vivo study confirmed that albumin is the main determinant of redox species distribution of thiols in plasma, acting by oxidations and thiol exchange reactions. The methionine metabolism, stimulated by MLT and characterized by an increased cellular production of reduced thiols, was associated with plasma increases in Hcy redox forms, and intricate changes of redox forms of other plasma thiols. Decreases in XSSP (essentially bCys decreases) and parallel increases in reduced and oxidized (disulfides) species were observed. Although plasma is considered an oxidant environment where thiols are rapidly transformed into disulfides, redox changes caused by methionine administration were more plausibly related to SH/SS exchange reactions than to oxidations. The importance of thiol exchanges in plasma was directly confirmed by specific in vitro experiments in which solutions of reduced thiols were added to plasma (Fig. 5).

The methionine metabolism in cells and tissues (Finkelstein 2000; Brosnan and Brosnan 2006) produces cellular increases in rHcy, rCys, and other metabolites of the methionine cycle and transsulfuration pathways. As the plasma concentration of metabolites of the methionine cycle and transsulfuration pathway are increased after MLT (Doshi et al. 2005), we assume that MLT activates the following exchange reactions (5, 6) of albumin. The export of rHcy into plasma delivers PSH from bCys, the most concentrated XSSP in plasma, forming the asymmetric disulfide

$$\mathrm{rHcy} + \mathrm{bCys} \rightleftharpoons \mathrm{PSH} + \mathrm{HcySSCys}; \quad (5)$$

in turn, the thiolated anion of albumin (PS^-) reacts with HcySSCys, as proposed by other investigators (Sengupta et al. 2001b), forming bHcy and rCys:

$$\mathrm{HcySSCys} + \mathrm{PSH} \rightleftharpoons \mathrm{bHcy} + \mathrm{rCys}. \quad (6)$$

The reaction (6) is apparently responsible for increases in bHcy (Fig. 1) and rCys observed in both the groups after MLT (Fig. 2). Similarly, the rCys increase may contribute to increase oxCys according to reaction (4). The reactions (4–6) well explain all our in vivo data, in particular the relatively great PSH increase in control and MS groups (Table 2). The bCys trend of both groups over time (Fig. 2) is expression of

Table 3 Plasma variations of differences in total thiol concentrations with respect to fasting levels in patients with MS and controls after MLT

Hours	Control ($n = 11$)				MS ($n = 12$)			
	ΔtCys	ΔtHcy	ΔtCGly	ΔtGSH	ΔtCys	ΔtHcy	ΔtCGly	ΔtGSH
2	−19	21.9$^{###}$	−2.5	1.2	−2	20.2$^{###}$	−5.7	−0.2
4	−3	25.8$^{###}$	−3	0.6	−10	28.5$^{###}$	−4.7	0.4
6	15	31.6$^{###}$	−2.8	0.1	8	33.0$^{###}$	−4.3	0.6

Mean values are expressed as micromolarity, standard deviations were omitted for clarity. Δ represents the difference between mean at each time and baseline. No significant differences between MS and control groups were found for each compound at all time points (one-way ANOVA). Significant differences from baseline within each groups are reported (### $P < 0.001$)

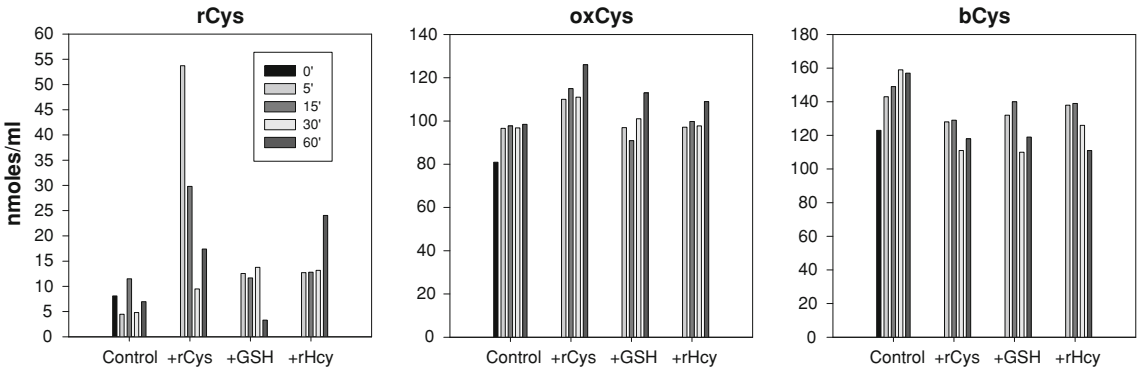

Fig. 5 Time course of plasma redox species of Cys after addition of reduced species (rCys, GSH, and rHcy) to plasma of healthy subjects. Blood from healthy volunteers (2 females, 2 males) was drawn and plasma was immediately treated with 60 μM (final concentration) of reduced specified species. Variations of redox species over time were measured by HPLC and rCys, oxCys, and bCys are here reported (results of a typical experiment; similar results were obtained for all subjects). Zero time of control (0′) represents the redox species concentration in the original plasma. Values within the time range of 5′–60′ are the concentrations measured after addition of same volume of buffer or reduced thiol

intricate dethiolation/thiolation phenomena involving all thiol species when the reduced species are increased. In this context, bCys dethiolation, described by reaction (5), resulted faster than the rethiolation to bCys. Similarly, a maximum of dethiolation was confirmed at 2 h, while the return of XSSP to normal levels occurred at 6 h (Fig. 3). In our opinion, remodeling processes of XSSP serve to delay the spontaneous XSSP accumulation observed in pathological subjects (patients with cardiovascular disease with or without kidney failure) as well as in healthy individuals by age (El-Khairy et al. 1999; Di Giuseppe et al. 2004).

Elevated plasma tHcy levels after fasting have been reported in MS patients (Lucchinetti et al. 1998; Ramsaransing et al. 2006; Besler and Čomoğlu 2003; Vrethem et al. 2003), but these findings were not confirmed in this study. Decreases in tCys and tCGly (data not shown) after MLT of healthy subjects are in line with findings of other authors (Mansoor et al. 1995; Obeid et al. 2004; Doshi et al. 2005; Ubbink et al. 1992).

The significant increase in tCys and bCys in MS patients at fasting are new findings whose origin is of difficult explanation. tCys plasma concentrations of MS patients persisted in being increased with respect to controls after MLT (Fig. 4), whereas the difference of bCys concentrations between groups became non-significant. In contrast to this, oxCys concentrations of both the groups, not significantly different under fasting conditions, became significant at 2, 4, and 6 h (Fig. 2). Moreover, bCys was more prone to be dethiolated in MS patients than in controls. All these changes are an evident expression of activation of SH/SS exchange reactions. This stimulus is strictly related to the methionine metabolism and to the plasma increase in circulating thiols (Hcy and Cys). However, while tHcy was increased, tCys was unchanged in both the groups after MLT. Since plasma cystathionine, a metabolite of the transsulfuration pathway, is augmented after MLT (Doshi et al. 2005), the observation that tCys did not change in both the groups after MLT was an unexpected result. Since tCys plasma concentrations in both the groups after MLT are not increased (Table 3), this observation does not consent to support the hypothesis of an up-regulation of transsulfuration reactions in MS patients.

The increase in plasma tCys in MS at fasting might also be linked to decreased levels of plasma cobalamin or methylene tetrahydrofolate reductase (MTHFR) activity. In

the methionine cycle, cobalamin and MTHFR are important to regenerate methionine from rHcy. If the remethylation pathway were deficient, the cellular growth of rHcy might be compensated by the activation of transsulfuration reactions with rCys formation and release of rHcy and rCys excesses from cells into plasma. No difference in expression of the MTHFR gene between controls and MS patients (data not shown), according to Tajouri et al. (2006) was observed under fasting conditions. Even folic acid and cobalamin levels were within a normal range in MS subjects at fasting (folic acid of MS group = 9.1 ± 4.2 ng/ml; B12 vitamin = 733 ± 230 pg/ml). Besides same concentrations of albumin in plasma of MS and control groups were measured (albumin concentration of MS group = 45.5 ± 1.6 mg/ml). Therefore, these data do not support the possibility of a reduced remethylation of rHcy of MS patients.

In an animal model of EAE, the importance of the methionine cycle and of *S*-adenosyl-homocysteine (AdoHcy) hydrolase, as influencing enzyme of the T cell activation and other autoimmune/inflammatory processes (such as those occurring in MS), has been described (Fu et al. 2006; Lawson et al. 2007). AdoHcy hydrolase is the enzyme responsible for Hcy and Ado production. Inhibition of AdoHcy hydrolase, and therefore of the transmethylation mediated by AdoHcy (a potent feed-back inhibitor of all *S*-adenosyl-methionine-dependent cell transmethylation) is an important step for immunosuppression and block of EAE induction (Fu et al. 2006; Lawson et al. 2007). The loss of inhibition of AdoHcy hydrolase should generate an up-regulation of AdoHcy hydrolase activity and an increase in cellular rHcy. In turn, the growth of cell rHcy could be controlled by remethylation to methionine or metabolism to rCys by transsulfuration reactions. These interesting observations of the literature require further studies to verify whether a relationship exists between these activities and increased tCys levels at fasting in MS patients.

Originally this study was carried out supposing that MLT of MS patients could reveal possible disfunctions at level of the intricate methionine metabolism and explain the reason of increased tHcy levels in MS patients (Lucchinetti et al. 1998; Ramsaransing et al. 2006; Besler and Čomoğlu 2003; Vrethem et al. 2003). By contrast, we found that plasma levels of Cys at fasting and after MLT were augmented in these patients, whereas those of tHcy were always unchanged with respect to controls (Fig. 4). Although increased tCys levels in MS patients at fasting could suggest an up-regulation of the transsulfuration pathway in these subjects, the hypothesis was denied by data of MLT experiments.

In conclusion, increases in plasma XSSX and XSSP are a recurrent finding in various pathological disorders of free-radical origin and are also occurring in MS patients after MLT. Oxidation and SH/SS exchange reactions work together to cause increases in plasma XSSX and XSSP and the redox form variation of the main plasma thiols is greatly stimulated after MLT. The present results emphasize the regulatory role exerted by albumin-mediated SH/SS exchange reactions. We found increases in bCys and tCys levels in plasma of MS patients at fasting, but at the moment we can not explain their origin.

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