Erythrocyte Glutathione Transferase Over-Activity and its Correlation with Plasma Homocysteine in Chronic Kidney Diseases

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Recieved : 29/03/2011 Accepted : 29/05/2011

ABSTRACT

Previous study reported increased expression of erythrocyte glutathione transferase (e-GST) in end-stage renal disease patients on maintenance hemodialysis (MHD), and physiological e-GST level in chronic kidney diseases
Erythrocyte Glutathione Transferase Over-Activity and its Correlation with Plasma Homocysteine in Chronic Kidney Diseases

Kutayba

patients under conservative therapy (CKD). Hyperhomocysteinemia is present in more than 90% uremic patients. In present study we re-evaluated the e-GST levels in 72 CKD patients divided into four stages according to the Kidney Disease Outcomes Quality Initiative (K-DOQI) guidelines staging, 62 MHD patients and 80 healthy controls and studied the correlation between e-GST expression and plasma homocysteine (Hcy) levels in MHD patients. The e-GST activity was assayed using a new automated procedure. A new automated spectrophotometric procedure for e-GST activity, validated by intra-day, inter-day and recovery experiments, confirmed an increased e-GST activity in MHD patients (10.2 ± 0.4 U/g Hb) compared with controls (5.8 ± 0.4 U/g Hb). A surprising significant increase of e-GST activity was observed in predialysis patients related to K-DOQI stages (7.4 ± 0.5 U/g Hb, 8 ± 1 U/g Hb, 9.5 ± 0.6 U/g Hb, 12 ± 1 U/g Hb in stages from I to IV, respectively). A direct correlation between plasma Hcy and e-GST expression was found in MHD patients. e-GST proposes as new biomarker for MHD and CKD patients.

INTRODUCTION

Glutathione transferases (GSTs) represent a superfamily of enzymes devoted to the cell protection (1). A prominent function of these enzymes is the conjugation of glutathione (GSH) to a lot of toxic hydrophobic compounds provided of an electrophilic center (2). This reaction facilitates their inactivation and renal elimination (1). Conversely, red blood cells express almost exclusively a single GST isoenzyme (e-GST) which has been identified as GSTP1-1 (3) and represents more than 95% of the erythrocyte GST pool. Over-expression of e-GST has been found only in uremic patients under maintenance hemodialysis (MHD) (4) and in subjects affected by hyperbilirubinemia (5). e-GST expression has been proposed as possible marker to check the accumulation of uremic toxins and to probe the efficiency of dialytic procedures (4). In these previous studies e-GST levels has been checked only in a few chronic kidney disease (CKD) patients under conservative therapy and the expression of this enzyme appeared not different from the one found in the controls (4). The first aim of the present study is to develop a simple method for the determination of e-GST activity and re-evaluate the e-GST activity on a more representative number of
CKD patients divided according to K-DOQI staging (6). High-sensitive C-Reactive Protein (hs-CRP) (7), prognostic inflammatory nutritional index (PINI) (8), alpha-1 acid glycoprotein, fibrinogen, and beta-2 microglobulin (9) were also measured to check a possible correlation between e-GST over-activity versus systemic inflammation (10).

Hyperhomocysteinemia is commonly found in renal patients and attracted a lot of attention because its relation to renal dysfunction (11). In non-uremic subjects homocysteine (Hcy) metabolism is under genetic control. Increased total plasma Hcy is often caused by genetic defects of metabolic enzymes (12, 13) such as 5,10- methylenetetrahydrofolate reductase. In renal patients the total plasma Hcy is significantly increased regardless of genetic defects (12). In fact, hyperhomocysteinemia is common in uremic MHD patients (14) with > 90% of dialysis patients having increased plasma Hcy. Elevated plasma Hcy may promote endothelial dysfunction, which is probably the consequence of the oxidative inactivation of endothelium-derived nitric oxide (NO) (12). Hcy may also cause oxidative stress by inhibiting the expression or the activity of cellular antioxidant enzymes such as glutathione peroxidase-1 (GPx-1) (15). Thus a second target of this study is to verify a possible relation between the increase of plasma Hcy level and eGST activity in uremic MHD patients. The direct correlation found here discloses an interesting scenario where eGST may act as a new marker complementary or substitutive of Hcy assay for oxidative stress in MHD patients.

MATERIALS AND METHODS

Patients and Study Design

All experiments in the present study were conducted at the University of Rome “Tor Vergata”, Italy. Blood samples were obtained from 72 CKD patients under conservative therapy (33 men, 39 women, mean age 54.5 years, range 24-80 years), 62 MHD patients (29 men, and 33 women, mean age 58.0 years, range 36-81 years) were on maintenance renal replacement therapy since six months at least, and 80 healthy controls (35 men, and 45 women, mean age 46.1 years, range 23-75 years) with normal renal function. K-DOQI CKD staging system was used to group pre-dialysis patients according to their estimated glomerular filtration rate (GFR). Exclusion criteria for both patients and control
Erythrocyte Glutathione Transferase Over-Activity and its Correlation with Plasma Homocysteine in Chronic Kidney Diseases

Kutayba

subjects were a clinical history virus hepatitis B and C or serum AST and/or ALT ≥ twice the upper limit of normal values, morbid obesity, rheumatologic disorders as systemic lupus erythematosus and active malignancy. All 62 MHD patients were treated with either standard bicarbonate dialysis with 1.5 to 2.0 square-meter hollow fiber low flux polysulphone membranes (or ‘on line’ hemofiltration with 1.5 to 2.0 square-meter hollow fiber high flux polysulphone (B. Braun GMBH, Melsungen, Germany)) four hours, three times weekly through a well functioning native A-V fistula or a cuffed internal jugular indwelling venous catheter. The vascular access performance was satisfactory with a blood flow of at least 300 ml/min and Kt/V ratio > 1.2. Underlying disease was nephroangioscleriosis in 20 patients, chronic primary glomerulonephritis in 23 patients, chronic interstitial nephritis in 10 patients, polycystic kidney disease in 6 patients and diabetes mellitus in 3 patients.

Analytical Procedures

All the reagents in the present study were from Sigma-Aldrich (St. Louis, USA) and used without further purification. Blood samples were collected from the antecubital vein in healthy subjects and in CKD patients under conservative therapy, from the arterial site of the vascular access before dialysis in MHD patients at the end of the long interval for Hcy determinations. Blood samples were collected into K3EDTA vacutainer tubes as well, put on ice and immediately centrifuged and stored at -20°C until analysis. For e-GST activity determinations, blood drawn was collected on EDTA and stored until used at 4°C for no more than four days. One volume (20 μl) of whole blood was diluted in 20 volumes (0.5 ml) of bi-distilled water and after five minutes introduced into the Modular P800 (Roche, Switzerland) automated apparatus for GST activity determination. GST activity was assayed spectrophotometrically at 340 nm (37°C) using 20 μl of hemolyzed sample in 0.2 ml final volume containing 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in 0.1 M potassium phosphate buffer, pH 6.5. Time run was one minute. The automated analyzer uses an optical path length of 0.5 cm and it can perform up to 800 tests per hour. Hemoglobin was determined with an automated haematology analyzer XE-2100 (Dasit, Milano, Italy). Results were expressed as enzyme units (U) per
gram of Hb. One unit represents the amount of enzyme that catalyzes the conjugation of 1 micromole of GSH to CDNB in one min at 37°C. Recombinant human GSTP1-1 expressed in *E. coli* and purified as described previously (16) was used as internal standard. All routine laboratory measurements were performed by nephelometric methods (BN IIITM BNHTM nephelometer, Siemens Healthcare Diagnostics, Milano, Italy), except for fibrinogen that was quantified by phototurbimetric method (Ca 7000 Sysmex, Japan). Plasma total Hcy was assayed by a fully automated HPLC method using reversed-phase separation and fluorescence detection as reported previously (17). Cystatine C and creatinine were determined as described previously (18). prognostic inflammatory nutritional index (PINI), alpha-1 acid glycoprotein and beta-2 microglobulin were determined as described previously (8, 9).

RESULTS AND DISCUSSION

*Simplified Procedure for e-GST Activity Determination*

Previous studies finalized to quantify e-GST activity needed time consuming erythrocyte isolation and conspicuous blood volumes (3, 4). Our simplified procedure, adapted to an automated apparatus (Modular P800), requires only 20 μl of whole blood and no erythrocyte purification step. Actually, the amount of extra-erythrocyte GST is always negligible and no relevant spectrophotometric artifacts occur in the presence of broken erythrocytes. Furthermore GSTP1-1 represents more than 95% of all GST isoenzymes found in the red blood cells. This enzyme homogeneity makes the activity value directly related to the level of expression of GSTP1-1. Linearity and recovery experiments performed on the automated apparatus using blood samples implemented by authentic GSTP1-1 are shown in fig. 1a and b. Intra-day precision and inter-day precision were evaluated using four whole blood samples. e-GST activity was measured six times for each sample and the relative SEM was 2.9% and 3.5% for intra-day and inter-day assay, respectively. A surprising property of e-GST is represented by its strong stability in whole blood samples stored at 4°C without dilution. The loss of enzyme activity does not exceed 7% even after twelve days at 4°C (fig. 1c). Conversely, a relevant GST inactivation occurs during blood storage at -20°C or at 4°C after hemolysis. Thus, the stability of the GST activity is likely linked to erythrocyte integrity.
while it is lost when GST is under dilute conditions. This behavior is consistent with the observation that GSTP1-1 under dilution, undergoes a remarkable biphasic inactivation process due to a solvatation of the active site (19).

**Fig.-1:** Glutathione transferase assay.

(a) Linearity of the automated assay procedure. Recombinant human GSTP1-1 activity was measured on the automated Modular P800 apparatus set at 340 nm (37°C).

(b) Recovery experiments of GSTP1-1 have been performed using the standard assay procedure on the automated Modular apparatus using whole blood samples implemented by variable amounts of recombinant GSTP1-1. The unit values of the endogenous GSTP1-1 have been subtracted in each sample.

(c) Stability of e-GST in blood samples. Squares (4°C), circles (-20°C).
**Evaluation of e-GST Activity in Renal Failure Patients**

The aforementioned procedure was successfully employed to evaluate e-GST activity in CKD patients under conservative therapy, in MHD patients and in healthy subjects. Mean clinical features and laboratory findings of healthy subjects, MHD patients and CKD patients under conservative therapy are summarized in Table 1. e-GST is highly enhanced in MHD patients, according to previous studies (5, 6). However, in disagreement to a previous report (5), we observed an increase of e-GST activity also in pre-dialysis patients, and this seems to be related to CKD stage (Table 1 and Fig. 2).

**Table 1.** Main clinical features and laboratory findings in 72 pre-dialysis patients divided into four subgroups according to K-DOQI stage (stage I to IV) CKD, 62 ESRD patients on MHD and 80 healthy subjects (control group).

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Stage I (CKD)</th>
<th>Stage II (CKD)</th>
<th>Stage III (CKD)</th>
<th>Stage IV (CKD)</th>
<th>ESRD on MHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>e-GST (U/g Hb)</td>
<td>5.8 ± 0.4</td>
<td>7.4 ± 0.5</td>
<td>8 ± 1</td>
<td>9.5 ± 0.6</td>
<td>12 ± 1</td>
<td>10.2 ± 0.4</td>
</tr>
<tr>
<td>Hs-CRP (mg/l)</td>
<td>1.2 ± 0.7</td>
<td>3 ± 1</td>
<td>3.4 ± 0.4</td>
<td>4.0 ± 0.8</td>
<td>7 ± 2</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>GFR (ml/min) a</td>
<td>118 ± 2</td>
<td>109 ± 3</td>
<td>77 ± 2</td>
<td>42 ± 2</td>
<td>20 ± 1</td>
<td>&lt; 4.7 b</td>
</tr>
<tr>
<td>PINI</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.3</td>
<td>1.0 ± 0.6</td>
<td>0.5 ± 0.1</td>
<td>1.3 ± 0.4</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>alpha-1 acid glycoprotein (g/l)</td>
<td>0.70 ± 0.03</td>
<td>0.90 ± 0.06</td>
<td>0.95 ± 0.04</td>
<td>0.92 ± 0.06</td>
<td>1.21 ± 0.09</td>
<td>1.24 ± 0.05</td>
</tr>
<tr>
<td>beta-2 microglobulin (mg/l)</td>
<td>0.90 ± 0.08</td>
<td>1.84 ± 0.09</td>
<td>2.2 ± 0.1</td>
<td>5.6 ± 0.5</td>
<td>12 ± 1</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Mean Age (years)</td>
<td>46.1</td>
<td>45.8</td>
<td>51.1</td>
<td>64.4</td>
<td>62.3</td>
<td>58.0</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.

* GFR was calculated on the basis of MDRD equation.

* b only two MHD patients showed a residual renal function while 60 were totally anuretic.
The difference is statistically significant between healthy subjects and all CKD patients (P < 0.0001 for groups I to IV and P < 0.0001 versus MHD patients). Among pre-dialysis patients the difference is significant between groups I and III (P = 0.016), I and IV (P < 0.0001), II and IV (P = 0.015) and III and IV (P = 0.04). e-GST activity is significantly higher in MHD patients than in CKD patients of groups I and II, (P < 0.0001 and P = 0.016) and in healthy
controls (P < 0.0001); surprisingly, it is significantly lower in MHD patients than in CKD patients of group IV (P = 0.034).

**e-GST Activity Does not Correlate to Clinical Inflammation Markers**

When e-GST activity was related to a few markers used to monitor the progress and gravity of the renal disease, we invariably observed a parallel increase (table 1). However, a more stringent statistical analysis demonstrated the absence of any direct correlation between e-GST activity and conventional markers of either acute phase (i.e. alpha-1 acid glycoprotein) or chronic inflammation (i.e. hs-CRP), or chronic inflammation/kidney disease (i.e. beta-2 microglobulin) and malnutritioninflammation (i.e. PINI) (table 1).

**e-GST Activity Correlates to Plasma Hcy in MHD Patients**

According to previous studies, almost all MHD patients display increased levels of plasma Hcy (table 2). Interestingly, we found that either mean e-GST activity and mean plasma Hcy were significantly increased in MHD patients compared to controls (10.2 ± 0.4 U/g Hb versus 5.8 ± 0.4 U/g Hb, P < 0.0001; 52 ± 4 μmol/l versus 13.6 ± 0.8 μmol/l, P < 0.0001) (table 2). Furthermore, a significant direct correlation was also found between plasma Hcy and e-GST activity (r = 0.796), as represented in fig. 3. A possible direct interaction between Hcy and e-GST has been checked. e-GST seems to be unable to use Hcy as substrate as demonstrated by the absence of transferase activity using 1 mM CDNB and 1 mM Hcy. On the other hand no detectable inhibition has been found using up to 1 mM Hcy or Hcy thiolactone in the presence of the classical GST substrates (1mM GSH and 1 mM CDNB) (not shown). Previous studies have shown a significant relationship between uremic toxins (such as Beta-2 microglobulin) and residual renal function (20). This has also been established for homocysteine (21). Therefore, residual renal function might be an important confounder for the observed relation between e-GST and plasma homocysteine. However, the multi-regression analysis that includes the residual GFR (assayed on the basis of cystatine C and creatinine ) did not show any correlation between e-GST activity and these markers (r2 = 0.338 and 0.230 for cystatine C and for creatinine respectively).
Table-2: Mean e-GST activity and plasma Hcy in MHD patients versus controls

<table>
<thead>
<tr>
<th></th>
<th>MHD patients</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>e-GST activity (U/g Hb)</td>
<td>10.2 ± 0.4</td>
<td>5.8 ± 0.4</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>plasma Hcy (μmol/l)</td>
<td>52 ± 4</td>
<td>13.6 ± 0.8</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. P < 0.05 is considered statistically significant.

Fig. -3: Correlation between plasma Hcy levels (μM) and e-GST activity (U/g Hb).

In the previous study and before eGST activity was measured, eGST was purified from a human RBCs by affinity chromatography on Sephadex G25. This technique requires a long time and cause a loss of amount of enzyme compared with the rapid and simple method used in our study. A simple procedure to measure the activity of the e-GST on an automated spectrophotometric apparatus has been optimized and employed to evaluate e-GST activity in pre-dialysis patients under conservative therapy and in MHD...
patients. The use of very small amounts of whole blood that avoids the time-consuming erythrocyte isolation represents a certain improvement over the usual procedures. This simplification is possible because the presence of extra-erythrocyte GST in the blood is always negligible, and broken erythrocyte membranes in the assay mixture do not perturb the spectrophotometric measurements. The simplified assay requires only 20 μl of whole blood which can be collected after a non-invasive pinprick. Furthermore, e-GST activity does not change appreciably during prolonged storage of the blood samples (4-6 days at 4°C) (see fig. 1a, and b). Overall, this procedure, validated by classical recovery experiments, as well as by low intra-day and inter-day variations could be of interest for routinely controls to test the efficiency of therapeutic procedures on patients with CDK. Actually, our results confirm previous reports of an enhanced e-GST activity in MHD patients (4, 5); conversely, in opposition to previous reports (4), in CKD patients under conservative therapy we observed for the first time a significant and progressive increase of e-GST activity that in likely related to the K-DOQI stage (fig. 2 and table 1). Interestingly, we also found a significantly higher activity of e-GST in pre-dialysis patients of K-DOQI stage IV compared to MHD patient. This finding is possibly explained by considering that GST expression reflects the abundance of circulating uremic toxins and that the hemodialytic procedure likely lowers the level of these toxic compounds. Both e-GST and inflammation/malnutrition markers are all significantly increased in CKD and MHD patients compared to controls (table 1). However, we did not find any significant correlation between the increased level of e-GST and all these markers. This finding is not surprising. In fact not all toxins ignite chronic or acute inflammation and GSTs are enzymes able to sweep or inactivate a lot of toxic compounds irrespectively of their inflammatory action. In this line the level of e-GST may fulfill a snapshot of the amount of circulating toxins complementary to the classical markers used to test the severity of chronic inflammation, malnutrition and kidney dysfunction. The correlation between the increased level of e-GST and plasmatic Hcy concentration in MHD patients is of particular interest. Plasma hyperhomocysteinemia is considered a cardiovascular risk factor and it is often associated to renal failure (11). The autoxidation of this sulfur-containing amino acid produces hydrogen peroxide. Moreover, high levels of Hcy reduces the
bioavailability of nitric oxide forming S-nitrosohomocysteine and inhibiting NOS. Thus, it has been proposed that high homocysteine levels are deleterious leading to endothelial dysfunctions and oxidative stress (12). In this view, homocysteine may represent the active biochemical factor that triggers these metabolic dysfunctions. However, a very different scenario is possible where Hcy is not the cause but the consequence of toxic dysmetabolisms, so Hcy could be only a marker of blood toxicity. Indeed, no effect on mortality and vascular diseases had been found in MHD patients by lowering their homocysteine level with folic acid (22). Actually, a paradoxical reverse relationship has been reported between Hcy and mortality in non-treated patients (23). The correlation found in the present study between hyperhomocysteinemia and e-GST supports the proposal that high levels of Hcy may be merely the consequence of high circulating toxins. In fact, increased levels of e-GST are certainly the effect (and not the cause) of an increased cell toxicity. In addition, our observation that the increase of e-GST in CKD patients does not correlate to inflammatory markers parallels a similar observation found for Hcy (23). The absence of any significant correlation between e-GST and cystatine C or creatinine means that the residual renal activity has no or little effect on the e-GST expression. No causative relation can be supposed for e-GST expression and plasmatic Hcy levels. It is well known that GST may bind to its active site hundred different toxic compounds but up today nobody of them is known to interact with the Hcy metabolism. In addition, we can leave out the possibility that Hcy itself or Hcy thiolactone may interact directly with e-GST because no detectable transferase activity is present using 1 mM Hcy as substrate nor relevant inhibition is caused by 1 mM Hcy or Hcy thiolactone. The possible oxidative interaction of Hcy with the essential sulfhydryl group of e-GST (Cys47) would be signaled by an inverse relation between Hcy concentration and GST activity, given that any chemical modification of this residue strongly inhibits the transferase activity (24). In conclusion, the present findings suggest that e-GST level could be a marker for toxin exposition and its determination may fulfill a useful probe to assess the efficiency of dialytic procedures. The e-GST assay may substitute or be complementary to the standard time consuming and expensive Hcy assay.
REFERENCES


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