

Diurnal variation in glutathione and cysteine redox states in human plasma¹⁻³

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ABSTRACT

Background: Plasma glutathione/glutathione disulfide (GSH/GSSG) and cysteine/cystine (Cys/CySS) couples are oxidized in humans in association with oxidative stress and cardiovascular disease risk. Animal studies show that both pools undergo diurnal variations associated with dietary intake of sulfur amino acids.

Objective: The objective of this study was to determine whether the redox state of GSH, Cys, GSH/GSSG, or Cys/CySS undergoes diurnal variation in healthy adults.

Design: Plasma samples were collected every hour for 24 h from 63 persons aged 18–86 y who were consuming normal food (protein, $0.8 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$; sulfur amino acids, $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) at standardized mealtimes. Measurements of Cys, CySS, GSH, and GSSG were used with the Nernst equation to calculate the redox states.

Results: Plasma Cys and GSH concentrations varied with the time of day. The highest values for plasma Cys occurred ≈ 3 h after meals. Glutathione was maximal 6 h after peak plasma Cys. The calculated redox states of the GSH/GSSG and Cys/CySS couples varied in association with the concentrations of the thiol forms. Maximal reduction and oxidation of the Cys/CySS couple occurred at 2130 and 0630, whereas the respective values for the GSH/GSSG couple occurred at 0330 and 1330. The mean diurnal variation for Cys/CySS redox in persons aged ≥ 60 y was 1.8-fold that in persons aged < 40 y.

Conclusions: Cys/CySS and GSH/GSSG redox states in human plasma undergo diurnal variation with an increased magnitude of variation in Cys/CySS redox state in older persons. This variation could alter sensitivity to oxidative stress over a course of hours. *Am J Clin Nutr* 2007;86:1016–23.

KEY WORDS Amino acids, oxidative stress, aging, sex-dependent differences, thiol

INTRODUCTION

The sulfur-containing amino acids, methionine and cysteine (Cys), are the only amino acids that undergo reversible oxidation-reduction (redox) changes under physiologic conditions. Such oxidative changes provide mechanistic switches to control protein conformation, catalytic activity, protein-protein interactions, protein-DNA interactions, and protein trafficking, and increased concentrations of oxidized forms of Cys and methionine provide indicators of oxidative stress (1, 2). The redox states of glutathione/glutathione disulfide (GSH/GSSG) and Cys/cystine (Cys/CySS) are oxidized in association with risk factors for cardiovascular disease (CVD), including age (3), cigarette smoking (4), type 2 diabetes (5), and carotid intima-media

thickness (6). It is important that recent studies using a model to study early events of atherogenesis showed that an oxidized extracellular Cys/CySS redox state is sufficient to stimulate monocyte binding to endothelial cells (7). Thus, if there is a causal relation of redox state and CVD development, a transient oxidation of Cys/CySS or GSH/GSSG could define the period of greatest risk.

Diurnal variation of hepatic GSH in rodents is linked to sulfur amino acid intake (8, 9), and plasma GSH varies in association with hepatic GSH (10, 11). Cys, a precursor for GSH, also undergoes a diurnal variation in mouse liver and pancreas, and the maximum and minimum values precede the respective values for GSH (12). Thus, thiol concentrations in rodents vary as a function of time of day linked to the dietary intake of sulfur amino acids. In humans, nonprotein thiols and GSH vary in bone marrow samples taken at 12-h intervals (13, 14), but no diurnal change in GSH was detected in skeletal muscle obtained by biopsy (15). Plasma Cys/CySS redox became more reduced after an oral load of Cys (16), but such data do not show the magnitude of oxidation and reduction with the intake of normal food.

The current study was designed to determine whether diurnal or meal-associated variations in Cys/CySS and GSH/GSSG redox states occur in the plasma of healthy adults consuming normal food (protein: $0.8 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$; sulfur amino acids: $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) at standardized mealtimes. We previously found that persons > 61 y old had significantly more oxidation than did persons < 43 y old (5) and that GSH/GSSG redox state had a biphasic curve in relation to age with a transition in the region of 45–50 y (3). Because nonlinear models are difficult to interpret with small sample size, we designed the study with age

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as a categorical factor and using 3 groups—<40, 40–59, and ≥ 60 y old—and approximately equal numbers of men and women to allow an analysis of time effects by sex. Subjects were asked to abstain from dietary supplements for 2 wk to avoid short-term effects of antioxidants (eg, vitamin C) on the GSH system. Differences in diet, exercise, and environment were minimized during the 24-h study period by conducting the study in the Emory General Clinical Research Center (GCRC).

SUBJECTS, MATERIALS, AND METHODS

Materials

Sodium heparin, bathophenanthroline disulfonate sodium salt (BPDS), sodium iodoacetate, dansyl chloride, L-serine, GSH, GSSG, Cys, CySS, and sodium acetate trihydrate were obtained from Sigma Chemical Corp (St Louis, MO). γ -Glutamylglutamate was obtained from MP Biomedicals Corp (Irvine, CA). The mixed disulfide of Cys and GSH (CySSG) was obtained from Toronto Research Chemicals (Toronto, Canada). Boric acid, sodium tetraborate, potassium tetraborate, perchloric acid, and acetic acid were reagent grade and were purchased locally. Methanol, acetone, and chloroform were HPLC grade.

Human subjects

This study was reviewed and approved by the Emory Investigational Review Board. A total of 63 volunteers, self-described as healthy, were recruited by posting fliers in public locations in the Atlanta/Emory University community. After providing written informed consent, subjects were admitted to the outpatient unit of the GCRC, given a brief questionnaire for demographic information, and screened via medical history, physical examination, urinalysis, standard chemistry profile, and complete blood count. Eligibility was established by the absence of evidence of acute or chronic illness (other than a history of well-controlled hypertension), no current smoking, a body mass index (BMI; in kg/m^2) < 30, and willingness to withdraw from nutritional supplements, if consumed, for 2 wk before entry. Subjects were then scheduled for a 24-h inpatient visit within 2 wk of screening in the GCRC. Premenopausal females were scheduled for study between days 2 and 14 of the follicular phase of the menstrual cycle; no females in the study were taking oral contraceptives. Although an attempt was made to obtain equal distribution among the age groups—18–39, 40–59, and 60–89 y old—a change in sample collection procedure (see Sample collection and analysis, below) and the inclusion of a nonrepresentative number of self-described vegans in the younger group resulted in excess numbers in the younger group.

For the evening before GCRC admission, subjects were instructed not to eat after 2200 to standardize baseline fasting levels. Subjects were admitted to the GCRC at 0700 on day 1, and a heparin-lock catheter was placed in a forearm vein for blood sampling at 0800. After a 30-min supine resting period, 3-mL blood samples were drawn hourly for 24 consecutive hours (from 0830 to 0830). Subjects were given breakfast at 0930, lunch at 1330, dinner at 1730, and an evening snack at 2130, just after the timed blood draw for that hour. The composition of the 3 meals and the snack was standardized for all subjects. The diet consisted of nutritionally balanced foods providing total daily energy intake of 30% over basal energy expenditure (calculated by the Harris-Benedict formula). Total energy intake was provided

as 15% protein (based on 0.8 g protein/kg/d), 30% fat, and 55% carbohydrate, and the distribution was 30%, 30%, 30%, and 10% at breakfast, lunch, dinner, and snack, respectively. Water was provided ad libitum throughout the admission. A few vegan subjects ($n = 8$) were included in the study. They received the same proportions of macronutrient and energy intake but were fed a diet that lacked animal-derived foods: eg, protein-fortified whole-grain cereal, soy milk, fruit juice, vegetable sandwich, hummus, tabouli, lasagna, salad and dressing, and beverage. Subjects were allotted 45 min to complete their meals and 15 min to complete the snack. Activity was confined to walking at limited intervals. Otherwise, subjects remained in their rooms, either lying in bed or sitting in a chair.

Sample collection and analysis

Blood samples were collected via a heparinized 5-mL syringe with the use of a needleless system. Samples were collected from the first 15 subjects, mostly in the younger age group, with 0.5 mL preservative solution and 0.5 mL blood, as previously described (17). Because of variability in GSSG measurements, the collection procedure was modified to use 0.15 mL of a more concentrated preservative solution and 1.35 mL blood. This procedure improved the accuracy and reproducibility of GSSG measurements but had no detectable effect on the average GSH/GSSG redox value. Consequently, for the overall analyses of time-of-day variation, these data were included; because of the unequal distribution by age, however, data from the first 15 subjects were excluded from comparisons based on age.

For the modified collection procedure, 1.35 mL blood was immediately transferred to a microcentrifuge tube containing 0.15 mL of a preservative solution consisting of 0.5 mol L-serine/L, 9.3 mmol BPDS/L, 0.165 mol γ -glutamylglutamate/L, 0.4 mol boric acid/L, 0.1 mol sodium borate/L, 0.144 mol sodium iodoacetate/L, and 2.5 mg sodium heparin/mL. Samples were treated with dansyl chloride and analyzed by HPLC with fluorescence detection (17, 18). Plasma GSH/GSSG and Cys/CySS redox states were calculated from the respective concentrations by using the Nernst equation with E_o values at pH 7.4 for GSH/GSSG of -264 mV and for Cys/CySS of -250 mV (17).

Statistical analysis

We use SAS software (version 8; SAS Institute Inc, Cary, NC) for all analyses. A small number of outliers (principally GSH and GSSG) were identified as values >3 SDs from the mean at the respective time point, and they were replaced by the mean of the values from the preceding and following time points for that person. Analyses on effects of time were performed via PROC MIXED, using repeated-measures 1-, 2-, and 3-factor analyses of variance (ANOVAs). In each of the models, time was the repeated-measures factor, and age, sex, or both were fixed effects. Within the 3-factor ANOVA setting, various time effect slices were calculated, with stratification by age, sex, and age by sex. Analysis of covariance was used to investigate how BMI confounds the effects of age, sex, or both. Results were considered significant at $P \leq 0.05$.

RESULTS

Subject characteristics

Demographic characteristics of the 63 subjects are summarized in **Table 1**. The overall population was 54% female, and the



TABLE 1

Characteristics of the total study population ($n = 63$) and a controlled subsample ($n = 38$) for analysis according to sex, age, race, and BMI¹

	Subjects	Age	Percentage European		BMI
			white	BMI	
	<i>n</i> (%)	<i>y</i>	%	<i>kg/m</i> ²	
Total	63 (100)	44 ± 19 ²	67	24.6 ± 3.6	
Total males	29 (46)	45 ± 19	55	26.4 ± 3.3	
Male	21	46.9 ± 18.7	57	26.3 ± 3.3	
<40 y old	8	27.8 ± 6.1	38	25.9 ± 3.4	
40–59 y old	7	48.6 ± 4.6	71	26.0 ± 3.3	
≥60 y old	6	70.3 ± 8.8	67	27.1 ± 3.6	
Total females	34 (54)	43 ± 20	76	23.1 ± 3.1	
Female	17	51.5 ± 20.7	76	23.3 ± 3.9	
<40 y old	5	25.2 ± 3.9	60	21.7 ± 1.0	
40–59 y old	6	51.5 ± 7.7	67	25.7 ± 5.3	
≥60 y old	6	73.5 ± 5.7	100	22.2 ± 3.0	

¹ Using Fisher's exact test in a comparison of race versus age and sex and of BMI versus race and sex, race was not significant for race versus age, race versus sex, race versus age stratified by sex, or race versus sex stratified by age. Using 2-way ANOVA, there was no age or age × sex interaction; only males > females ($P = 0.0001$).

² $\bar{x} \pm SD$ (all such values).

mean ± SD age was 43 ± 20 y for women and 45 ± 19 y for men. Most of the subjects ($n = 42$) were European white, 15 were African American, 3 were Asian, 2 were Hispanic, and 1 was Middle Eastern. African Americans were evenly distributed among the age groups, whereas the Asian, Hispanic, and Middle Eastern subjects were only in the younger group (aged 18–39 y). No one currently smoked, but 8 were former smokers; all but 1 of these 8 were in the older group (≥60 y old). Most subjects (39/63) consumed <1 alcoholic beverage per week, and only 2 reported usual consumption of >1 alcoholic drink/d. Most subjects reported good-to-excellent sleep habits; 4 subjects in the younger group reported poor sleep, and 1 subject in the middle-aged group and 2 subjects in the older group reported having intermittent symptoms compatible with sleep apnea. Attempts were made to avoid sleep disruption in the GCRC, and nursing reports indicated only transient arousal during the night of study in all subjects. Most subjects (50/63) reported regular use of multivitamins, other nutritional supplements, or both. Approximately one-half of the subjects in the younger and middle-aged groups reported taking no regular exercise, whereas 12 of the 14 subjects in the older group reported regular exercise ≥3 times/wk. Of the subjects in the younger and middle-aged groups, ≈50% indicated moderate-to-strenuous exercise. Regular exercise in the older group was almost exclusively walking or other low-impact activity.

A subset of 38 subjects (Table 1) was used for comparisons for age and sex. This subset was created because the distribution of subjects studied with the original preservative solution ($n = 15$) and vegans ($n = 8$) was unequal among the age groups. Although we had no reason to believe that the original preservative solution provided data that were not equivalent to those obtained with the modified solution, this exclusion eliminated the change in analytic procedures as a possible contributing factor to observed age-dependent changes. In contrast, preliminary analyses showed that plasma Cys for vegans differed from that for non-vegans, and thus the exclusion of vegans was necessary. There

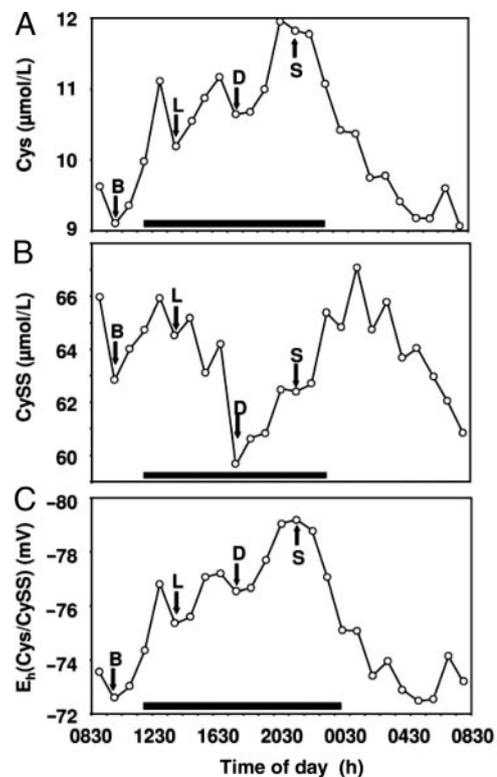


FIGURE 1. Diurnal variations in plasma cysteine (Cys; A), cystine (CySS; B), and the redox state of Cys/CySS [$E_h(\text{Cys/CySS})$; C] in 63 healthy persons aged 18–86 y. B, breakfast; L, lunch; D, dinner; S, snack. Subjects were studied in an inpatient metabolic unit and received ordinary food, as outlined in Subjects, Materials, and Methods. The scale for $E_h(\text{Cys/CySS})$ is oriented with the most reduced (most negative) values on top and the most oxidized (most positive) values on bottom. Values expressed are means for all subjects at each respective time point; SDs were omitted for clarity. The time-of-day effect on the biomarkers of oxidative stress [Cys, CySS, and $E_h(\text{Cys/CySS})$] in the total population ($n = 63$) was significant, $P < 0.0001$ for all.

were too few vegans to include them in a 4-way repeated-measures ANOVA and obtain any reliable information. We made the decision to delete them from the age and sex analysis. One subject who took acetaminophen during the study period and 1 subject whose hypertension was poorly controlled during the inpatient study period also were excluded from those analyses.

Diurnal variations in plasma Cys, CySS, and plasma Cys/CySS redox

The average Cys values for the total group of 63 human subjects over a 24-h period showed a significant time-dependent variation ($P < 0.0001$; **Figure 1**). During the day, plasma Cys increased with time, reaching a mean maximum value (12 $\mu\text{mol/L}$) at 2030. After this time point, the plasma Cys decreased to a minimum value (9 $\mu\text{mol/L}$) at 0430–0630. Increases in plasma Cys were closely related to mealtimes (0930, 1330, and 1730), occurring ≈2–3 h after meals.

The pattern of plasma CySS also showed a diurnal variation (Figure 1); the mean concentrations ranged from 59 $\mu\text{mol/L}$ (at 1730) to 67 $\mu\text{mol/L}$ (at 0130). The absolute magnitude of change in CySS (7.5 $\mu\text{mol/L}$) above the minimum value was considerably greater than that for Cys (3 $\mu\text{mol/L}$), but in a relative sense, the change in CySS was only 12%, whereas the variation in the



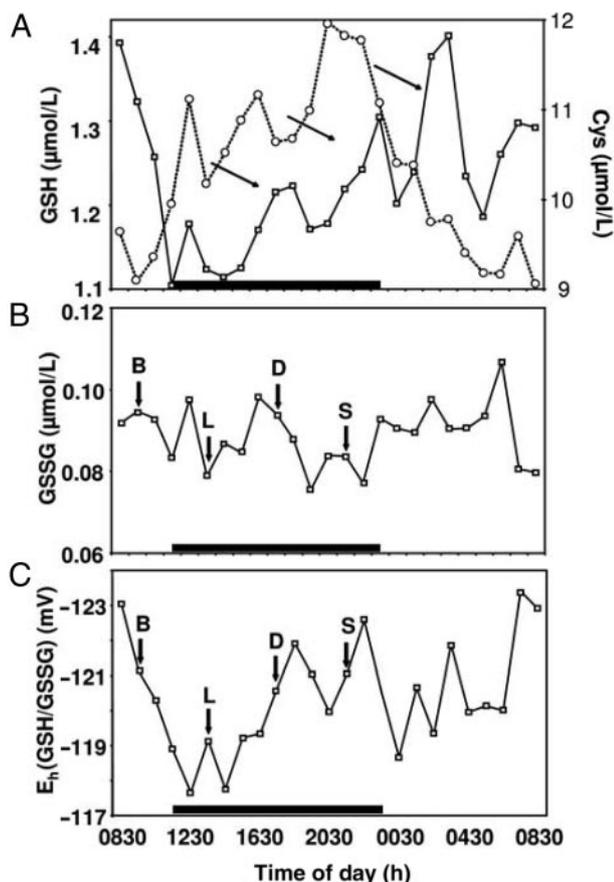


FIGURE 2. Diurnal variations in plasma glutathione (GSH; □; A) glutathione disulfide (GSSG; B), and the GSH/GSSG redox state (C) in 63 healthy persons aged 18–86 y. B, breakfast; L, lunch; D, dinner; S, snack. Studies were conducted and analyzed as described in Figure 1 and in Subjects, Materials, and Methods. A) ○, the corresponding variations in cysteine (Cys) concentrations from Figure 1A for comparison. Diagonal arrows indicate corresponding but delayed increases in GSH that follow meal-associated increases in Cys. Values expressed are means; SDs were omitted for clarity. The time-of-day effect on the biomarkers of oxidative stress was $P = 0.05, 0.33,$ and 0.02 for GSH, GSSG, and $E_h(\text{GSH}/\text{GSSG})$, respectively.

Cys concentration was 33%. Visual inspection of the pattern of plasma CySS suggested a 12-h cycle rather than a 24-h cycle, with each cycle reaching the zenith around midday or midnight. The 0830 time point immediately after admission was greater than the 0830 time point at the end of the study (Figure 1B), which may have been due to differences in activity level or in other dietary or behavioral variables between the period immediately before the study and that in the research center.

Plasma Cys/CySS redox [$E_h(\text{Cys}/\text{CySS})$] showed a significant time-of-day effect with a range from -72 mV (most oxidized at 0630) to -79 mV (most reduced at 2130) (Figure 1C). Thus, the general pattern of $E_h(\text{Cys}/\text{CySS})$ was to become reduced during the day and to become oxidized during the evening. As indicated by the mealtimes shown in Figure 1C, the pattern of $E_h(\text{Cys}/\text{CySS})$ was closely associated with the meals.

Variation in plasma GSH and GSSG

Plasma GSH variation with time had borderline significance ($P = 0.05$) and was shifted to the right compared with the Cys curve (Figure 2). The maximal plasma GSH concentration occurred 6 h after the maximal plasma Cys concentration. The

TABLE 2

Values for a 3-way interaction and BMI and an estimate of the SDs for plasma cysteine (Cys) and glutathione (GSH) variables in the controlled subsample¹

Biomarker	<i>P</i>		
	Three-way interaction ²	BMI	SD ³
Cys	0.0944	0.1193	1.83
CySS	0.9099	0.2849	8.73
$E_h(\text{Cys}/\text{CySS})$	0.0748	0.0731	4.27
GSH	0.3514	0.6666	0.52
GSSG	0.9882	0.1487	0.05
$E_h(\text{GSH}/\text{GSSG})$	0.8250	0.3559	8.96

¹ CySS, cysteine; $E_h(\text{Cys}/\text{CySS})$, redox state for plasma Cys/CySS couple; glutathione disulfide, GSSG; $E_h(\text{GSH}/\text{GSSG})$, redox state for plasma GSH/GSSG couple.

² Sex \times age \times time interaction analyzed in a controlled subsample ($n = 38$) with characteristics given in Table 1. There were no 2-way interactions in the 3-way ANOVA.

³ Estimates of the pooled within-subject SD from the 3-way ANOVA.

minimal plasma GSH value occurred in the middle of the day (1130–1530, centered at 1330)—ie, ≈ 7 h after the minimum for plasma Cys. The plasma GSSG showed no significant time-dependent variation ($P = 0.33$; Figure 2B). The plasma GSH/GSSG redox [$E_h(\text{GSH}/\text{GSSG})$] showed a diurnal variation of 6 mV ($P < 0.05$; Figure 2C); the greatest oxidation of $E_h(\text{GSH}/\text{GSSG})$ occurred when the plasma GSH was at a minimum. In contrast, the most reduced $E_h(\text{GSH}/\text{GSSG})$ values were found in the early waking hours (0730–0830) and in the evening (1730–2130). The pattern of the $E_h(\text{GSH}/\text{GSSG})$ also had an appearance of a 12-h periodicity, which may be noteworthy because that pattern mirrors the 12-h periodicity seen with CySS (Figure 1B) and because it is consistent with a previously observed correlation of CySS with $E_h(\text{GSH}/\text{GSSG})$ (19).

Sex-dependent effects on diurnal variations in the plasma Cys/CySS and GSH/GSSG redox states

Results for the sex \times age \times time interaction, P values for BMI interactions, and estimates of the within-subject SD from the 3-factor ANOVA are presented in Table 2. No values were significant at $P < 0.05$, but several variables showed a trend, which indicated that an investigation of subgroup responses may be appropriate. Because an a priori purpose of this study was to determine the effect over time in men and women separately, specific effects for age and sex versus time were determined. BMI was never a significant covariate. The time-of-day variations of Cys concentration, CySS concentration, and $E_h(\text{Cys}/\text{CySS})$ in men and women are shown in Figure 3. A significant time effect \times sex interaction was observed for Cys concentration and $E_h(\text{Cys}/\text{CySS})$ in men and women (Table 3); however, the time-of-day effect for CySS was significant only in women (Table 3). The shapes of the curves for the plasma Cys showed that men had significantly higher values for most time points than did women (Figure 3A). CySS concentrations were lower in men than in women (Figure 3B). The resulting $E_h(\text{Cys}/\text{CySS})$ was significantly more reduced in men than in women (Figure 3C; $P < 0.001$).

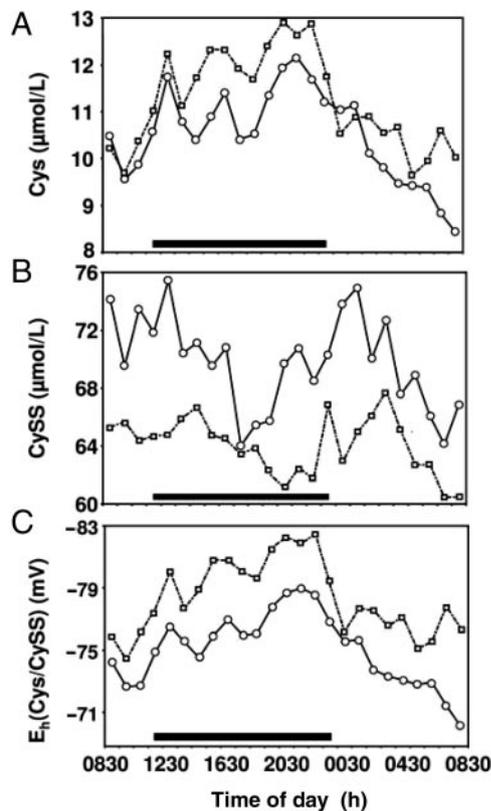


FIGURE 3. Diurnal variations in plasma cysteine (Cys; A), cystine (CySS; B), and the Cys/CySS redox state (C) in women (○; $n = 17$) and men (□; $n = 21$). Values expressed are means; SDs were omitted for clarity. An estimate of the pooled within-subject SD for each variable is provided in Table 2. The time-of-day effect on the biomarkers of oxidative stress in the controlled subsample ($n = 38$) was $P < 0.0001$ for Cys, CySS, and $E_h(\text{Cys}/\text{CySS})$ and $P = 0.12$, 0.78 , and 0.61 for GSH, GSSG, and $E_h(\text{GSH}/\text{GSSG})$, respectively. Because an a priori purpose of the present study was to determine the effect over time, and because the male and female subjects differed in the 2-way ANOVA for the entire study population (Table 1), specific effects for age and sex versus time were examined. Significant time-of-day effects were seen for Cys and $E_h(\text{Cys}/\text{CySS})$ in both sexes. For men ($n = 21$), the time effect by sex was $P < 0.0001$ for Cys and $E_h(\text{Cys}/\text{CySS})$ and $P = 0.31$ for CySS. For women ($n = 17$), the time effect by sex was $P < 0.0001$ for Cys, CySS, and $E_h(\text{Cys}/\text{CySS})$.

For GSH, GSSG, and $E_h(\text{GSH}/\text{GSSG})$, there were no significant time-dependent effects as a function of sex (Table 3). However, women showed a trend ($P = 0.088$) for time-of-day effect on GSH concentration.

Age-dependent effects on diurnal variations in the plasma Cys/CySS and GSH/GSSG redox states

Time-dependent effects were seen for Cys in all 3 age groups (Figure 4 and Table 3). To estimate the magnitude of diurnal variation, values for 2030, 2130, and 2230 were averaged to obtain a mean maximal value for each subject, and those for 0430, 0530, and 0630 were averaged to obtain a mean minimal value. The difference between the maximal and minimal values in the younger group (4.5 ± 3.5 mV) was significantly ($P < 0.001$) less than that in the older group (8.3 ± 6.1 mV). For CySS, only the older age group showed a significant time-of-day variation in concentrations ($P < 0.001$; Table 3). The plasma CySS concentration in the older age group was significantly higher than that in the younger and middle-aged groups at all time points

TABLE 3

Time-of-day effect on the plasma cysteine (Cys)/Cystine (CySS) redox pool in the controlled subsample stratified by sex and age¹

Time effect by sex	Cys	CySS	$E_h(\text{Cys}/\text{CySS})$
Males ($n = 21$)			
P	<0.0001	0.31	<0.0001
Females ($n = 17$)			
P	<0.0001	<0.001	<0.0001
Time effect by age group	Cys	CySS	$E_h(\text{Cys}/\text{CySS})$
<40 y old ($n = 13$)			
P	<0.0001	0.66	<0.0001
$40\text{--}59$ y old ($n = 13$)			
P	<0.0001	0.26	<0.0001
≥ 60 y old ($n = 12$)			
P	<0.0001	<0.001	<0.0001

¹ $E_h(\text{Cys}/\text{CySS})$, redox state for plasma Cys/CySS couple. P values are from various slices of 3-way ANOVA; note that these analyses were based upon the experimental design to study age as a categorical factor; results for the sex \times age \times time interaction are presented in Table 2. There were no significant 2-way interactions. For 3-way analysis, no values showed significance at $P < 0.05$, but several variables showed a trend ($P < 0.10$), which indicates that subgroup analyses may be appropriate.

($P < 0.0001$; Figure 4B). The pattern of variation in $E_h(\text{Cys}/\text{CySS})$ over the 24-h period did not differ among the age groups except that the older group had significantly more oxidization at most time points (Figure 4C).

No significant time-of-day effects were observed for GSH, GSSG, or GSH/GSSG redox when data were analyzed by age group. However, a trend was observed for GSH among women (Table 3), and P values for GSH and $E_h(\text{GSH}/\text{GSSG})$ were relatively small only in the younger group.

DISCUSSION

Previous studies established that GSH concentration undergoes a diurnal variation in rodent liver that is linked to the dietary intake of sulfur amino acids (8, 9). Rodent studies also show that GSH is continuously released from hepatic and other tissues, and biochemical studies show that GSH undergoes thiol-disulfide exchange with plasma CySS to form CySSG, the disulfide of Cys and GSH. In circulation in vivo, GSH, CySSG, and GSSG are hydrolyzed in kidney to release Cys and CySS, thereby maintaining total Cys supply in postabsorptive periods (1). The present study shows that human plasma Cys concentrations vary in a diurnal fashion: peak values occur 3 h after the beginning of mealtime, and maximal values occur in the evening, between 2030 and 2230. The plasma Cys/CySS redox closely mirrored the changes in Cys concentration: CySS concentrations were much higher under all conditions, had less percentage change, and did not change in association with Cys concentration.

GSH concentration showed a time-of-day variation with an oscillatory pattern resembling that for Cys, but with a phase delay of 6 to 7 h. Maximal values occurred in the middle of the night (0230–0330). No significant diurnal variations in GSSG concentrations were detected, and, as did plasma Cys/CySS redox, plasma GSH/GSSG redox mirrored the change in GSH concentration.

The mean differences between maximal and minimal redox state values for Cys/CySS and GSH/GSSG were 6 and 4.5 mV, respectively. In the context of redox-sensitive proteins, a 6-mV difference is equivalent to a 1.6-fold increase in the ratio of

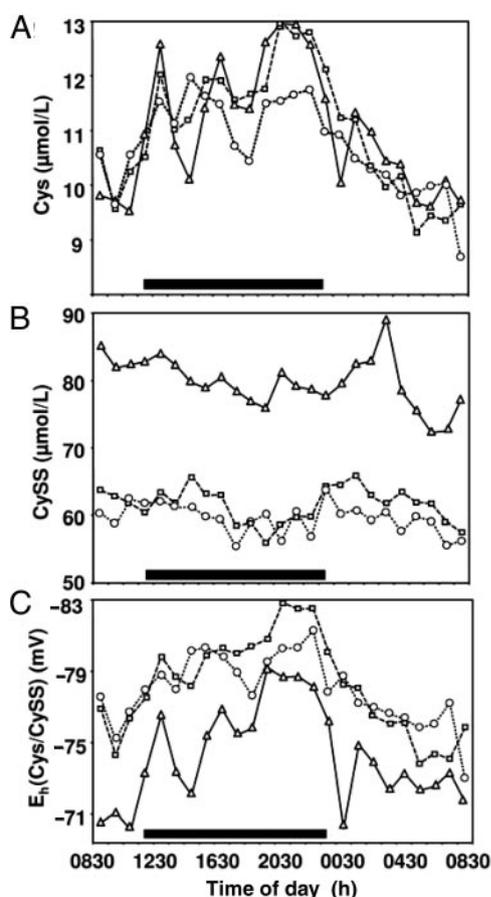


FIGURE 4. Diurnal variations in cysteine (Cys; A), cystine (CySS; B), and the Cys/CySS redox state (C) in the study population stratified according to age. \circ , subjects aged 18–39 y ($n = 13$); \square , subjects aged 40–59 y ($n = 13$); and \triangle , subjects aged >60 y ($n = 12$). Values expressed are means; SDs were omitted for clarity. An estimate of the pooled within-subject SD for each variable is provided in Table 2. Because an a priori purpose of this study was to determine the effect over time in different age groups, specific effects for age versus time were examined. Significant time effects were observed for Cys and $E_h(\text{Cys/CySS})$ in all age groups. In the group aged <40 y ($n = 13$), the time effect by age group was $P < 0.0001$, $P = 0.66$, and $P < 0.0001$ for Cys, CySS, and $E_h(\text{Cys/CySS})$, respectively. In the group aged 40–59 y ($n = 13$), the time effect by age group was $P < 0.0001$, $P = 0.26$, and $P < 0.0001$ for Cys, CySS, and $E_h(\text{Cys/CySS})$, respectively. In the group aged ≥ 60 y ($n = 12$), the time effect by age group was $P < 0.0001$, $P < 0.001$, and $P < 0.001$ for Cys, CySS, and $E_h(\text{Cys/CySS})$, respectively.

dithiol to disulfide forms (dithiol:disulfide). Thus, even a 6-mV difference could be functionally important in redox-dependent processes such as monocyte adhesion to endothelial cells. In the older group, the mean difference for Cys/CySS was 8 mV, which is equal to nearly a 2-fold increase in dithiol:disulfide. This change could contribute to age-dependent redox-sensitive pathophysiological processes. Superimposed on oxidation associated with cigarette smoking [9 mV for both Cys/CySS and GSH/GSSG redox states (4)] and type 2 diabetes [15 mV for GSH/GSSG (5)], the time-of-day variations could increase the risk of events that contribute to disease. Although no causal link has been established, oxidation of plasma Cys/CySS redox (odds ratio, 13.6) and GSH/GSSG redox (odds ratio 6.1) has recently been associated with persistent atrial fibrillation in a cross-sectional, case-control study of males matched for other known risk factors (20).

The mechanisms underlying cell responses to extracellular Cys/CySS and GSH/GSSG redox are only beginning to be elucidated. Monocyte adhesion to endothelial cells in response to oxidized Cys/CySS is signaled through nuclear factor- κ B-dependent activation of cell adhesion molecule expression, mediated by a sensing mechanism that is sensitive to nonpermeant alkylating agents (7). Human retinal pigment epithelial cells under more oxidized Cys/CySS are more sensitive to oxidant-induced apoptosis, which is mediated by a mitochondrial mechanism (21). Proliferation of colon carcinoma CaCo-2 cells is doubled by a reducing Cys/CySS redox (22, 23); this process is mediated by the activation of a metalloproteinase that releases an epidermal growth factor receptor ligand and activates the mitogen-activating protein kinase (24). Thus, the accumulating data indicate that multiple mechanisms are involved in controlling processes that are sensitive to the extracellular redox state.

The transport and enzymatic systems functioning in the control of Cys and GSH homeostasis have been extensively studied, but the key determinants of plasma Cys/CySS and GSH/GSSG redox states are not well established. Studies of Cys turnover measured with stable-isotope tracer methods showed that Cys flux is $0.63\text{--}1.3 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ($38\text{--}80 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) (25–27). The intake of Cys during the present study was $0.17 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, so that, if one assumes uniform absorption for 18 h, the rate of Cys absorption is $\approx 0.16 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, or 12%–25% of the total flux. This magnitude of effect on flux is similar to that found for tracer analyses comparing fed with fasted conditions (25). Kinetic analysis of the hourly Cys concentrations (Figure 1A) can be compared only roughly with the tracer studies because there is no way to evaluate effects on individual rate components affecting the steady-state plasma concentrations. However, the summed increase in the area under the curve for Cys ($29 \mu\text{mol/L}$) was small compared with the oral intake, as expected from extensive clearance from plasma at rates comparable to absorption. Consistent with this, the average rate of increase in plasma Cys between 0930 and 2030 was $0.0038 \mu\text{mol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$, a value indicating that the stimulated clearance of Cys from circulation (or the altered rate of tissue release of Cys plus GSH, or both) very closely matched the rate of Cys absorption. The whole-body rate of clearance of Cys, estimated from the apparent first-order elimination rate constant (slope of a semilog plot of Cys concentration versus time over the period from 2330 to 0530; Figure 1A) was $1.3 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. This value does not differ significantly from the Cys turnover measured by tracer methods (25–27). One must be aware, however, that the rate estimates from the changes in plasma Cys concentration do not take into account the rates of conversion of Met to Cys or the oxidative catabolism of Cys.

It is of considerable interest that the plasma component present at highest concentration, CySS, did not have any apparent association with meals. CySS transport occurs by x_c^- , an Na^+ -independent cystine-glutamate exchange transport system with expression controlled by the antioxidant response element (28) and responsive to amino acid deprivation (29). Variation in CySS concentration contributed little to diurnal changes in redox state, but differences in CySS concentrations between males and females and between the age groups appeared to largely account for sex and age differences in Cys/CySS redox. The expression of x_c^- is regulated by the transcriptional regulator nuclear factor erythroid 2-related factor 2 (Nrf2), which declines with age (30) and which could contribute to the age-dependent increases in

CySS. Consistent with a function of x_c^- in the control of plasma Cys/CySS redox, x_c^- knockout mice have a more oxidized plasma redox state (31). Thus, the CySS transport and reduction systems (separately or together) may be important in determining age and sex differences, but they are not primary determinants of diurnal variations in Cys/CySS redox states. Oxidative degradation of Cys by cysteine dioxygenase is highly inducible (32) and likely to change during the time of day, but the contribution of this system to homeostatic regulation of the redox state cannot easily be inferred from the present data.

The plasma GSH/GSSG redox state is likely to be affected by multiple processes, including synthesis of GSH from its constitutive amino acids, cyclic oxidation and reduction involving GSH peroxidases and GSSG reductase, transport of GSH and GSSG into the plasma, reaction of GSH with CySS, and degradation of GSH and GSSG by γ -glutamyltranspeptidase. Glutamate-cysteine ligase, also known as γ -glutamylcysteine synthetase, is the most important enzyme for GSH synthesis. Glutamate-cysteine ligase is induced by various oxidants, electrophiles, hormones, and heavy metals (33), and glutamate-cysteine ligase activity has been shown to decline with age (34), at least in part because of the decline in nuclear factor erythroid 2-related factor 2 (30). Enzymes needed for the conversion of methionine to Cys also decline with age (35). Additional studies will be needed to understand the relative contributions of these processes to the time-of-day- and age-dependent differences in GSH/GSSG redox control observed in the present study.

In summary, the data show that GSH/GSSG and Cys/CySS redox states exhibit diurnal variations in healthy adults in a pattern indicating that meal intake acutely influences these major antioxidant systems. Furthermore, the data show that homeostatic regulation of Cys and GSH pools declines with age and that this change appears at a younger age in men than in women. Thus, the data indicate that variations in sensitivity to oxidative stress could occur within the time frame of a day as a function of the timing and the quantity and quality of food intake.

The authors' responsibilities were as follows—RAB: participated in the design of the study, subject recruitment, and coordination of the study's clinical aspects under the direction of TRZ; TRZ: participated in clinical design, supervision, data interpretation, and manuscript preparation; BAC: participated in initial data analysis; YP: performed pharmacokinetic analyses of data for discussion of results in the context of previous tracer studies; PYC and GAC: performed statistical analyses; CJA: participated in data interpretation and project supervision; and DPJ: participated in study design, supervision of the analyses, data interpretation, and manuscript preparation. None of the authors had a personal or financial conflict of interest.

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