Homocysteine and red blood cell glutathione as indices for middle-aged untreated essential hypertension patients
Piibe Muda, Priit Kampus, Mihkel Zilmer, Kersti Zilmer, Ceslava Kairane, Tiina Ristimäe, Krista Fischer and Rein Teesalu

Objective Intracellular glutathione in its reduced state is a principal cellular biomolecule with antioxidant activity. Glutathione and homocysteine metabolism are closely associated. As both oxidative stress and hyperhomocystinemia are associated with hypertension, we assessed the relationships between these variables.

Design and setting An observation-based case-control study, performed at a university teaching hospital

Patients Middle-aged male patients with untreated uncomplicated essential hypertension (mean ± standard deviation age 53.0 ± 7.2 years, n = 48) before any treatment and controls with similar age distributions (age 51.6 ± 5.5 years, n = 28) were evaluated.

Methods In all subjects, the plasma levels of homocysteine, lipids, creatinine, protein, and glucose were measured. Reduced and oxidized glutathione and folic acid were measured from red blood cells (RBC).

Results The hypertensive patients had decreased levels of red blood cell reduced glutathione (RBC-GSH) and increased levels of oxidized glutathione, which resulted in elevated ratio of oxidized/reduced glutathione as compared to controls (P < 0.001). Plasma homocysteine levels were significantly higher in the hypertensive patients versus the age-matched controls (P < 0.004). In the hypertensive patients, RBC-GSH correlated inversely with systolic blood pressure, serum creatinine, protein and RBC folic acid. No correlation was detected between RBC-GSH and homocysteine. In the controls, RBC-GSH correlated inversely with homocysteine, RBC folic acid and creatinine. According to multiple regression, RBC-GSH was related to systolic blood pressure (SBP), hemoglobin, plasma homocysteine, creatinine and protein. Such a relationship was not detected for the controls.

Conclusion In untreated hypertensive patients both homocysteine and systolic blood pressure are associated with intracellular oxidative stress as determined by RBC-GSH. J Hypertens 21:1–5 © 2003 Lippincott Williams & Wilkins.

Keywords: folic acid, glutathione, homocysteine, hypertension, redox ratio

Introduction Hypertension is a well-established and independent risk factor for development and progression of atherosclerosis [1]. The mechanisms predisposing hypertensive subjects to organ injury and atherosclerosis are multifactorial. The mechanisms that are implicated in essential hypertension include prolonged high-grade oxidative stress and elevation of plasma homocysteine (Hcy) level [2–5]. Additionally, several links between hypertension and red blood cell glutathione (GSH) as the principal cellular antioxidant have been reported recently [6,7].

There exist reciprocal links between oxidative stress, GSH and Hcy. Namely, cellular GSH is involved in protection of endothelial cells of reactive oxygen species, leading to prevention of endothelial dysfunction in the arteries exposed to severe oxidative stress [8,9]. Hcy metabolism provides about half of GSH. The Hcy is produced during methionine metabolism and is converted either via transmethylation (back to methionine) or transsulfuration reactions. The latter pathway is catalyzed by the vitamin B6-dependent enzymes and heme, and yields cystathionine and, subsequently, cysteine and GSH. Hence transsulfuration provides a direct link between Hcy and GSH, the major redox buffer in mammalian cells [8,10]. According to experimental studies, increased oxidative stress appears to play a pathophysiological role in the deleterious endothelial effects of Hcy [4–5]. The Hcy may also induce intracellular suppression of the glutathione antioxidant defense system and possibly reduce Hcy-derived GSH synthesis [10].
To the best of our knowledge, the relationships between the levels of plasma Hcy and red blood cell glutathione (RBC-GSH) have not been investigated in human subjects previously. The aim of the present study was to estimate the relationships between these two variables in hypertensive subjects without any other traditional cardiovascular risk factors in order to find out whether experimental data are valid in clinical setting.

**Methods**

**Study population**

The study population consisted of 48 male out-patients (mean age 53.0 ± 7.2 years) with untreated mild to moderate essential hypertension (mean duration of hypertension 12.6 years, range 1–36). All subjects who responded to the advertisement and in whom the inclusion criteria were met were recruited on consecutive basis between September 2000 and December 2002 at the Department of Cardiology, University of Tartu, Estonia. The diagnosis of hypertension was established on the basis of systolic blood pressure > 140 mmHg and/or diastolic blood pressure > 90 mmHg measured during three different visits. Patients with previous antihypertensive treatment had been free of medication for at least two months. A total of 32 patients had never been treated (66.7%), nine (18.8%) not had been on an antihypertensive treatment during the previous 2 months and seven (14.5%) patients had not been treated for the last 6 months to 2 years. All patients were clinically stable. We excluded patients with diabetes (based upon glucose tolerance test), history of cardiac or cerebrovascular disease, heart failure (left ventricular ejection fraction < 50%), hypercholesterolemia (total cholesterol > 6.5 mmol/l), other systemic diseases, recent/current infection, anemia and secondary hypertension. Routine clinical, hematological and radiological examinations excluded the secondary forms of hypertension.

The control group consisted of 28 healthy sex- and age-matched consecutive volunteers (mean age 51.6 ± 5.5 years) who responded to the advertisement. All controls demonstrated normal findings at physical and biochemical examinations, and had normal blood pressure (BP) values (systolic blood pressure (SBP) lower than 136 mmHg and diastolic blood pressure (DBP) lower than 88 mmHg).

All study subjects were non-smokers with a body mass index (BMI) of < 30 kg/m². None of the patients had clinical evidence suggestive of coronary artery disease, based upon history, electrocardiography, exercise test and echocardiography. The subjects who were taking any medical preparations of vitamins or drugs were not included. No dietary restrictions were imposed. The information of the family history of hypertension and other cardiovascular diseases was collected for all study subjects. Hypertension was present in the first-degree relatives in 30 (62.5%) of the hypertensive patients and in 15 (53.6%) in normotensive controls, but the difference was not significant (P = 0.56). The family history of cardiovascular diseases (stroke, myocardial infarction) was positive in 15 (31.2%) hypertensive subjects and in nine (32.1%) controls (P = 0.94). The Ethics Committee of the Medical Faculty, University of Tartu, approved the study protocol and informed consent was obtained from all participants before the study.

**Study protocol**

The subjects were studied and the plasma samples were collected between 0800 and 0900 h, after an overnight fast. BP was measured in both arms in a sitting position after 10 min rest using a conventional mercury sphygmomanometer and a normal size cuff: the mean of three readings with 2 min intervals was taken, with DBPs at Korotkov phase versus. Thereafter, blood samples were drawn from the antecubital vein for the measurement of the oxidative stress markers, Hcy, red blood cell (RBC) folate acid, blood glucose, blood lipids, creatinine, protein, hemoglobin, hematocrit and RBC count. The blood samples were processed within 30 min. The height and weight were recorded, and the BMI calculated.

**Biochemical methods**

The RBC-GSH was measured by using an enzymatic method as described previously [11]. Briefly, protein was removed from 0.3 ml of heparinized whole blood by adding an equal volume of a 10% solution of metaphosphoric acid in water, leaving the mixture at room temperature, and then centrifuging it (4°C, 1200 X g, 10 min). The supernatant was carefully collected and stored at −20°C. The sample was divided into two parts for measurement of the total amount of red blood cell glutathione (RBC-TGSH) and RBC-GSSG. To assay RBC-TGSH or RBC-GSSG, the supernatant was mixed with 0.895 ml of 0.2 mol/l sodium phosphate buffer (pH 7.5) containing 0.01 mol/l ethylenediaminetetraacetic acid and with 0.5 ml the same buffer containing 0.5 U GSH-reductase and 0.3 mmol/l NADPH. The reaction was initiated by the addition of 0.1 ml of 1 mmol/l 5,5′-dithiobis-(2-nitrobenzoic acid). The change in optical density was measured at 412 nm after 10 min and quantitated by comparison with the standard curve. The concentration of RBC-GSH was calculated as the difference between RBC-TGSH and RBC-GSSG.

Hcy was measured by using enzyme immunoassay method (Axis-Shield Diagnostics Ltd, Dundee, UK). For Hcy measurement, blood was drawn into tubes containing EDTA, placed on ice and then centrifuged.
The plasma samples were stored at –70°C until analysis.

The concentration of RBC folic acid was measured both in the whole blood and in the sera by chemiluminescence method with the Immulite 2000 Analyzer (Diagnostic Products Corporation, California, USA), and RBC folic acid in ng/ml was calculated according to the formula: RBC folic acid = (R – [S × (100 – H)/100]) × (100/H), where R denotes whole blood folic acid, H is the hematocrit and S is the level of the patient’s serum folic acid.

Statistical analysis
The normally distributed data are presented as mean ± standard deviation; the non-normally distributed data are presented as the geometric mean with the 95% confidence intervals (CI). Since the distributions of Hcy, RBC count, RBC folic acid and creatinine were skewed, they were log-transformed for analysis as required to improve normality. Unpaired two-tailed t-test was performed to compare the means of the cases and the controls for the study variables. Univariate linear correlation between RBC-GSH and the other variables was used. Linear correlation coefficients were used to estimate the relationships between the variables. Stepwise multiple regression analysis was performed to assess the relationships between RBC-GSH as the dependent variable and the other study parameters as independent variables. All statistical analyses were conducted using the software R, version 1.6.0. for Windows. The level of significance was defined as $P < 0.05$.

Results
Table 1 shows that the untreated hypertensive patients and the normotensive controls did not differ with regard to age, total cholesterol, HDL- and LDL-cholesterol, serum creatinine, protein, RBC count, hematocrit and hemoglobin. The patients with essential hypertension had significantly higher BMI as well as elevated triglycerides and blood glucose. The RBC-GSH levels were significantly lower and the RBC-GSSG (oxidized glutathione) levels higher in subjects with essential hypertension when compared with the age-matched controls (Table 2). After adjusting the RBC-GSH level for age and body mass index, the difference between the patients and the controls remained significant ($P < 0.04$). In the hypertensive patients, the RBC glutathione redox status, expressed as GSSG/GSH was twice as high as that of the control subjects (Table 2) indicating to cellular oxidative stress.

Table 1 Clinical characteristics of the study groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients n = 48</th>
<th>Controls n = 28</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>53.0 ± 7.2</td>
<td>51.6 ± 5.5</td>
<td>0.33</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27.7 ± 2.0</td>
<td>24.7 ± 3.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>147.9 ± 12.9</td>
<td>117.9 ± 10.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>97.1 ± 7.3</td>
<td>76.9 ± 6.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.6 ± 0.8</td>
<td>5.3 ± 0.8</td>
<td>0.14</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.7 ± 0.7</td>
<td>3.4 ± 0.8</td>
<td>0.06</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.3 (1.2; 1.4)</td>
<td>1.4 (1.2; 1.8)</td>
<td>0.13</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.4 (1.3; 1.8)</td>
<td>0.9 (0.8; 1.1)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.6 ± 0.4</td>
<td>5.0 ± 0.4</td>
<td>0.0001</td>
</tr>
<tr>
<td>Protein (g/l)</td>
<td>73.0 ± 5.4</td>
<td>71.3 ± 3.8</td>
<td>0.11</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>102.2 (98.8; 107.8)</td>
<td>96.9 (93.3; 101.6)</td>
<td>0.08</td>
</tr>
<tr>
<td>RBC count ($\times 10^{12}$/l)</td>
<td>4.9 (4.8; 5.0)</td>
<td>4.8 (4.6; 4.9)</td>
<td>0.06</td>
</tr>
<tr>
<td>Hemoglobin (g/l)</td>
<td>147.6 ± 8.2</td>
<td>143.6 ± 13.9</td>
<td>0.18</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>43.7 ± 2.4</td>
<td>42.3 ± 3.6</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Normally distributed data are presented as mean ± standard deviation; non-normally distributed data are presented as the geometric mean with the 95% confidence intervals. The level of significance was defined as $P < 0.05$. BP, blood pressure.

Table 2 Plasma homocysteine, red blood cell glutathione and folic acid in hypertensive versus normotensive subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients n = 48</th>
<th>Controls n = 28</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine (μmol/l)</td>
<td>10.7 (9.7; 12.7)</td>
<td>9.2 (8.5; 10.2)</td>
<td>0.02</td>
</tr>
<tr>
<td>Reduced glutathione (GSH) (μmol/l)</td>
<td>744.7 ± 298.4</td>
<td>1001.1 ± 286.2</td>
<td>0.0005</td>
</tr>
<tr>
<td>Oxidized glutathione (GSSG) (μmol/l)</td>
<td>108.6 (104.7; 145.2)</td>
<td>81.3 (72.9; 103.0)</td>
<td>0.008</td>
</tr>
<tr>
<td>GSSG/GSH</td>
<td>0.16 (0.15; 0.22)</td>
<td>0.09 (0.08; 0.13)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Folic acid in RBCs (ng/ml)</td>
<td>250.6 (243.3; 322.3)</td>
<td>211.4 (183.3; 269.3)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Normally distributed data are presented as mean ± standard deviation; non-normally distributed data are presented as the geometric mean with the 95% confidence intervals. The level of significance was defined as $P < 0.05$.
The untreated hypertensive patients had significantly higher Hcy levels compared with the healthy controls (Table 2). After adjusting Hcy for age and BMI, difference between cases and controls became more pronounced ($P < 0.008$).

In the hypertensive subjects, RBC-GSH concentration showed a negative correlation with SBP ($r = -0.44$, $P < 0.004$, Fig. 1), creatinine ($r = -0.43$, $P < 0.002$), protein ($r = -0.41$, $P < 0.004$) and RBC folic acid ($r = -0.30$, $P < 0.04$). No correlation was detected between RBC-GSH and Hcy, ($r = 0.25$, $P < 0.09$, Fig. 2). For the controls, RBC-GSH correlated negatively and significantly with Hcy ($r = -0.41$, $P < 0.03$, Fig. 2), RBC folic acid ($r = -0.49$, $P < 0.047$) and creatinine ($r = -0.45$, $P < 0.02$).

The results of multiple regression for the hypertensive patients are given in Table 3. Hcy and hemoglobin were positively and significantly correlated with RBC-GSH level, SBP, protein and creatinine were negatively correlated with RBC-GSH level.

**Discussion**

The results of this study confirmed that RBC-GSH level was significantly lower in the middle-aged untreated essential hypertension patients compared with the age-matched controls (Table 2). This correlation was independent of age and BMI. This and the about two-fold elevated glutathione redox ratio could indicate the disbalanced/alterated cellular glutathione system in the hypertensive patients. Our data support the findings of several previous studies. First, it was shown that hypertension is directly associated with the elevated RBC glutathione redox ratio both in gestational hypertension [6] and in juvenile hypertensive patients [7]. Second, considering middle-aged persons, intracellular glutathione, but not plasma glutathione, is characterized by a markedly lower level [12]. In our study including middle-aged persons, we found an inverse correlation between SBP and RBC-GSH. It is plausible that RBC-GSH may have relevance in the pathogenesis of hypertension. Glutathione depletion is known to result in perturbation of the nitric oxide system and causes severe hypertension in normal animals [13]. Thiol supplementation with GSH, given by intravenous infusion, selectively improves human endothelial dysfunction by enhancing nitric oxide effects [14–15]. Moreover, GSH infusion causes reduction of BP in adult hypertensive patients [16]. Besides antioxidant activity, GSH also has an impressive spectrum of
biological functions, including stabilization of biomembranes, detoxification, glutathionylation of proteins, metabolism of nitric oxide, etc [17–19].

Several previous studies have suggested an impact of elevated Hcy on hypertension [4–5,20]. However, its precise role remains to be elucidated. We propose that RBC-GSH and plasma Hcy should be assessed together in middle-aged hypertensive patients. It is known that approximately half of GSH is derived via Hcy metabolism in the human body. Although univariate analysis did not reveal a linear significant correlation between RBC-GSH and Hcy, in multiple regression analysis the Hcy level was significantly correlated with RBC-GSH for the hypertensive patients. In accordance with previous studies [4–5,20], this study demonstrated significantly higher plasma Hcy concentration. At the same time, the elevated Hcy level could not be explained by the effect caused by RBC folic acid deficit (Table 2). An elevated level of Hcy leads to reduced GSH content, probably through different pathways. It has been reported that Hcy may induce intracellular suppression of the glutathione antioxidant defense system, and possibly also reduce Hcy-derived-GSH synthesis [10]. In the control group, a negative linear correlation was observed between RBC-GSH and Hcy. This is in accordance with in vitro studies, where increasing Hcy concentrations reduced intracellular GSH concentration in endothelial cells [21].

Conclusion
Statistical analysis revealed that there existed significant differences between the untreated patients with essential hypertension and the controls with respect to the parameters of RBC-GSH. These changes are consistent with the view that oxidative stress is present in hypertensive patients. In the determination of RBC-GSH, both SBP and plasma Hcy were important indicating a metabolic link between RBC-GSH and plasma Hcy in untreated hypertensive patients.

References