Research Article

Dilber Çoban Ramazan, Ülker Anadol, A. Destina Yalçın and A. Süha Yalçın*

Plasma homocysteine and aminothiol levels in idiopathic epilepsy patients receiving antiepileptic drugs

Antiepileptik ilaç alan idiyopatik epilepsi hastalarında plazma homosistein ve aminotiyol düzeyleri

Abstract

Objective: Homocysteine is a sulfur containing amino acid that is formed during methionine metabolism. Patients under long-term antiepileptic drug treatment often have hyperhomocysteinemia. These patients have low levels of serum folate, vitamin B12 and vitamin B6, all of which are associated with homocysteine metabolism. We have investigated the effects of valproic acid and new generation antiepileptic drugs (lamotrigine and levetiracetam) on plasma levels of homocysteine and aminothiols as well as serum vitamin B12 and folic acid.

Materials and methods: Forty-seven idiopathic epileptic patients on antiepileptic drugs were compared with 38 age-matched healthy controls. Commercial immunoassay methods were used for vitamin B12 and folic acid analyses. Homocysteine, cysteine, cysteinylglycine and glutathione levels were determined by high performance liquid chromatography.

Results: There was no significant difference in patient and control values in terms of vitamin B12, folic acid and homocysteine. Valproic acid and lamotrigine seemed to effect aminothiol redox status. Glutathione levels of epileptic patients receiving valproic acid and lamotrigine were higher than controls.

Conclusion: Our results suggest that redox homeostasis may be impaired and glutathione synthesis increased in response to the oxidative stress caused by antiepileptic drug use.

Keywords: Antiepileptic drugs; Vitamin B12; Folic acid; Homocysteine; Glutathione; Idiopathic epilepsy patients.

*Corresponding author: Prof. Dr. A. Süha Yalçın, Department of Biochemistry, School of Medicine, Marmara University, Başıbüyük, Maltepe, 34854 İstanbul, Turkey, e-mail: asyalcin@marmara.edu.tr. https://orcid.org/0000-0002-3527-631X

Dilber Çoban Ramazan: Department of Biochemistry, School of Medicine, Marmara University, Maltepe, Istanbul, Turkey, e-mail: dr.dilber1985@hotmail.com

Ülker Anadol and A. Destina Yalçın: Department of Neurology, Ümraniye State Hospital, School of Medicine, Health Sciences University, Ümraniye, Istanbul, Turkey, e-mail: unadol@hotmail.com (Ü. Anadol); destinayalcin@yahoo.com (A.D. Yalçın)
etkilediği görüldü. Bu ilaçları alan hastalarda glutatyon değerleri kontrollerden yüksek bulundu.

Sonuç: Bulgularımız antiepileptik ilaç kullanımı-nın oksidatif strese yanıt olarak redoks dengesinde bozukluga ve glutatyon sentezinde artışını düşündüremektedir.

Anahtar Kelimeler: Antiepileptik ilaçlar; B12 Vitamini; Folik asit; Homosistein; Glutatyon; İdipatik epilepsi hastaları.

Introduction

Homocysteine is a sulfur containing amino acid formed during methionine metabolism. Plasma homocysteine level is an important risk factor for many diseases such as arterial and venous thrombosis, myocardial infarction and chronic renal insufficiency. Accordingly, hyperhomocysteinemia has been associated with cardiovascular diseases and is regarded as an independent risk factor for atherosclerosis [1]. Patients with long-term antiepileptic drug treatment often have hyperhomocysteinemia and low levels of serum folate, vitamin B12 and vitamin B6, all of which are associated with homocysteine metabolism [2, 3]. On the other hand, some antiepileptic drugs, i.e. carbamazepine, phenytoin, phenobarbital and primidone, have inducing effects on hepatic enzymes [4, 5], and may stimulate hepatic cytochrome P450 and glucuronyl transferase [6]. However, many of the lately developed antiepileptics are less likely to influence the activity of hepatic enzymes when compared with older drugs. The risk of hyperhomocysteinemia can be regarded negligible or even absent with these new drugs [7].

In the present study, our aim was to investigate the effects of valproic acid and two new generation antiepileptic drugs (lamotrigine and levetiracetam) on plasma homocysteine and aminothiols. We have used the commercial homocysteine method to measure plasma aminothiols namely cysteine, cysteinylglycine and glutathione.

Materials and methods

Subjects and study design

Patients who were referred to the epilepsy outpatient clinic (May 2016–May 2017) were included in the study which was approved by the institutional Ethics Committee (6907-06.05.2016). All subjects gave their written and informed consent. The patient group comprised 47 idiopathic epilepsy who were under valproic acid, levetiracetam or lamotrigine monotherapy. Controls were 38 healthy subjects aged 15–65 years without any health problems. Gender distribution was 24 women (63.16%) and 14 men (36.84%) in the control group, and 33 women (70.21%) and 14 men (29.79%) in the patient group. The mean age of the controls was 28.71 ± 8.90 and that of the patients was 26.06 ± 8.61. Family history was null in controls and 58% in patients. Patients were divided into three groups according to their medications. Group 1 was valproic acid (n = 26), Group 2 was lamotrigine (n = 8), and Group 3 was levetiracetam (n = 13). Duration of drug use was 7.90 ± 6.13 years for valproic acid, 9.50 ± 5.81 years for lamotrigine and 7.25 ± 5.47 years for levetiracetam. The presence of diabetes, hypertension, hypothyroidism, liver and kidney disease, malignancy, vitamin use and vegetarian diet were questioned in both patient and control groups. People with these were excluded from the study. Following an overnight fasting period venous blood was taken from the antecubital vein. Blood samples were transferred to the laboratory on ice within 1 h. After centrifugation at 3400 × g for 5 min plasma and serum was separated and samples were stored at −80°C until the corresponding assays were conducted.

Methods

Commercial immunoassay kits were used for serum folic acid and vitamin B12 determinations (Beckman Coulter UniCel Dxi 800, USA). Plasma homocysteine was assayed by HPLC with fluorescence detection (ImmuChrom GmbH, Lot. No. LC2801, Germany). Cysteine, cysteinylglycine and glutathione were assayed from the same chromatogram of homocysteine. Cysteine (CAS No. 168149-25G, Sigma-Aldrich, USA), cysteinyl-glycine (CAS No. C0166-25MG, Sigma-Aldrich, USA) and glutathione (CAS No. G4251-10G, Sigma-Aldrich, USA) were purchased and used for standards.

Validation studies

Stock solutions of cysteine, cysteinylglycine and glutathione (10 mmol/L) were prepared and used in validation studies. To demonstrate the linearity of the method, to draw calibration graphs and to determine the analytical measurement range serial dilutions (50, 100,
200 μmol/L for cysteine; 1, 5, 10, 50 μmol/L for glutathione and cysteinylglycine) were made and applied to the homocysteine sample preparation procedure. The area under the curve (AUC) of the peak retention times were calculated from the chromatograms. Cysteine, cysteinylglycine and glutathione concentrations were calculated from the corresponding concentration-AUC plots. A representative chromatogram and the concentration-AUC plots for cysteine, cysteinylglycine and glutathione is shown in Figure 1. To determine the limit of detection (LOD) values, pretreatment was carried out in accordance with the homocysteine sample preparation procedure. Twenty repetitive measurements were performed. LOD values were determined as 3.19 μmol/L for cysteine and 0.43 μmol/L for homocysteine. For glutathione and cysteinylglycine LOD values could not be calculated because their retention times were very close to each other and the peaks were not completely separated in all runs. For reproducibility studies, working standards (cysteine: 50, 100, 200 μmol/L, cysteinylglycine: 5, 10, 50 μmol/L, glutathione: 1, 5, 10 μmol/L) were used. Appropriate samples were prepared from these standards according to the homocysteine sample preparation procedure. Each concentration was studied 12 times (3 times on the first day, 3 times on the second day and 6 times on the third day). Percentage CV values for intra and inter day measurements were calculated. They were less than 10% except for two concentrations (Table 1). For the recovery study, a plasma pool was prepared from the leftover blood specimens. Recovery values were calculated as 97.7% for homocysteine and 97.9% for cysteine. Recovery values for glutathione and cysteinylglycine were not calculated because as mentioned above their retention times were very close and the peaks were not completely separated in all runs.

Table 1: Intra day and inter day CV values for cysteine, cysteinylglycine and glutathione.

<table>
<thead>
<tr>
<th>Concentration (μmol/L)</th>
<th>CV (%) Intra day</th>
<th>CV (%) Inter day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>9.64</td>
<td>10.20</td>
</tr>
<tr>
<td>100</td>
<td>6.68</td>
<td>3.10</td>
</tr>
<tr>
<td>200</td>
<td>6.55</td>
<td>9.64</td>
</tr>
<tr>
<td>Cysteinylglycine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.08</td>
<td>5.23</td>
</tr>
<tr>
<td>10</td>
<td>6.15</td>
<td>3.02</td>
</tr>
<tr>
<td>50</td>
<td>7.70</td>
<td>4.10</td>
</tr>
<tr>
<td>Glutathione</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8.05</td>
<td>8.02</td>
</tr>
<tr>
<td>5</td>
<td>6.89</td>
<td>2.52</td>
</tr>
<tr>
<td>10</td>
<td>5.22</td>
<td>10.60</td>
</tr>
</tbody>
</table>

Figure 1: A representative chromatogram and calibration plots for cysteine, cysteinylglycine and glutathione.
**Statistical analysis**

SPSS v.15.0 package was used for statistical evaluation. Data on continuous variables were given as mean and standard deviation. The significance level for all analyses was \( p < 0.05 \).

**Results**

Our controls were 38 healthy subjects aged 15–65 years without any health problems and the patient group comprised 47 idiopathic epilepsy who were under valproic acid, levetiracetam or lamotrigine monotherapy. No significant gender difference was present. Also there was no significant age difference between control and patient groups, and there was no significant difference in duration of drug use between different patient groups. We have used commercial immunoassay kits for serum folic acid and vitamin B12 determinations. Plasma aminothiols were assayed by the homocysteine method using HPLC with fluorescence detection where cysteine, cysteinylglycine and glutathione were assayed from the same chromatogram obtained for homocysteine. However, as mentioned in validation studies, cysteinylglycine and glutathione peaks could not be separated completely in all chromatograms. Therefore, in future studies the chromatography conditions used in measuring aminothiols needs to be improved by changing chromatography conditions such as flow-rate and pH.

Table 2 shows vitamin B12, folic acid and aminothiol levels of healthy controls and patients with idiopathic epilepsy receiving different antiepileptic drugs. There was no significant difference in vitamin B12 and folic acid levels of antiepileptic drug users compared to controls. Also, no significant difference was found for homocysteine, cysteine and cysteinylglycine levels between patients and controls. However, although there was no significant difference in glutathione levels of patients using levetiracetam compared to the controls, glutathione levels of lamotrigine and valproic acid users were significantly increased. Table 3 shows comparison of plasma aminothiol levels of controls and all idiopathic epilepsy patients. No significant difference was found in homocysteine and cysteinylglycine levels but cysteine and glutathione levels of antiepileptic drug users were significantly different than controls.

**Discussion**

In early studies on plasma homocysteine levels of epilepsy patients receiving phenytoin [8–10] fluctuations in homocysteine levels were reported. Further studies after phenytoin have been performed on patients receiving carbamazepine and valproic acid in which almost all patients were observed to have elevated homocysteine levels [3, 11–16]. Later, effects of newer antiepileptics were studied. Gidal et al. [17] examined homocysteine, vitamin B12 and folic acid in patients who received valproic acid and lamotrigine monotherapy before and after treatment. Chronic treatment with lamotrigine did

| Table 2: Vitamin B12, folic acid and aminothiol levels of controls and idiopathic epilepsy patients receiving different antiepileptic drugs. |
|-----------------|-----------------|-----------------|-----------------|
|                 | Control (n=38)  | Valproic acid (n=26) | Lamotrigine (n=8) | Levetiracetam (n=13) |
| Vitamin B12 (pg/mL) | 209.21±98.67 | 189.12±58.26 | 198.63±42.65 | 168.85±60.30 |
| Folic acid (ng/mL)    | 6.67±2.98     | 7.44±2.32      | 7.44±2.32      | 6.91±2.26      |
| Homocysteine (μmol/L)  | 14.39±7.55    | 13.78±2.79      | 15.48±13.55 | 13.54±3.60      |
| Cysteine (μmol/L)       | 171.44±43.86  | 158.55±42.43    | 137.38±44.68  | 146.86±40.17 |
| Cysteinylglycine (μmol/L) | 46.23±17.35  | 39.13±8.71     | 46.24±22.06  | 38.57±8.35     |
| Glutathione (μmol/L)     | 2.42±2.53    | 3.71±1.75      | 4.57±1.29*  | 3.54±1.84    |

Values represent mean± standard deviation.

*p < 0.05 compared to the control group.

| Table 3: Plasma aminothiol levels of controls and all idiopathic epilepsy patients. |
|-----------------|-----------------|-----------------|
|                 | Controls (n=38)  | Patients (n=47) | p-Value |
| Homocysteine (μmol/L) | 14.39±7.56   | 14.00±6.00    | 0.396 |
| Cysteine (μmol/L)    | 171.44±43.86  | 151.71±42.58  | 0.028 |
| Cysteinylglycine (μmol/L) | 46.23±17.35 | 40.17±11.82   | 0.197 |
| Glutathione (μmol/L) | 2.42±2.53     | 3.80±1.71     | 0.001 |

Values are expressed as μmol/L and represent mean± standard deviation.
not effect blood concentrations of homocysteine, folic acid and vitamin B12. On the other hand, valproic acid caused increased vitamin B12 and decreased homocysteine concentrations whereas folic acid was not changed. Belcastro et al. [18] examined 259 epileptic patients and 231 healthy controls and reported that plasma homocysteine levels were significantly higher and folate levels were lower in patients. Patients who were treated with oxcarbazepine, topiramate, carbamazepine, and phenobarbital exhibited mean plasma homocysteine levels above the physiologic range. Conversely, homocysteine concentrations were observed to be unchanged in lamotrigine and levetiracetam groups [18].

Antiepileptic drugs may interfere with the absorption and metabolism of folate [19, 20]. It is unclear how antiepileptics cause changes in vitamin levels. One mechanism is by decreasing the activity of the enzyme required for re-methylation of methionine [8, 21, 22]. Other possible mechanisms involved are activation of the cytochrome P450 system, reducing absorption of vitamins by increasing intestinal pH, and interacting with the metabolism of vitamins [23–25]. While carbamazepine, phenytoin and phenobarbital induce cytochrome P450 system, recent reports on newly developed antiepileptics such as lamotrigine, topiramate, levetiracetam and oxcarbazepine suggest that they do not induce enzymes. Drugs that do not induce hepatic enzymes also have no effect on serum folic acid levels [17].

No significant difference between vitamin B12, folic acid and homocysteine levels of controls and patients under valproic acid, lamotrigine and levetiracetam monotherapy was observed in our study. However, valproic acid and lamotrigine users had higher glutathione levels than controls. Glutathione is a tripeptide synthesized from glutamate, cysteine and glycine. It protects cells against free radical related oxidative damage and is present at high levels in many tissues especially in the liver [26]. Glutathione is also involved in the transport of amino acids via gamma-glutamyl cycle. Therefore, it is thought that it serves as the storage and transport form of cysteine.

There is limited information on the relationship between homocysteine and aminothiols in epilepsy patients treated with different antiepileptic drugs. Apeland et al. [5] studied patients with hyperhomocysteinemia receiving antiepileptics who were also given pyridoxine, riboflavin, folic acid as supplements for 1 month. They observed that all redox products were decreased. Homocysteine will transform into cysteine and glutathione by trans-sulfuration pathway. This is not only a compensatory mechanism that prevents accumulation of homocysteine, but also provides cysteine and glutathione to the body. Thus the trans-sulfuration pathway assumes a protective role against oxidative stress while reducing homocysteine levels. As suggested by Vitvitsky et al. [27], the significant link between the trans-sulfuration pathway and the redox buffering capacity warrants investigations into potential neuroprotective benefits of nutritional modulation of redox homeostasis.

We admit that the chromatography conditions we have used in measuring aminothiols needs to be improved by changes such as flow-rate and pH. In any case, antiepileptic drugs resulted in increased glutathione compared to the control values. This may suggest impaired redox homeostasis causing increased glutathione synthesis in response to oxidative stress induced by these drugs. Since glutathione synthesis occurs in the cytoplasm with the aid of two enzymes, namely gamma-glutamylcysteine synthetase and glutathione synthetase, future studies determining the activities of these enzymes will be useful to clarify the underlying mechanism of the changes observed.

Acknowledgement: This study was financially supported by Marmara University Scientific Research Projects Unit (Project no: SAG-C-TUP-200716-0366).

Conflict of interest statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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