Free Radical Research, 2002 Vol. 36 (2), pp. 151–156

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healthsciences

The Redox State of Glutathione, Cysteine and Homocysteine in the Extracellular Fluid in the Skin

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Accepted by Professor H. Sies

(Received 9 May 2001; In revised form 23 August 2001)

Glutathione, the most abundant low-molecular weight thiol in the skin, has been shown to protect the skin from both photobiological and chemical injury. The thiols, glutathione in particular, have also been shown to be crucially involved in defence against contact allergens. Since the levels of extracellular thiol concentrations are important determinants of intracellular thiol status, we have compared the normal concentrations and the redox status of the main low-molecular weight thiol components in the extracellular fluid at the dermo-epidermal junction with the corresponding plasma levels. In their sulfhydryl form, all three thiols, i.e. glutathione, cysteine and homocysteine, were more abundant in experimental skin blister fluid than in plasma, as were the free disulfides of glutathione and homocysteine, whereas the free disulfides of cysteine were about the same in blister fluid and in plasma. Protein mixed disulfide levels were higher in plasma than in blister fluid. The present results provide information concerning the extracellular defence in the skin.

Keywords: Thiol; Disulfide; Blister fluid; Plasma; Oxidative stress

INTRODUCTION

Thiols constitute one of the major cellular defence systems against toxic substances in all mammalian cells, and thus also in the skin. Glutathione (GSH) is the most important compound among these thiols.[1] Its concentration in mammalian cells ranges from 0.1 to 10 mM,[2] and intracellularly it exists mainly in the reduced form,[3] a prerequisite for its protective properties. Reduction of its disulfide, GSSG, is catalyzed by the enzyme glutathione reductase. Glutathione conjugation to xenobiotics is normally catalyzed by glutathione transferases, although strong electrophilic reagents such as quinones may react with thiols without enzymatic catalysis.

Glutathione is the most abundant low-molecular weight thiol in the skin and a crucial determinant of cellular redox status. Its concentration in the skin has been found to decrease in response to UV radiation,[4–6] and it has been found to fulfil a protective function against UV-induced cytotoxic free radicals.[7,8] The skin is exposed to an abundance of xenobiotics, which may react with thiols. In the stratum corneum, most thiols are oxidized, probably by atmospheric oxygen, to the corresponding disulfide, unable to function as a nucleophile. A gradient of GSH as well as of other antioxidants was recently described in the stratum corneum.[9]

The plasma levels of GSH and GSH disulfides have been determined by several authors,[10–12] who found them to be several orders of magnitude lower than the respective intracellular levels and therefore more difficult to determine. The levels of GSH in specimens of epidermal and dermal origin have been reported.[13] The intracellular concentrations of GSH, cysteine and their disulfides in cultured melanoma cells have been determined.[14] To the best of our knowledge, however, the thiol system in extracellular fluid surrounding the cells of the skin has not been investigated earlier. The skin is exposed to a

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/10715760290006448

large number of reactive substances, which often do not reach the blood. Among these there are also electrophilic compounds which are reactive with thiols. The aim of the present work was to define how the extracellular thiol defense system in normal, human skin differs from the thiol system in plasma.

In this study, we determined the levels of thiols and their redox status in plasma and in extracellular fluid from experimental skin blisters in healthy human subjects. The blisters were induced at the border between the dermis and epidermis, using the technique developed by Kiistala.[15] We compared the findings in extracellular fluid with those in plasma from the same healthy subjects. As blister fluid is easily available, its analysis might be an alternative to plasma analysis in certain conditions.[16] Recently blister fluid analysis has been found superior to analysis of plasma in the determination of locally produced cytokines in some skin diseases.[17] Extracellular glutathione has been found to be important in the protection of cell surface proteins such as receptors and transporters.[18]

MATERIALS AND METHODS

Materials

5-Sulfosalicylic acid dihydrate was obtained from Pierce Europe B.V. (Buchs, Netherlands), *tris* (hydroxymethyl)aminomethane (Trizmao® Base) from Sigma Chemical Co. (St Louis, USA), dithiothreitol (DTT) Ultrole® grade from Calbiochem (San Diego, USA), and hydrochloric acid and sodium hydroxide from Merck (Darmstadt, Germany). Solutions with basic or acidic properties used for preparation of plasma or blister fluid samples were designed to give the samples specific pH values (i.e. after protein precipitaion pH 1.7–1.9, during reduction pH 7.5–8.5 and at HPLC-injection pH 2.0–2.3) and they were prepared exactly as described in Material and Methods without any adjustments of pH.

RSH, free and total glutathione, cysteine and homocysteine are the fractions measured with the present method.† The free disulfide level is calculated as the difference between free and RSH levels, and the protein mixed disulfide level as the difference between total and free thiol levels.

Test Subjects and Statistics

Ten healthy Caucasians (five men, five women), ranging in age from 28 to 53 years, underwent suction-induced blistering and blood sampling. A mean value of each pair of samples was determined and used in the calculation of the mean value of each parameter. Correlations were determined using Spearman's rank correlation method. The study was approved by the Ethics Committee of the Medical Faculty, University of Lund, Sweden.

Plasma Samples

Blood samples, obtained by superficial venepuncture in the antecubital fossa 1 h after commencing blister-induction in the contralateral arm, were collected in pre-chilled, EDTA-containing 7 ml Vacutainer tubes® (Becton Dickinson, Rutherford, NJ, USA) which were placed in ice-water for 5 min and then turned once. For analysis, 1.8 ml of blood was transferred to each of two pre-chilled Eppendorf tubes which were centrifuged (10,000 *g*) for 3 min at 4°C. For analysis of the plasma content of RSH and free thiols, 500 μl of plasma was transferred from each Eppendorf tube to two new 1.5 ml Eppendorf tubes and immediately mixed with 125 μl of 15.0% sulfosalicylic acid (SSA). These protein-precipitated samples were kept at −70°C until analysed (within four days). At analysis, the preparations were thawed and centrifuged (10,000 *g*) for 3 min at ambient temperature. To determine the concentrations of sulfhydryls (RSH), 80 μl of the supernatant was subjected to HPLC-analysis. To determine the concentrations of free (non-protein-bound) thiols, 200 μl of the supernatant was mixed with 32 μl of 0.80 mol/l boric acid and 13.0 μl of 2.00 mol/l NaOH. After the addition of 8.0 μl of 0.10 mol/l DTT (dithiothreitol), the sample was incubated for 15 min at 37°C. Before HPLC, 20.0 μl of 1.00 mol/l hydrochloric acid was added for acidification and the sample was centrifuged (10,000 *g*) for 3 min at room temperature.

To determine total plasma thiol content, 100 μl of plasma from each of the original centrifuged blood-samples was mixed with 25 μl of 0.10 M DTT, and incubated for 10 min at room temperature, 25.0 μl of 15.0% SSA then being added and mixed. The samples were frozen at −70°C until HPLC-analysis was performed (within four days). After thawing and centrifugation (10,000 *g*, 3 min), 30 μl of the supernatant was applied to the HPLC assay. Further processing and the HPLC system used were as previously described.[10]

Suction Blister Samples

Suction blisters were produced on normal skin on the ventral aspect of the upper forearm, using eight 1.5 ml disposable Eppendorf tubes with the bottom cut off, fixed in a polythene sleeve and connected to a

†Definition of different quantities of thiol derivatives exemplified for glutathione. GSH: thiol with intact sulfhydryl group, generally RSH; Free disulfides of glutathione: GSSG+GSSX (XSH=low-molecular weight thiol); Free glutathione: GSH+GSSG+GSSX; Protein glutathione mixed disulfide: GSSPr (protein bound glutathione); Total glutathione: GSH+GSSG+GSSX+GSSPr.

vacuum pump giving a pressure of −200 mm Hg. Disposable Eppendorf tubes were used to avoid the risk of virus contamination from the blister fluid. We found it much easier to induce blisters in older than in younger persons, and no macroscopic bleeding was observed when the blisters were produced at this moderate pressure (−200 mm Hg), findings all in agreement with those of Kiistala.[15] After about 2 h of constant suction, blisters of 5–9 mm diameter were developed. Suction blister fluid was obtained using a 1 ml fine needle syringe (Becton Dickinson). Fluid from four blisters was immediately pooled in a pre-chilled 1.5 ml Eppendorf tube, thus the eight blisters yielded two samples which were analysed separately. The samples were immediately centrifuged (10,000 *g*) at 4°C for 2 min, the supernatant being used for further analysis. The residual pellet was counted and the total amount of cells never exceeded 7000.

To analyse RSH and free thiols, 130 μl of blister fluid from each of the centrifuged samples were mixed with 32.5 μl 10.0% SSA and then frozen until analysed. After thawing and centrifugation (10,000 *g*, 3 min), 40 μl of the supernatant was applied to the HPLC for assay of RSH. For the analysis of free thiols, 60 μl of the supernatant was transferred to a tube containing 20.5 μl reducing solution (117 mmol/l *tris*(hydroxymethyl)aminomethane, 270 mmol/l NaOH and 14.6 mmol/l DTT), mixed and incubated at ambient temperature for 8 min. The samples were acidified with 10.0 μl 0.50 mol/l hydrochloric acid, mixed and centrifuged (10,000 *g*, 3 min). Forty microlitres were applied to the HPLC for assay of free thiols. Total thiol content was analysed by letting 60 μl of centrifuged blister fluid be mixed and incubated with 15.0 μl of 0.10 M DTT at room temperature for 10 min. Fifteen microlitres of 10.0% SSA were then added and the samples were stored at −70°C until analysed (within four days). At analysis they were thawed, centrifuged (10,000 *g*, 3 min) and 30 μl supernatant was applied to HPLC for determination of total thiols. The HPLC analyses of these thiols in blister fluid were performed using previously described plasma methods.[10]

RESULTS

The concentrations of RSH, free, and total fractions of thiols in blister fluid and plasma were determined in duplicate, while free disulfides and protein-mixed disulfide fractions were calculated. The free disulfide fractions consisted of symmetric as well as mixed low-molecular weight disulfides. The results are presented in Table I.

The concentrations of the RSH forms of all thiols investigated, i.e. glutathione, cysteine and homocysteine were higher in blister fluid than in plasma, as

TABLE I Mean ± SD concentrations (μmol/l) of RSH, free and total thiols and calculated values for free disulfides and protein-mixed disulfides in blister fluid and plasma from ten healthy subjects ($n = 10$).

	Blister fluid thiol					Plasma thiol				
	RSH	Free-disulfide	Protein mixed	Free	Total	RSH	Free-disulfide	Protein mixed	Free	Total
Cys	23.7±4.8*	126±29	28.0±6.8*	149±32*	178±30*	11.8±3.9	119±22	142±23	130±25	272±42
GSH	4.59±0.99*	17.3±3.4*	0.64±1.2*	21.9±4.0*	22.5±3.7*	1.84±0.36	2.20±0.41	1.75±0.49	4.0±0.5	5.8±0.8
Hcy	0.29±0.11*	3.22±1.0*	0.86±0.48*	3.51±1.12*	4.43±1.6*	0.18±0.06	2.84±1.09	7.13±3.11	2.9±1.1	10.0±4.1

Cys, cysteine; GSH, glutathione; Hcy, homocysteine; *Significant differences: $p < 0.05$ between blister fluid and plasma.

were those of free disulfide forms of glutathione and homocysteine. The free disulfide cysteine concentration was about the same in blister fluid and in plasma. The concentrations of all protein-mixed disulfides were significantly higher in plasma than in blister fluid. Total homocysteine and total cysteine concentrations were also higher in plasma than in blister fluid, but not the total glutathione concentration which was markedly higher in blister fluid than in plasma (22.5 vs 5.8 μmol/l). The plasma levels were in accord with earlier presented results.[10–12]

The ratios of the RSH, free disulfide and protein-mixed disulfide fractions, to the total glutathione, cysteine and homocysteine levels are presented in Table II. In plasma, the protein glutathione mixed disulfide fraction of the total glutathione concentration was lower than the corresponding fractions of homocysteine and cysteine (30 vs 70 and 52%, respectively); the free disulfide glutathione fraction was between the corresponding fraction of homocysteine and cysteine (39 vs 28 and 44%) whereas the GSH fraction was markedly greater than the corresponding fractions of either cysteine or homocysteine (32 vs 4.3 and 1.8%, respectively).

The protein glutathione mixed disulfide fraction of the total glutathione concentration in blister fluid was markedly lower than that in plasma (1.6 vs 30%) and markedly lower than the corresponding fraction of cysteine or homocysteine (1.6 vs 15 and 18%, respectively). The GSH fraction of the total glutathione concentration in blister fluid was not as great as the corresponding plasma fraction (20 vs 32%), but was greater than the corresponding RSH blister fluid fraction of homocysteine or cysteine (20 vs 6.8 and 13%, respectively). The free disulfide fractions of all three thiols in blister fluid were much the same.

Most of the variables of cysteine and homocysteine were characterized by strong correlation between their respective plasma and blister values (Table III). No correlation was found between the plasma and the blister fluid values of any of the glutathione forms.

DISCUSSION

Glutathione has been shown to manifest numerous protective and regulatory functions in the skin. Depletion of cellular glutathione is an important toxicological mechanism of cell injury,[19] and tumour cells that have become insensitive to cytostatic agents have been shown to have twice the normal GSH content.[20,21] A large number of photobiological studies have been made on glutathione in relation to the skin. It has been established that PUVA (psoralen+UV radiation A) treatment depletes both dermal and epidermal glutathione in a dose-dependent manner, and no phototoxic reaction appears unless more than 50% of glutathione has disappeared. The peak phototoxic effect appears after 24–48 h, i.e. at the time of maximum glutathione depletion.[5,6]

Blister fluid ought to be almost equivalent to the extracellular fluid at the dermo-epidermal junction. The suction blister technique is simple and the capillary basement membrane remains intact during the blister formation.[22] Furthermore Dabbagh and Frei investigated the redox status of fluid from experimental blisters and compared it to the redox status of plasma. No significant difference was found.[23] They concluded that suction technique does not cause oxidation artefacts. Extracellular GSH has been determined in different pools. Its concentration is generally lower than the intracellular values.[24] However rather high concentration (200–800 μM, was registered in alveolar lining fluid[25] compared to plasma values (about 2 μM). In the present investigation of the normal levels of thiols and their redox status in extracellular fluid, we found the total value of glutathione, but not those of homocysteine and cysteine, to be higher in blister fluid than in plasma. The concentration of protein in plasma is about three times higher than that in blister fluid.[26] A high amount of sulfhydryl-containing proteins in plasma favours the formation of low-molecular weight thiol–proteinthiol mixed disulfides. Compared to those of the other two thiols investigated, the level of protein glutathione mixed disulfide was less both in plasma and in blister fluid, which in part explains the lower level of total glutathione in plasma than in blister fluid, However, less utilisation of the protein binding capacity in plasma by glutathione does not explain the several-fold higher amount of total glutathione in blister fluid. The content of the free disulfide form of

TABLE II Mean ± SD ratios (RSH/total, free disulfide/total and protein-mixed disulfide/total) of thiol content in blister fluid and plasma from ten healthy subjects ($n = 10$)

	Blister fluid ratio, %±SD			Plasma ratio, %±SD		
	RSH/total	Free disulfide/total	Protein mixed/total	RSH/total	Free disulfide/total	Protein mixed/total
Cys	13±2*	71±7*	15±7*	4.3±1	44±4	52±5
GSH	20±3*	78±8*	1.6±7*	32±6	39±5	30±6
Hcy	6.8±2*	75±5*	18±5*	1.8±0.6	28±4	70±4

*Significant differences: $p < 0.05$ between blister fluid and plasma.

TABLE III Correlations between total, free and RSH thiol concentrations in blister fluid and in plasma ($n = 10$). Only significant ($p < 0.05$) correlation coefficients (r values) are shown. No correlation was found between the plasma and the blister fluid values of any of the glutathione forms

Plasma thiol	Blister fluid thiol					
	Total		Free		RSH	
	Cys	Hcy	Cys	Hcy	Cys	Hcy
Total Cys	0.92	–	0.93	–	0.72	–
Total Hcy	–	0.94	–	0.94	–	0.79
Free Cys	0.99	–	0.98	–	0.79	–
Free Hcy	–	0.98	–	0.98	–	0.85
Cys (RSH)	0.65	–	0.72	–	–	–
Hcy (RSH)	–	–	–	–	–	0.71

glutathione was several times higher in blister fluid than in plasma (17.3 vs 2.2 μmol/l), and thus responsible for the higher total amount. We also showed free disulfide glutathione to constitute about 80% of the total blister fluid glutathione, but only 39% of total plasma glutathione (Table II). The level of GSH in blister fluid was about twice that in plasma. GSH is present at millimolar concentrations intracellularly, only a minor fraction being oxidized.[27] It has been shown that cells export GSH,[1] and the extracellular glutathione disulfides may have been formed extracellularly by oxidation. However, it is also possible that some of it is of intracellular origin. Our finding of a very low fraction of protein glutathione mixed disulfide in blister fluid indicates a low level of extracellular oxidation. Intracellular oxidized glutathione has been shown to be actively transported out of cells.[28,29]

The atherogenic amino acid, homocysteine, was characterized by strong correlation between its RSH, free and total concentrations in plasma and the corresponding fractions in blister fluid. The determinants of the concentration would thus seem to be the same in plasma as in blister fluid. No correlation was found between the plasma and the blister fluid values of any of the glutathione forms. Free glutathione in blister fluid in healthy individuals mainly consists of free disulfide glutathione (mixed or unmixed), and the determinants of the concentrations in plasma and blister fluid seem in part to differ. Local conditions more than simple perfusions of plasma components are probably of importance for the blister fluid values, and in this respect blister fluid and plasma are not equivalent moieties. In a recent paper Jones *et al*. reported a lack of correlation between GSH and GSSG in normal human plasma in accordance with our results.[12]

This investigation was conducted as a part of our attempt to elucidate the involvement of glutathione in contact dermatitis. In 1980, Summer and Göggelmann showed that the strongly mutagenic contact allergen dinitrochlorobenzene (DNCB) depleted glutathione in rat skin,[30] and we believe that the ability of DNCB to deplete glutathione is a prerequisite for its effect as a strong contact allergen. Schmidt and Chung reported the glutathione concentration in mouse skin to be increased 12 h after hapten challenge, and suggested this increase to be due to enzyme induction stimulating glutathione synthesis.[31] Recently, Hirai and colleagues showed allergic inflammatory reactions in mice to be exacerbated when glutathione was depressed.[32] Senaldi and co-workers showed inflammatory reactions in mouse skin to be reduced by treatment with *N*-acetylcysteine before exposure to allergic or irritant xenobiotics.[33] Thus, the thiol system in the skin has been shown to interact with haptens, and the extracellular part of this thiol system is defined in the present study.

Acknowledgements

This investigation was supported by grants from the Swedish Foundation for Health Care and Allergy Research, the Swedish Council for Work Life Research, the Påhlson Foundation, and the Alfred Österlund Foundation for Scientific Research.

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