Elevated homocysteine, glutathione and cysteinylglycine concentrations in patients homozygous for the Chuvash polycythemia *VHL* mutation

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ABSTRACT

In Chuvash polycythemia, homozygous von Hippel-Lindau (VHL) 598C>T leads to increased hypoxia inducible factor-1α and 2α, thromboses and lower systemic blood pressures. Circulating homocysteine, glutathione, γ-glutamyltransferase and cysteinylglycine concentrations were higher in 34 *VHL598C>T* homozygotes than in 37 normal controls and cysteine was lower. Multivariate analysis showed elevated homocysteine independently associated with higher mean systemic blood pressures and elevated glutathione was associated with lower pressures to a similar degree. Among *VHL598C>T* homozygotes, homocysteine was elevated with low and normal folate concentrations, consistent with a possible defect in the remethylation pathway. The elevated glutathione and γ-glutamyltranserase levels correlated positively with cysteinylglycine, consistent with possible upregulation of a glutathione synthetic enzyme and γ-glutamyltransferase. Cysteinylglycine correlated inversely with cysteine, consistent with possible reduced cysteinyldipeptidase activity. We conclude that upregulated hypoxia-sensing may influence multiple steps in thiol metabolism. The effects of the resultant elevated levels of homocysteine and glutathione on systemic blood pressure may largely balance each other out.

Key words: VHL, polycythemia, homocysteine, folate, glutathione.

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Introduction

Chuvash polycythemia, the first recognized congenital defect of augmented hypoxia-sensing, is characterized by homozygous von Hippel-Lindau germ-line mutation *(VHL598C>T)*[1], upregulation of hypoxia inducible factor (HIF)-1α, HIF-2α and several target genes under normoxia,[1,2] arterial and venous thrombosis,[2] lower systemic blood pressures,[2] elevated pulmonary artery pressures[3] and other abnormalities in respiratory and pulmonary vascular regulation.[4] Many other genes are regulated by HIF-1[5] and might have altered expression. Mutations of prolylhydroxylase-2 also lead to upregulation of HIF in normoxia.[6] Elevated plasma homocysteine levels are associated with an increase in blood pressure and with thrombosis.[7,8] Lower cellular glutathione levels have been associated with hypertension.[9] Homocysteine levels depend on remethylation of homocysteine to methionine (with methylene tetrahydrofolate reductase (MTHFR) and methionine synthase) and/or transsulfuration to cysteine (with cystathionine beta-synthase and cystathionine gamma-lyase).[10]

Cysteine is the rate-limiting amino acid for synthesis of the antioxidative tripeptide, glutathione, which involves gamma-glutamylcysteine synthetase and glutathione synthetase. Catabolism of glutathione leads to formation of cysteinylglycine through the action of gamma-glutamyl transpeptidase (GGT). Cysteinylglycine can be catabolized through the action of a dipeptidase to cysteine.

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Design and Methods

The research was approved by Howard University's Institutional Review Board. All study subjects provided written informed consent. Serum folic acid, vitamin B12 and GGT concentrations were determined by Quest Diagnostics (Baltimore, MD, USA). Total plasma cysteine, glutathione and cysteinylglycine concentrations were determined by high performance liquid chromatography (HPLC) with fluorescence detection.[11] Reference ranges are: homocysteine: 3.3-13.9 uM; cysteine: 136-308 uM; glutathione: 4.1-9.9 uM; cysteinylglycine: 20.5-41.0 uM.[11,12] Polymerase chain reaction PCR for the *VHL598C>T* mutation was performed as reported.[3] *MTHFR677T>C* was detected by allele-specific polymerase chain reaction. Amplification of *MTHFR* exon 4 was carried out in 50 μL PCR master mix containing 1.0 U of Taq DNA Polymerase (Promega, USA), 150 ng DNA and 1.5 pmol of each of the following primers: 5'-CTCTCTCTCTGAAGGAGAAG-GTGTCTGCGGGA-3' and 5'-AGGACGGTGCGGTGA-GAGTG-3'. Each reaction was controlled by a pair of primers that amplified a 429 bp fragment of human growth hormone gene. Conditions were denaturation for three min, at 95°C, followed by 35 cycles at 95°C for 15 sec, 61°C for 15 sec, 72°C for 15 sec, and a final extension step of ten min at 72°C. PCR products were separated by electrophoresis on 2% agarose gel.

Results were compared according to *VHL* or *MTHFR* genotype using ANOVA (continuous variables) or Pearson's χ^2 test (categorical variables). Linear regression was used to evaluate relationships among homocysteine, cysteine, glutathione, cysteinylglycine, GGT and other important variables. Systat® software (San José, CA, USA) was used. Outlying values or values with large leverage were excluded from the ANOVA and linear regression models.

Results and Discussion

General characteristics and thiol concentrations of study participants

Thirty-five patients with the diagnosis of Chuvash polycythemia (n=16, <20 years of age) and 40 Chuvash control subjects without polycythemia (n=17, <20 years) were studied. Thirty-four patients with a diagnosis of Chuvash polycythemia were *VHL598C>T* homozygotes and one had normal *VHL* alleles. Thirty-six controls had normal VHL alleles and four were *VHL598C>T* heterozygotes. Fourteen out of 34 VHL *598C>T* homozygotes had a history of phlebotomy therapy and six had normal or low hemoglobin concentrations at the time of study. One of the *VHL 598C>T* homozygotes had a history of peripheral thrombosis and one participant with normal alleles had a history of myocardial infarction. None of the study subjects had a history of cerebral vascular accident. Serum vitamin B12 levels did not differ significantly according to *VHL* genotype but folate levels were significantly lower in *VHL598C>T* homozygotes. Plasma homocysteine, glutathione and cysteinylglycine concentrations were higher in *VHL598C>T* homozygotes than in subjects with normal *VHL* genotype and plasma cysteine concentrations were lower both in unadjusted analysis and after adjustment for significant covariates. Adjusted serum GGT concentrations were significantly higher in *VHL598C>T* homozygotes (*Table 1, online supplement*).

Relationship of plasma thiol concentrations to systemic blood pressure

In a linear regression analysis, mean systemic blood pressure correlated independently and positively with plasma homocysteine concentration (p=0.039) as well as with age (p<0.001) and hemoglobin concentration (p=0.013). At the same time, mean pressure correlated independently and negatively with VHL598C>T (p=0.008) and plasma glutathione concentration (p=0.015) (*Table 2, online supplement*). After adjustment for the other covariates, a 6.6 uM increase in plasma homocysteine concentration (the average increase between subjects with normal alleles and VHL598C>T homozygotes in the present study shown in *Table 1, online supplement*) was associated with an estimated 3 mm Hg increase in mean blood pressure (95% confidence interval of 0.1 to 5 mm Hg increase in mean blood pressure). After adjustment for the other covariates, a 6.8 μM increase in plasma glutathione concentration (the average increase between participants with normal alleles and VHL598C>T homozygotes in the present study) was associated with an estimated 3 mm Hg (0.5 to 5 mm Hg) decrease in mean blood pressure.

Homocysteine

Univariate analysis of subjects with normal *VHL* genotype, showed plasma homocysteine concentrations to have the expected inverse correlations with both serum vitamin B12 levels (n=33, R=-0.627, p<0.001) and serum folate levels. Among *VHL* 598C>T homozygotes, plasma homocysteine concentrations had an inverse correlation with serum vitamin B12 (n=31, R=-0.389, p=0.031) but serum folate did not. Indeed, homocysteine tended to be elevated regardless of the folate concentration. There was no significant difference between plasma homocysteine concentrations according to *MTHFR677C>T* polymorphism status (p=0.4).

Cysteine

Plasma cysteine concentration had a positive relationship with homocysteine concentration in both subjects with normal *VHL* genotype (n=36, R=0.66, p<0.001) and VHL598C>T homozygotes (n=32, R=0.29, p=0.1). After adjustment for cysteinylglycine concentration and age category, the positive relationship between cysteine

and homocysteine was maintained in both *VHL 598C>T* homozygotes (p=0.0002) and subjects with normal *VHL* alleles (p=0.0004). In contrast, cysteine had a positive relationship with cysteinylglycine in *VHL* wildtype subjects (n=37, R=0.33, p=0.047) and a negative relationship in VHL598C>T homozygotes (N=34, R=-0.27, p=0.1). After adjustment for homocysteine concentration and age category, there was a significant inverse relationship between cysteinylglyine and cysteine in VHL598C>T homozygotes (p=0.0005) but not in subjects with normal alleles (p=0.9).

Glutathione

There was no significant association between plasma glutathione concentration and cysteine concentration in either wildtype subjects or *VHL598C>T* homozygotes. Glutathione correlated significantly with cysteinylglycine in *VHL598C>T* homozygotes (n=34, R=0.60, p=0.0002) but not in subjects with normal *VHL* alleles (n=36, R=0.04, p=0.8).

Cysteinylglycine

Plasma cysteinylglycine concentration did not correlate significantly with either glutathione or GGT in subjects with wildtype *VHL* alleles. However, cysteinylglycine correlated positively with glutathione after adjustment for GGT level and age category (p=0.001) and with GGT after adjustment for glutathione and age category (p=0.023) in *VHL598C>T* homozygotes. Cysteinylglycine levels correlated significantly with homocysteine levels in both VHL598C>T homozygotes (n=34, R=0.54, p=0.001) and individuals with normal VHL genotype (n=36, R=0.54, p<0.001).

Discussion and Results

Circulating concentrations of homocysteine, glutathione, γ-glutamyltransferase and cysteinylglycine were elevated and cysteine concentrations were reduced in homozygotes for the *VHL598C>T* Chuvash polycythemia mutation. Our observations suggest that multiple steps in thiol metabolism may be influenced by HIF-mediated hypoxia sensing. HIF-1 leads to up regulation of certain classes of genes and down regulation of others.[5] We are not aware of published evidence that the promoter regions of the genes involved in thiol metabolism have HIF recognition elements.

Elevated homocysteine concentrations are associated with an increased risk of arteriosclersosis, thrombosis, hypertension and renal dysfunction.[7,8] Thrombosis is a common complication of Chuvash polycythemia. However, we were unable to test for an association between homocysteine levels and thrombosis in the present study because of the low prevalence of thrombotic events. Another characteristic of Chuvash polycythemia is a tendency to low rather than elevated blood pressures.[2] Increased homocysteine[7] and depletion of glutathione[9] have been associated with an increase in blood pressure. In the present study, higher homocysteine concentrations were associated with higher systemic blood pressures while higher glutathione concentrations were associated with lower pressures to a similar degree (*Table 2, online supplement*). Therefore, the effects of the elevated levels of homocysteine and glutathione on systemic blood pressure may have largely balanced each other out.

In the present study serum folate levels were significantly lower in *VHL598C>T* homozygotes (Table 1) but their elevated plasma homocysteine concentrations in *VHL598C>T* homozygotes were maintained after adjusting for serum folate and vitamin B12 concentrations. An expected inverse correlation between serum folate concentration and plasma homocysteine concentration was found in *VHL* normal subjects but not *VHL598C>T* homozygotes, in whom homocysteine levels were elevated with the entire spectrum of folate concentrations. Multivariate analyses showed significant positive correlations between plasma homocysteine and cysteine concentrations in both subjects with *VHL* wildtype alleles and *VHL598C>T* homozygotes. Therefore, the elevated homocysteine concentrations might be due to a reduction in the activity of a remethylation pathway enzyme rather than a transsulfuration enzyme.

Elevated homocysteine is usually associated wih increased plasma homocysteine concentration and decreased cysteine concentration due to defects in either the remethylation or transsulfuration pathways.[13-15] Elevated plasma homocysteine concentrations have been reported in polycythemia vera and other myeloproliferative disorders,[16] but a number of *VHL598C>T* homozygotes in the present study had normal hematocrits maintained by therapeutic phlebotomy, and homocysteine levels remained elevated after adjustment for hemoglobin concentration (p=0.011).

Plasma glutathione is mainly found in the liver.[17-19] The increased glutathione and cysteinylglycine concentrations (Table 1) and the positive correlation of cysteinylglycine with both glutathione and GGT in *VHL598C>T* homozygotes in the present study suggest that a glutathione synthetic enzyme and GGT may be up-regulated. Notably, GGT levels are elevated in patients with chronic obstructive lung disease,[20] GGT is necessary for the normal respiratory response to hypoxia,[21] and *VHL598C>T* homozygotes have both a reduced PCO2 set point for respiratory control and an abnormally high acute hypoxic ventilatory sensitivity.[4]

The significant negative correlation of plasma cysteine with cysteinylglycine in *VHL598C>T* homozygotes but not subjects with normal alleles in the present study might be explainable by a reduction in the activity of a cysteinylglycine dipeptidase. The strong positive relationships between plasma homocysteine and plas-

ma cysteinylglycine concentrations suggest a possible common or parallel regulatory mechanism.

Authorship and Disclosures

AIS developed the hypothesis for this study and participated in designing the study, conducting the study, interpreting the results and drafting the manuscript; GYM participated in developing the hypothesis of the study, designing the study, conducting the study, interpreting the results and drafting the manuscript; DJO participated in designing the study, conducting the study and drafting the manuscript; AAL participated in developing the hypothesis, designing the study, conducting the study, interpreting the results and drafting the manuscript; ZD, TA, XN, SN: participated in conducting the study, interpreting the results and drafting the manuscript; EAR participated in developing the hypothesis, conducting the study, interpreting the results and drafting the manuscript; PMDB participated in developing the hypothesis, conducting the study, interpreting the results and drafting the manuscript; DWJ participated in developing the hypothesis, conducting the study, interpreting the results and drafting the manuscript; JTP participated in designing the study, developing the hypothesis, interpreting the results and drafting the manuscript; VRG designed the study and drafted teh manuscript: he participated in developing the hypothesis, conducting the study and interpreting the results.

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