Impairment of glutathione biosynthetic pathway in uraemia and dialysis

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Abstract

Background. Glutathione (GSH), the predominant intracellular antioxidant, reportedly has been shown to be decreased in chronic renal failure patients, which renders these patients more susceptible to oxidative damage by free radicals. To our knowledge, the ability of erythrocytes to normalize the GSH level by \textit{de novo} synthesis in uraemic and dialysis patients has not been studied previously. The main goal of the present study was to measure the activities of the enzymes that are responsible for \textit{de novo} GSH generation, namely \(\gamma\)-glutamylcysteine synthetase (\(\gamma\)-GCS) and glutathione synthetase (GSH-S), in erythrocytes from uraemic and dialysis patients.

Methods. Erythrocyte total GSH level and \(\gamma\)-GCS and GSH-S activities as well as plasma malondialdehyde (MDA) levels were measured in 19 non-dialysis patients (ND), 34 haemodialysis patients (HD), 22 continuous ambulatory peritoneal dialysis patients (CAPD) and 21 normal healthy controls. The effect of a single haemodialysis session was determined in 16 HD patients.

Results. Significant decreases in GSH levels and \(\gamma\)-GCS activity but not GSH-S were observed in ND, HD and CAPD patients compared with controls. However, GSH levels as well as \(\gamma\)-GCS and GSH-S activities were not different among the ND, HD and CAPD patients. The decrease in GSH was strongly and positively correlated with the decrease in \(\gamma\)-GCS in ND, HD and CAPD patients \((r = 0.717, P < 0.001; r = 0.854, P < 0.001; \text{and } r = 0.603, P < 0.01, \text{respectively})\). In addition, plasma MDA was negatively correlated with \(\gamma\)-GCS in ND, HD and CAPD patients \((r = 0.721, P < 0.001; r = 0.560, P < 0.01; \text{and } r = 0.585, P < 0.01, \text{respectively})\). A single dialysis session had no effect on GSH level or on \(\gamma\)-GCS and GSH-S activities. Only a significant reduction in MDA was observed at the end of dialysis.

Conclusions. The activity of the rate-limiting enzyme in GSH biosynthesis, \(\gamma\)-GCS, was significantly decreased in uraemic and dialysis patients, which explains, at least in part, frequent reports of reduced GSH levels in these patients. The decrease in \(\gamma\)-GCS activity may have been secondary to inhibitory effects from uraemic factors that are not removed by standard dialysis. However, this assumption does not exclude the possibility of down-regulation of \(\gamma\)-GCS protein expression and further studies in this context are recommended.

Keywords: \textit{de novo} glutathione; dialysis; erythrocyte; \(\gamma\)-glutamylcysteine synthetase; uraemia

Introduction

Uraemic patients, especially those on regular dialysis treatment, are at high risk for oxidative damage caused by free radicals [1]. It has been suggested that impairment of various intra- and extracellular antioxidant systems, which protect against harmful effects of free radicals, plays a significant role in the development, exacerbation or both of oxidative damage in uraemia and dialysis [2]. The glutathione antioxidative system is among the antioxidant mechanisms frequently investigated in uraemia [3–6]. Glutathione is a sulphydryl tripeptide (\(\gamma\)-glutamyl-cysteinyl-glycine) that acts as antioxidant, antitoxin and enzyme co-factor [7]. Glutathione is present in cells as reduced glutathione (GSH), the predominant form, and as oxidized glutathione (GSSG), which together attain millimolar concentrations within cells, making this peptide one of the most highly concentrated intracellular antioxidants. The level of GSH is controlled homeostatically, being continually self-adjusting with respect to the balance between GSH synthesis (controlled by the GSH-synthesizing enzymes \(\gamma\)-glutamylcysteine...
De novo GSH in uraemia and dialysis

synthetase (γ-GCS) and glutathione synthetase (GSH-S), its recycling from GSSG (by glutathione reductase) and its utilization (by peroxidases, transferases, transhydrogenases and transpeptidases). GSH is transported out of specific cells, such as erythrocytes, either as GSSG or GSH-conjugates and this transport accounts for most of GSH turnover in these cells [7].

De novo synthesis of GSH is controlled by two consecutive steps that both utilize ATP. First, cysteine and glutamate are combined to produce γ-glutamylcysteine by the action of the enzyme γ-GCS. Second, γ-glutamylcysteine combines with glycine to generate GSH by the action of the enzyme GSH-S. The first reaction, catalysed by γ-GCS, is the rate-limiting step in GSH synthesis and is under feedback inhibition by GSH [8].

Numerous studies have shown that uraemia and dialysis patients have significant reductions in red-cell total GSH levels [2–6], as well as impairments in GSH-metabolizing enzymes [3,4]. The decrease in GSH level has been explained by an enhancement in the rate of GSH turnover [2,5,6]. In addition, the depletion of other important antioxidants, such as vitamins C and E, adds cumulative effects that burden GSH antioxidant activity and accelerate its depletion in uraemia and dialysis patients [9]. However, the rate of GSH biosynthesis in uraemia and dialysis has not been studied. To our knowledge, there has been no previous work to evaluate the status of the enzymatic machinery that is directly involved in GSH synthesis (i.e. the enzymes γ-GCS and GSH-S) in erythrocytes of uraemic and dialysis patients.

The main purpose of this study was to investigate the activities of erythrocyte γ-GCS and GSH-S in relation to GSH level in order to gain more insight regarding the factors that affect intracellular GSH concentration and its de novo biosynthesis in patients with uraemia.

Subjects and methods

Subjects

A total of 75 uraemic patients (43 males and 32 females) from Al-Karamma, Al-Yarmouk and Al-Kadhemia teaching hospitals were enrolled in this study. Uraemic patients were categorized into three groups: non-dialysed patients (ND), haemodialysis patients (HD) and a continuous ambulatory peritoneal dialysis (CAPD) group. Patient characteristics are summarized in Table 1. None of the patients had diabetes, liver disease, pulmonary diseases, malignancies or infectious diseases or had undergone recent transfusion of blood or any of its components. Informed consent was obtained from all patients and the study was approved by the local ethics committee. The HD patients received dialysis treatment three times per week for 4 h per session with the aid of low-flux cuprophane dialysers (Belleco, Italy) and bicarbonate as the dialysis buffer. The CAPD patients were treated with a standard peritoneal dialysis solution and glucose concentrations were adjusted in accordance with the excess fluid to be removed from patients.

Table 1. Clinical characteristics and biochemistries of the uraemic patient groups and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ND</th>
<th>HD</th>
<th>CAPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>19</td>
<td>34</td>
<td>22</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.7±21.6</td>
<td>60.2±15.9</td>
<td>54.4±10.1</td>
<td>63.6±18.8</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>13/8</td>
<td>12/7</td>
<td>19/15</td>
<td>12/10</td>
</tr>
<tr>
<td>BUN (mmol/l)</td>
<td>6.17±15.9</td>
<td>31.3±8.17</td>
<td>27.8±7.67</td>
<td>26.7±7.24</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>67.1±15.9</td>
<td>577±128</td>
<td>892±270</td>
<td>848±257</td>
</tr>
<tr>
<td>Hb (g/l)</td>
<td>144±12</td>
<td>98±17</td>
<td>110±23</td>
<td>103±21</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.3±3.51</td>
<td>23.6±2.90</td>
<td>24.1±3.34</td>
<td>23.8±2.10</td>
</tr>
</tbody>
</table>

Values are expressed as means±SD.

BUN, blood urea nitrogen; BMI, body mass index.

Twenty-one age- and sex-matched normal healthy volunteers served as controls. None of the control subjects were heavy smokers, alcoholic or on any special diet or taking any antioxidants before participating in the study.

Blood sampling

Five millilitres of blood were obtained from the uraemic patients and controls. To assess the effect of a single haemodialysis session, blood specimens were also collected from 16 HD patients immediately before (pre-HD) and at the end of a dialysis session (post-HD). Blood samples were transferred into heparinized tubes, centrifuged at low speed (700 g) for 10 min and plasma and buffy coats were removed by aspiration. The remaining red blood cells (RBCs) were washed three times with isotonic saline, centrifuged as described above and after the final wash were resuspended in an equal volume of isotonic saline. All of these operations were performed at 4°C.

Analytical procedures

Analytical procedures were performed within 1–2 h of blood collection. GSH was determined as total glutathione in the erythrocyte suspension as described previously [10]. Haemoglobin (Hb) concentration was obtained for the erythrocyte suspension and whenever required, according to Drabkin’s method. The level of GSH was expressed as μmol/g Hb. For the assay of erythrocyte γ-GCS and GSH-S activities, an aliquot of packed RBCs was lysed with 10 vol of 10 mmol/l Tris–HCl solution (pH 8.0), centrifuged at high speed to remove erythrocyte debris and dialysed for 3 h at 4°C against 10 mmol/l Tris–HCl (pH 8.0) to deplete intracellular GSH. All of the subsequent procedures were as described by Beutler and Gelbart [11] and the activities of erythrocyte γ-GCS and GSH-S were expressed as U/g Hb. The plasma malondialdehyde (MDA) level was determined according to the standard technique of Ohkawa et al. [12] with the application of butylated hydroxyanisole (BHA) to plasma samples (10 μl of 1% ethanolic solution of BHA for each 100 μl plasma) before assay to prevent the alteration of lipid oxidation.

Statistical analysis

Differences between the control and the uraemic patient groups were compared by analysis of variance (ANOVA). When ANOVA was significant at the P<0.05 level, Fischer’s
least significant test was applied to determine individual differences between the control and uraemic groups. The paired $t$-test was used to determine differences between the pre-HD and post-HD samples. Correlation studies were applied using Pearson’s correlation coefficient. All values are expressed as means ± SD unless otherwise stated.

**Results**

Table 2 illustrates erythrocyte GSH levels and activities of $\gamma$-GCS and GSH-S and plasma MDA levels in healthy controls and uraemic patients. A significant decrease in erythrocyte GSH level was observed in all uraemic groups as compared with the control ($P<0.05$). While the activity of GSH-S was not affected in the entire patient population, that of $\gamma$-GCS was significantly reduced in erythrocytes from all uraemic patients compared with controls ($P<0.05$). Although the decreases in GSH level and $\gamma$-GCS activity were more pronounced in HD patients than in the ND and CAPD groups, these differences did not reach statistical significance. Plasma MDA was significantly elevated in uraemic and dialysis patients compared with controls ($P<0.05$).

Correlation studies, shown in Figure 1, revealed strong and positive correlations between GSH and $\gamma$-GCS in ND, HD and CAPD patients ($r=0.717$, $P<0.001$; $r=0.854$, $P<0.001$; and $r=0.603$, $P<0.01$, respectively), contrasting with no relationship in controls ($r=-0.214$, $P=0.089$). From the entire uraemic population ($n=75$), 16 patients had GSH within the normal range combined with depressed $\gamma$-GCS activity (Figure 1). Inverse correlations were found between erythrocyte $\gamma$-GCS and plasma MDA in ND, HD and CAPD patients ($r=0.721$, $P<0.001$; $r=0.560$, $P<0.01$; and $r=0.585$, $P<0.01$, respectively). There were no correlations between $\gamma$-GCS and the clinical and demographic characteristics of uraemic patients, which included age, sex, blood urea nitrogen, creatinine, glomerular filtration rate and body mass index (data not shown). However, significant but weak correlations were observed between $\gamma$-GCS and each of Hb and GSH in ND patients ($r=0.396$, $P<0.05$; $r=0.424$, $P<0.05$, respectively).

A single haemodialysis session did not change GSH level or $\gamma$-GCS and GSH-S activities in erythrocytes from HD patients (Table 3). However, we found that haemodialysis produced a 43% reduction in plasma MDA ($P<0.001$).

**Discussion**

Maintenance of high GSH level and an elevated GSH/GSSG ratio are essential for overall cell health and may provide a good index for oxidative stress [7]. The mechanisms that govern these levels include the balance between various enzymatic and non-enzymatic reactions that control GSH synthesis and turnover. GSH generation is controlled by two linked, enzymatically controlled reactions catalysed by $\gamma$-GCS and GSH-S, respectively. Both reactions require ATP and the first is rate-limiting and under feedback inhibition by GSH. The turnover of GSH is either reversible or irreversible. Reversible utilization of GSH can come about through its consumption by glutathione peroxidase to neutralize $\text{H}_2\text{O}_2$ and organic peroxides with the liberation of GSSG. GSSG is recycled by the action of glutathione reductase. A portion of GSH may be oxidized non-enzymatically by the effect of reactive oxidizing species. Reversible interchange may also occur between GSH and protein thiol to form disulphide adducts in a reaction catalysed by the enzyme thiol transferase. Irreversible degradation of GSH can take place through several routes. By entering the $\gamma$-glutamyl cycle, GSH is degraded by removing its $\gamma$-glutamyl moiety in a reaction catalysed by $\gamma$-glutamyl transpeptidase. Glutathione S-transferase uses GSH to detoxify fat-soluble toxins via conjugation. The extrusion of GSH out of cells as GSH conjugates (GSH-X) or GSSG by the membrane enzymes GSSG/GSH-X pump and multidrug-resistance protein

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**Table 2.** Erythrocyte total GSH levels and $\gamma$-GCS and GSH-S activities and plasma MDA levels in uraemic patient groups and control subjects

<table>
<thead>
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<th>Control</th>
<th>ND</th>
<th>HD</th>
<th>CAPD</th>
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</thead>
<tbody>
<tr>
<td>GSH (µmol/g Hb)</td>
<td>8.25±2.54</td>
<td>6.48±2.30</td>
<td>6.24±2.36</td>
<td>6.77±2.51</td>
</tr>
<tr>
<td>$\gamma$-GCS (U/g Hb)</td>
<td>1.36±0.27</td>
<td>0.81±0.19</td>
<td>0.76±0.23</td>
<td>0.85±0.20</td>
</tr>
<tr>
<td>GSH-S (U/g Hb)</td>
<td>0.35±0.08</td>
<td>0.31±0.07</td>
<td>0.36±0.10</td>
<td>0.35±0.12</td>
</tr>
<tr>
<td>MDA (µmol/l)</td>
<td>2.22±0.35</td>
<td>3.50±0.62</td>
<td>5.41±1.03</td>
<td>4.65±0.89</td>
</tr>
</tbody>
</table>

*P<0.05 as compared with control.

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**Fig. 1.** Relation between GSH and $\gamma$-GCS in ND, HD and CAPD patients.
provides another route for intracellular depletion of GSH [7,8].

In brief, the enzymes glutathione peroxidase, glutathione reductase, glyoxalase and thiol transferase are responsible for a normal GSH/GSSG ratio, while γ-glutamyl transpeptidase, glutathione S-transferase, the GSSG/GSH-X pump, GSH-S and γ-GCS are responsible for the maintenance of normal total GSH level within cells.

Several reports have shown that uraemic patients, especially those on chronic dialysis, have low erythrocyte GSH concentrations [2–6]. Disturbances in GSH-metabolizing enzymes also have been documented [3,4]. It has been suggested that the decrease in the GSH level may occur as a result of inhibition of GSH reduction [2], an increase in GSH extrusion from erythrocytes as GSSG [5] or enhancement of consumption or loss during therapy [6]. Effects that exacerbate GSH consumption may also derive from the depletion of other important antioxidants, such as vitamins C and E [9]. However, the extent to which red cells of uraemic patients conserve normal total GSH levels by production of de novo GSH has not been elucidated previously.

In this study we demonstrated a significant decrease in erythrocyte γ-GCS activity in uraemic patients (Table 2). This decrease was strongly and positively correlated with low total GSH level in all uraemic groups (Figure 1). Decreased activity of erythrocyte γ-GCS has been reported in uraemic patients [13] and in hereditary γ-GCS deficiency, a very rare disorder with only a few cases reported [14]. The present observations suggest that the decrease in γ-GCS activity may contribute, in part, to the diminished GSH level frequently seen in uraemic patients. However, some of our patients (16 out of 75), regardless of treatment type, had a decreased γ-GCS activity combined with normal GSH levels. This may derive from the ability of erythrocytes to sequester their GSH content for a longer period of time before a substantial GSH depletion, caused mainly by efflux, could occur. It has been shown that administration of buthionine sulfoximine, the most commonly used inhibitor for γ-GCS in kidney, liver and pancreas, causes a rapid depletion of intracellular GSH (t0.5 = 30–60 min), whereas depletion occurs within a several hours in most tissues and too slowly (t0.5 = 4 days) in a few cell types, such as RBCs, for the loss to be measured easily [15].

The decrease in γ-GCS activity is probably due to an inhibitory effect exerted by uraemia and dialysis. Oxidative and nitrosative stress, which are known to be altered in uraemia and dialysis, can negatively affect γ-GCS activity [16,17]. In accordance with this, we found a negative correlation between the oxidative stress marker MDA and γ-GCS in all uraemic groups and this was especially strong in ND patients. The end products of oxidative and nitrosative stress (such as S-nitrosothiols), which have been reported to be elevated in uraemic patients [18], are potent in vitro inhibitors of γ-GCS and act in a dose- and time-dependent manner. Although the level at which S-nitrosothiols caused inhibition of γ-GCS was higher than the levels attained in uraemia [8,16], in vitro studies involved only short-term exposure to the inhibitor. It is possible that chronic exposure to lower levels, such as in uraemia, might still exert significant effects. Disturbances in thiol amino-acids manifested by a high level of blood cysteine have been reported in uraemia [19]. It was shown recently that cysteine can exert an inhibitory effect on γ-GCS [20]. S-sulphohomocysteine and S-sulphocysteine, known also as Bunte salts, are powerful inhibitors of γ-GCS [21] and there is indirect evidence that these species may accumulate in uraemia. High levels of inorganic sulphite also have been reported in uraemia [22]. The half-life of sulphite is too short and it circulates mainly as adducts bound to SH groups of thiol amino-acids and proteins [22]. Adducts of cysteine and homocysteine with sulphite have a similar structure to that of Bunte salts [21]. Interestingly, patients with sulphite oxidase deficiency excrete significant amounts of sulphite and S-sulphocysteine in their urine [23]. There is a similarity between sulphite oxidase deficiency syndrome and uraemia from the point of view of sulphite metabolism, with uraemia having in addition impairment in kidney excretory function and, consequently, retention of these metabolites in the circulation.

The results demonstrated in Table 3 show that a single dialysis session with a low-flux cuprophane dialyser did not replenish GSH levels nor restore γ-GCS activity. We found that incubation of partially purified γ-GCS with plasma obtained from patients having the lowest γ-GCS activity resulted in >63% inhibition of γ-GCS, an effect that was only abolished partially (26.4% ± 15.7% inhibition) by incubation with plasma after dialysis or with protein-free plasma ultrafiltrate (unpublished data). These findings suggest that either there is a persistent inhibitory factor in uraemic plasma which is non-removable by conventional dialysis treatment and that is most likely bound to plasma proteins or that inhibition is secondary to the irreversible inactivation of γ-GCS by post-translation modifications of the enzyme molecule. Modifications of proteins and enzymes by oxidation, nitrrosylation, carbamylation or reactive carbonyls such as MDA, acrolein, methylglyoxal and 4-hydroxynonenal have

Table 3. The effect of a single haemodialysis session on erythrocyte GSH level and γ-GCS and GSH-S activities and plasma MDA levels in HD patients (n = 16)

<table>
<thead>
<tr>
<th></th>
<th>Pre-HD</th>
<th>Post-HD</th>
<th>P-valuea</th>
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<tbody>
<tr>
<td>GSH (µmol/g Hb)</td>
<td>6.61 ± 2.41</td>
<td>6.47 ± 2.58</td>
<td>NS</td>
</tr>
<tr>
<td>γ-GCS (U/g Hb)</td>
<td>0.79 ± 0.21</td>
<td>0.82 ± 0.24</td>
<td>NS</td>
</tr>
<tr>
<td>GSH-S (U/g Hb)</td>
<td>0.34 ± 0.07</td>
<td>0.36 ± 0.12</td>
<td>NS</td>
</tr>
<tr>
<td>MDA (µmol/l)</td>
<td>5.26 ± 0.92</td>
<td>3.45 ± 1.25</td>
<td>&lt; 0.001</td>
</tr>
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</table>

aPaired t-test.

NS, not significant.
been reported in uraemia. A significant inhibition of 
γ-GCS by nitrosylation was reported in vitro [16]. However, there is no information on how the other factors affect γ-GCS kinetic behaviour, although extensive studies have shown that γ-GCS expression is affected significantly by these species.

In conclusion, patients with chronic renal failure exhibited low activity of γ-GCS, the rate-limiting enzyme of GSH biosynthesis. This low activity may, in turn, negatively affect de novo GSH synthesis and explain, in part, the decrease in GSH level frequently reported in these patients. γ-GCS activity was probably inhibited by a uraemic factor that itself was unaffected by dialysis type or by a single haemodialysis session. This hypothesis, however, does not rule out the possibility of decreased γ-GCS expression. Determinations of γ-GCS protein and mRNA levels might provide valuable information to understand the mechanism of γ-GCS inactivation. Further studies are recommended to evaluate the effect of administration of GSH pro-drugs that bypass de novo GSH biosynthesis, such as GSH ethyl esters and γ-Glu-Cys, in order to provide strategies to increase intracellular GSH levels in uraemia and dialysis.

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Conflict of interest statement. None declared.

References


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