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Biochemical Pharmacology 65 (2003) 741–746

Biochemical Pharmacology

www.elsevier.com/locate/biochempharm

Protein glutathiolation in human blood

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Received 29 May 2002; accepted 30 July 2002

Abstract

Glutathione (GSH) exists in both free and protein-bound (glutathiolated) forms (GSSP). Protein glutathiolation may represent an important post-translational regulatory mechanism for proteins. However, there are little data regarding the regulation of glutathiolation in blood. Our objectives were to examine GSSP levels of human blood by determining the distribution and variability of blood GSSP, as well as its relationship to free GSH and hemoglobin in healthy adults. To this end, we used a newly modified method allowing for rapid analysis of both GSH and GSSP in blood. GSSP was found in red cells with levels ranging from 4 to 27% of total (free + bound) GSH (mean ± SD: 12.1 ± 4.5%) with a concentration of 0.13 ± 0.05 μEq GSH/mL (mean ± SD). No correlations were observed between GSSP and either GSH ($r = -0.085$) or hemoglobin ($r = 0.086$). Together these results suggest that the extent of protein glutathiolation in blood is substantial (~0.1 mmol/L). While the interindividual variation in GSSP is large (34%), its levels are apparently not regulated by GSH content.

Keywords: Glutathione; Human blood; Protein glutathiolation; Protein thiolation

1. Introduction

GSH is a ubiquitous intracellular low-molecular-weight thiol (γ-glutamyl-cysteinyl-glycine) that functions as a major endogenous antioxidant and redox buffer. GSH plays numerous roles in cellular defense including the detoxification of xenobiotics and peroxides and the maintenance of immune function [1,2]. GSH is also an important regulator of cellular homeostasis through the regulation of protein synthesis and degradation, signal transduction, apoptosis, and gene expression via alteration of redox-sensitive *trans*-acting factors [3–5].

One mechanism by which GSH can regulate cellular functions is through protein thiolation, a post-translational modification involving the formation of protein mixed disulfides of low-molecular-weight thiols such as GSH and key Cys residues in proteins [6]. Cysteine is often a critical component of active sites or key regulatory sites on proteins, and maintenance of the redox status of these Cys groups is necessary for the maintenance of optimal activity. The modification of proteins by glutathiolation is believed to be an important regulator of many cellular functions [7]. To date, numerous proteins have been found to be regulated through this mechanism including c-Jun [8], spectrin [9], protein kinase C [10], ubiquitin conjugating enzymes [11], carbonic anhydrase III [12], and H-ras [13].

GSH in blood and tissues has been reported to occur in both free and protein-bound forms. Bound GSH (GSSP) has been found in a variety of tissues including rat liver [14], heart, kidney [15], and human lens [16]. In the liver, reported levels of GSSP can be as high as 30% of total (free + bound) GSH [17]. Previously, we observed in human erythrocytes that GSSP constituted a substantial fraction of total GSH levels (17%) [18]. Little is known regarding the factors that regulate protein glutathiolation in blood. The current objectives were to determine levels and degree of variation of GSSP in blood from healthy free-living adults. In addition, the relationships between the levels of GSSP and hemoglobin were examined.

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Abbreviations: GSSP, protein-bound glutathione; MPA, metaphosphoric acid.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01560-5

2. Materials and methods

2.1. Blood collection and processing

Study subjects were healthy adults (subject characteristics are provided in Table 1) who participated in an in-house health screening of American Health Foundation (AHF) employees. Subjects were recruited using flyers, postings, and staff memos announcing the screening. Refusal rates were low as a total of 105 of the 136 employees (77%) at the Valhalla, NY, site participated in the study. Study subject characteristics and health parameters were all within normal ranges and similar to those obtained in other studies of the surrounding communities with the exception of smoking prevalence, which was substantially lower in the study population. After the participants had provided informed consent according to the AHF Internal Review Board requirements, questionnaire data based on age, sex, height, weight, and smoking status were obtained. Capillary blood was drawn by finger puncture with a sterile lancet (Becton Dickinson) and collected into a 300-μL capillary tube (Sarstedt) containing lithium heparin as an anticoagulant. Optimal sample-processing procedures were utilized as determined previously for GSH and Cys [19,20]. Samples were immediately placed in an ice bath and processed within 30 min by deproteinization with 4 vol. of ice-cold 5% (w/v) MPA. After 10–20 min, acid extracts were obtained by centrifugation at 14,000 *g* for 2 min at room temperature, and supernatants were removed and stored at −20° until analyzed for free GSH. The pellets were also stored at −20° until further processing and analysis for protein-bound GSH.

Table 1
Characteristics of study subjects

	Number of subjects (%)		
	Males	Females	Total
N	55 (52.4)	50 (47.6)	105 (100)
Age[a] (years)			
<35	13 (24.1)	16 (32.6)	29 (28.2)
35–44	15 (27.8)	8 (16.3)	23 (22.3)
45–54	18 (33.3)	14 (28.6)	32 (31.1)
55–64	7 (13.0)	8 (16.3)	15 (14.6)
>64	1 (1.8)	3 (6.1)	4 (3.9)
BMI[b] (kg/m^2)			
<20.00	1 (1.8)	6 (12.5)	7 (6.9)
20.01–25.00	25 (46.3)	28 (58.3)	53 (52.0)
25.01–30.00	21 (38.9)	10 (20.8)	31 (30.4)
>30	7 (13.0)	4 (8.3)	11 (10.8)
Smoking status (cpd[c])			
Non-smokers	47 (85.5)	45 (90.0)	92 (87.6)
Smokers	8 (14.5)	5 (10.0)	13 (12.4)
≤5	5	2	7
5–10	0	2	2
10–20	3	1	4
Blood parameters[d]			
Total cholesterol (mg/dL)	194 ± 34.5	192 ± 37.0	193 ± 35.8
LDL (mg/dL)	122 ± 28.9	117 ± 34.1	120 ± 31.5
HDL (mg/dL)	42.8 ± 11.1	54.3 ± 12.0	48.4 ± 12.9
Triglycerides (mg/dL)	147 ± 103	99.8 ± 56.7	124 ± 86.6
Glucose (mg/dL)	107 ± 30.0	96.7 ± 9.96	102 ± 23.1
Hemoglobin (g/dL)	15.3 ± 2.54	13.8 ± 2.50	14.6 ± 2.62

[a] Age data were unavailable for two subjects.
[b] Weight data were unavailable for three subjects. BMI = body mass index.
[c] Cigarettes per day.
[d] Values are means ± SD. LDL = low-density lipoproteins; HDL = high-density lipoprotein.

2.2. Reduction of pellets for bound GSH analysis

Reductions were performed using a modification of our previous method [18]. The pellets were washed twice by resuspending in 800 μL of 5% MPA and centrifuging for 5 min at 14,000 *g* at room temperature. After a final wash with 800 μL of 1 mM EDTA, followed by centrifugation (14,000 *g*, 5 min, room temperature), the pellets were resuspended in 1 mL of 8 M urea/1 mM EDTA, transferred to Pyrex 13 × 100 tubes, shaken on an Ames aliquot mixer for 5–10 min and incubated for 10 min at 40°. Octanol (30 μL) was added to each tube (to prevent foaming), followed by 500 μL of 1.3 M potassium borohydride (KBH_4) (producing a final KBH_4 concentration of 0.38 M). The samples were incubated for 45 min at 40°, prior to the addition of 1 mL of 20% MPA. After 15 min at room temperature, the samples were centrifuged at 3000 *g* for 15 min and the supernatants were removed and filtered through a 25-mm 0.45-μm nylon Acrodisc syringe filter (Gellman Science). All chemicals were obtained through the Sigma Chemical Co.

2.3. GSH assay

GSH was determined using our microplate method [19], which is based on the DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)]/enzymatic recycling procedure of Tietze [21] and Owens and Belcher [22]. For measurement of free GSH, MPA extracts of whole blood were diluted 40-fold in assay buffer containing 100 mmol/L of NaH_2PO_4 and 5 mmol/L of EDTA, adjusted to pH 7.5 with NaOH. For measurement of GSSP, supernatants from KBH_4-reduced pellets were diluted 8-fold in assay buffer.

Fifty microliters of each working standard or diluted sample extract was added to the wells of a flat-bottomed 96-well microtiter plate (Immulon 1, Dynatech Laboratories, Inc.). All standards and samples were run in duplicate in adjacent wells. Fifty microliters each of 1.26 mM DTNB and 2.5 kU/L of glutathione oxidoreductase (EC 1.6.4.2) (type IV, Sigma) were then added to each well using an eight-channel pipette. After 15 min at room temperature, the reaction was started by the addition of 50 μL of 0.72 mmol/L of NADPH to each well, again using the multichanelled pipette. The plate was transferred immediately to a 96-well plate reader (MR5000, Dynatech Laboratories, Inc.), which monitored at 10-sec intervals for 2 min the rate of change in absorbance at 410 nm. Data were analyzed using Dynatech Biolinx version 2.1 software.

Table 2
Free and bound blood GSH concentrations by age and sex

Group	N	Free GSH[a] (μmol/mL)	Bound GSH[a] (μmol/mL)	Total GSH[a] (μmol/mL)	Bound GSH (% of total)
Sex					
Males	55	0.949 ± 0.182 (0.510–1.43)	0.138 ± 0.048 (0.048–0.320)	1.09 ± 0.183 (0.673–1.60)	13.0 ± 4.83 (3.98–26.2)
Females	50	0.925 ± 0.166 (0.49–1.34)	0.114 ± 0.034 (0.066–0.191)	1.04 ± 0.166 (0.678–1.47)	11.2 ± 3.89 (5.69–27.1)
Males and females	105	0.938 ± 0.174 (0.494–1.43)	0.126 ± 0.041 (0.048–0.320)	1.06 ± 0.176 (0.673–1.60)	12.1 ± 4.48 (3.98–27.1)
Age					
<35	29	0.919 ± 0.179	0.130 ± 0.038	1.05 ± 0.185	12.6 ± 4.11
35–44	25	0.918 ± 0.118	0.138 ± 0.040	1.06 ± 0.128	13.1 ± 3.50
45–54	30	0.950 ± 0.193	0.126 ± 0.054	1.08 ± 0.188	12.1 ± 5.54
55–64	15	0.964 ± 0.207	0.130 ± 0.030	1.07 ± 0.208	10.1 ± 4.20
>64	4	0.930 ± 0.191	0.126 ± 0.050	1.06 ± 0.214	11.9 ± 4.32

[a] Values are means ± SD (range).

To maintain assay precision and accuracy over long periods of time, quality control measures included the use of both external standards and control samples on each assay plate. Samples were run at different dilutions to ensure reproducibility and proportionality of assay responses. Control values were tracked over time to monitor assay performance and precision.

2.4. Statistical analyses

Data are expressed as means ± SD. Differences between groups were considered significant at $P < 0.05$ by Student's *t*-test or by ANOVA with Scheffe's post hoc test where appropriate. Correlations were determined using Pearson correlation coefficients.

3. Results

Our previously described method for analysis of GSSP [18] was modified for use in 96-well plates. This modified method provides both accurate and reproducible results based upon recovery studies and analysis of multiple samples. Recovery experiments were performed by adding 10 mmol/L of GSH or GSSG to samples of freshly obtained whole blood, giving final concentrations of 0 to 1 mEq/L, and processing blood as described above. Recovery values ranged from 98 to 102% for both GSH and GSSG. Likewise, when GSH or GSSG was added to samples prior to reduction, similar recoveries were obtained. Values for GSH and GSSG were confirmed by HPLC-DEC [23] with results from both methods being highly correlated ($r = 0.996$). Using this method, it is possible for a single investigator to process and analyze several hundred samples per day.

As observed previously, GSH and GSSP were found primarily in red cells with less than 1% occurring in plasma. The levels of free, bound, and total GSH in blood from the 105 healthy individuals are provided in Table 2. Free GSH levels were normally distributed and ranged from 0.49 to 1.43 μEq/mL (mean ± SD: 0.94 ± 0.17) (Fig. 1). This distribution is similar to results obtained

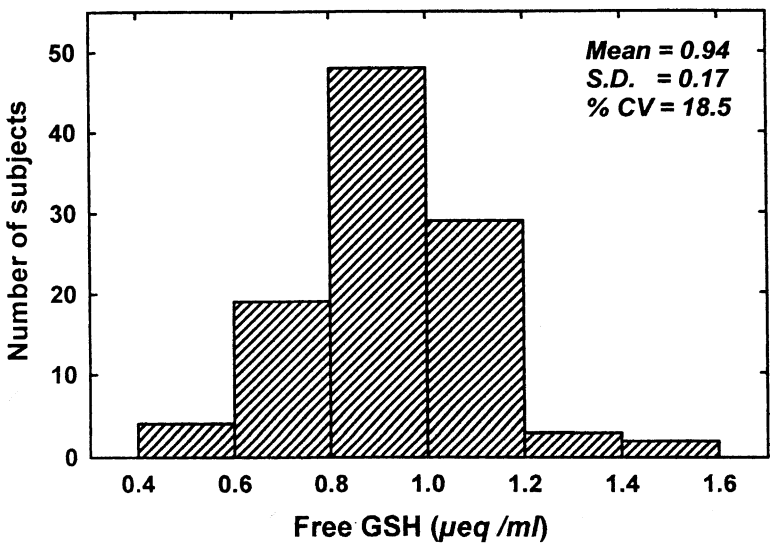

Fig. 1. Frequency distribution of free GSH measured in blood of 105 healthy individuals.

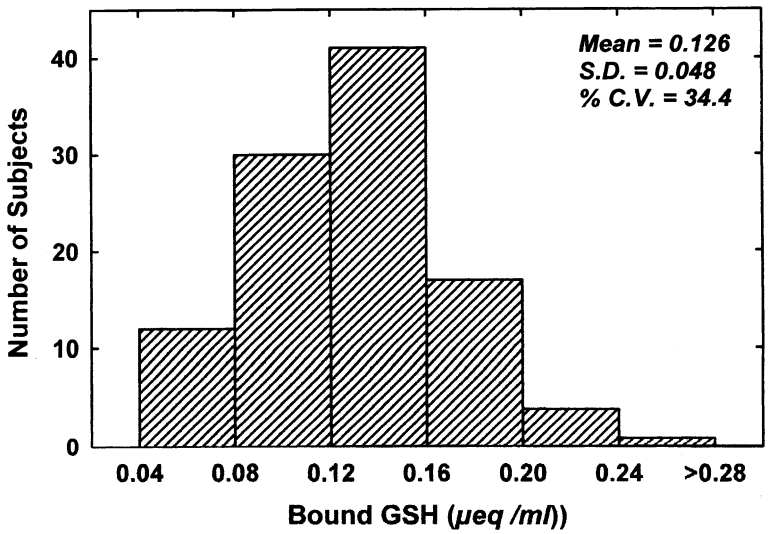

Fig. 2. Frequency distribution of bound GSH measured in blood of 105 healthy individuals.

in previous studies [19,24]. No age- or sex-related differences were observed.

GSSP levels were also normally distributed and ranged from 0.048 to 0.320 μEq/mL (mean ± SD: 0.126 ± 0.048) (Fig. 2). When calculated as a percentage of total GSH, GSSP values ranged from 3.98 to 27.1% (mean ± SD: 12.1 ± 4.48) (Table 2). The variation in GSSP (CV = 34.4%) was greater than that of free GSH

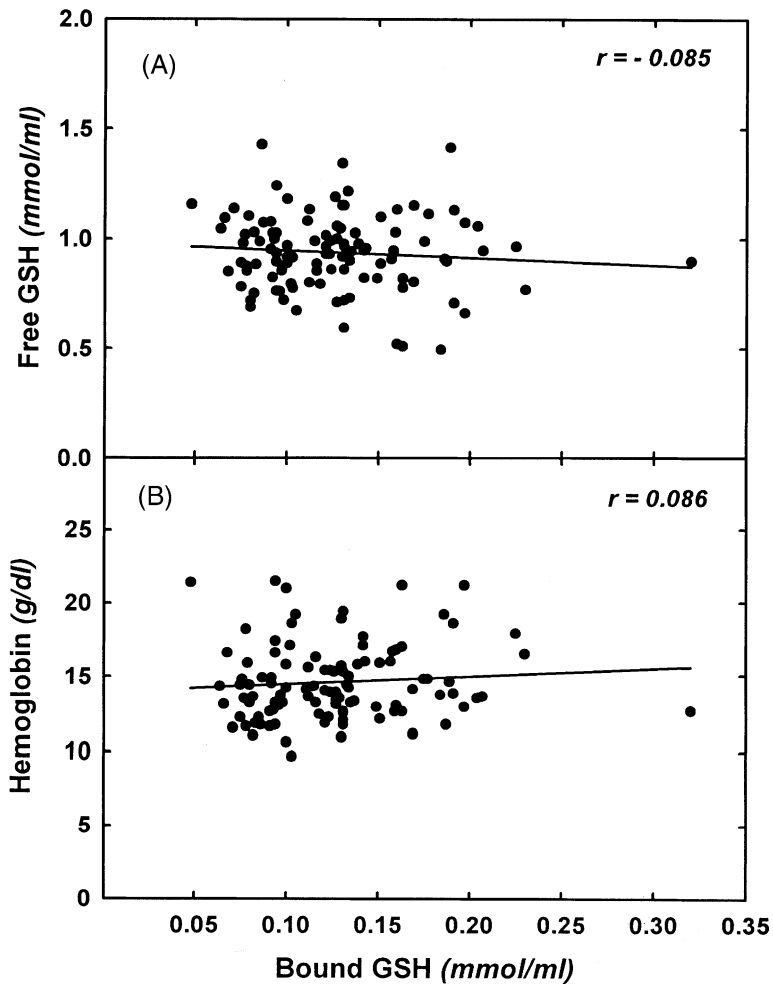

Fig. 3. Correlation of bound GSH levels with free GSH (A) and hemoglobin (B) concentrations in human blood.

(CV = 18.5%). The levels of GSSP calculated per mole of hemoglobin ranged from 0.014 to 0.163 mol GSH/mol hemoglobin with a mean ± SD of 0.057 ± 0.021 mol GSH/mol hemoglobin. When expressed by either amount or percent, there were no significant differences in GSSP relating to age and sex.

Although GSSP accounted for a significant portion of the total GSH content in human blood, its levels were not correlated with those of free GSH ($r = -0.085$) (Fig. 3A) or hemoglobin levels ($r = 0.086$) (Fig. 3B). In addition, there were no significant correlations between GSSP and other variables such as body mass index (BMI), age, or cholesterol levels (data not shown).

4. Discussion

The results demonstrated that the extent of protein glutathiolation in blood, as assessed by measurement of GSSP content, is large, averaging 0.1 mmol/L and accounting for 4–27% of total GSH in the 105 healthy subjects examined. The levels of GSSP were similar to those observed in our previous smaller study [18].

Similar to GSH, greater than 98% GSSP in blood was found in the red cells. While the specific proteins that are glutathiolated were not identified, it is likely that the majority of bound GSH is in the form of mixed disulfides with hemoglobin. The molar ratio of GSSP to hemoglobin ranged from 1 to 16%, with a mean of 5.7%, suggesting that a significant proportion of hemoglobin molecules are glutathiolated. GSH can form complexes specifically with Cys-93 of the β chain of hemoglobin *in vitro*, and this moiety is a likely site of glutathiolation *in vivo* [14,15]. While the functional role of this thiolation reaction is unknown, GSSP may play an important role in maintaining hemoglobin structure and function. GSH binding has been shown to protect hemoglobin against alkylating agents [25] as well as enhance oxygen affinity, and reduce cooperativity and Bohr effects [26]. Consequently, it is possible that the wide variations in glutathiolation between individuals may be associated with differences in hemoglobin activity in these subjects.

Concentrations of GSSP as well as GSH were normally distributed among individuals. Interindividual variation in GSSP (CV = 34%) was nearly 2-fold greater than that observed for GSH (CV = 18%). While the nature of this variation is unknown, it has been suggested that GSSP may be in equilibrium with free GSH, serving as a reserve pool to replenish intracellular GSH when required [14]. However, the present data do not support such a relationship as GSH and GSSP levels were not correlated between individuals. This lack of correlation suggests that GSSP and GSH represent two distinct and differentially regulated pools and contradicts the notion that bound GSH is in equilibrium with free GSH [14,15]. Additionally, the levels of GSSP do not appear to be dependent upon the availability of protein thiols in blood since GSSP levels were not correlated with the concentrations of hemoglobin or total proteins (data not shown).

GSSP formation is likely dependent upon the levels of oxidative stress. The main route of GSSP formation is thought to involve thiol/disulfide exchange between GSSG and Cys-derived thiol residues on proteins. Thus, during conditions associated with enhanced oxidative stress, enhanced formation of GSSG may be associated with a greater production of GSSP [27]. Many have suggested that GSSG/GSH ratios can serve as an accurate indicator of oxidative stress levels in blood. However, due to the high activity of the enzyme glutathione reductase within red cells, the permeability of the cell membrane to GSSG, and the reactivity of GSSG with protein thiols via thiol/disulfide exchange, levels of GSSG in blood remain quite low (<10%) and unstable, limiting their usefulness as a biomarker. Since GSSP appears to be fairly stable, unlike GSSG, we propose that the levels of GSSP or GSSP/GSH ratios may serve as a useful marker for oxidative stress. While a comparison of GSSP and GSH levels in smokers versus non-smokers would likely provide valuable information regarding this potential relationship, there were too few smokers in the study population to examine this aspect.

Free GSH in blood has been proposed as a useful indicator of susceptibility to disease and toxicity as well as the overall health status in humans [28]. Low GSH levels have been implicated in numerous pathologic conditions including diabetes [29], alcoholic liver disease [30], AIDS [31,32], acute hemorrhagic gastric erosions [33], cataracts [16], Parkinson's disease [34], xenobiotic-induced oxidative stress and toxicity [35], and aging [28,36,37]. Since oxidative damage is likely an important mechanistic factor in many of these conditions, it would be of interest to examine if GSSP concentrations are altered as well. To better understand the regulation of GSSP in blood and to evaluate its potential roles in oxidative stress and specific disease status, large-scale epidemiological studies measuring all blood GSH forms will be required. We have now shown that large numbers of bound as well as free GSH samples can be analyzed accurately, using our newly modified method adapted for use in a 96-well plate. The data reported here provide baseline levels of GSSP in a healthy population.

Acknowledgments

We thank Shelley Isaacs for her technical assistance, and Marie-Ange Brunnemann for her editorial assistance. This study was supported, in part, by NIH Grants CA17613 and CA68384.

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