Overhydrated hereditary stomatocytosis (OHSt), which is clinically characterized by a hemolytic anemia, is a rare disorder of the erythrocyte metabolism. We have developed a simple strategy for the extraction and global mass spectrometry-based analyses of red blood cells and erythrocyte metabolomics for a better understanding of the pathophysiological bases of hemolytic anemia associated with erythrocyte abnormalities.

Key words: metabolome, red blood cells, hemolytic anemia.


©2011 Ferrata Storti Foundation. This is an open-access paper.

Introduction

Overhydrated hereditary stomatocytosis (OHSt), which is clinically characterized by a hemolytic anemia, is a rare disorder of red blood cells (RBCs) associated with increased membrane permeability to monovalent cations and increased activity of the Na⁺K⁺-ATPase. The OHSt diagnosis is based on a hemolytic anemia associated with a massive right shift of the osmotic gradient ectectomy curve and a decreased osmotic resistance, together with a major increase in a monovalent cation leak. Blood smears show stomatocytes and bowl-shaped RBCs presenting a slit-like area. OHSt is associated with mutations in the gene encoding the Rh-associated glycoprotein (RhAG), a member of the Rh complex. The RhAG may function as an ammonium transporter and/or a gas channel. When expressed in Xenopus laevis oocytes, the human wild-type RhAG induces a monovalent cation leak considerably enhanced when mutated RhAG transporters are expressed instead. Among membrane abnormalities, OHSt RBCs display a sharp reduction or an absence of stomatin, an integral RBC membrane protein. In human RBCs, stomatin acts as a molecular switch that partly converts the glucose transporter 1 (Glut1) into a transporter for L-dehydroascorbic acid (DHA) that is metabolized producing ascorbate. In the absence of stomatin, the DHA transport by Glut1 undergoes a 2-fold decrease while glucose uptake is significantly increased, suggesting an enhanced glycolysis in OHSt RBCs. Altogether, these results indicate that the OHSt physiopathology affects several metabolic pathways in RBCs and may disrupt homeostasis of RBC metabolism. We have developed a simple strategy for the extraction and global mass spectrometry-based analyses of
metabolites from purified human RBCs. Here we use this strategy to distinguish metabolic changes in RBCs from OHS patients in order to fully characterize the metabolic bases of this disease.

**Design and Methods**

Blood samples were collected from 4 French patients (3 males and one female) from three different families carrying the Phe65Ser mutation in RhAG, and 24 healthy adults as controls, including 5 relatives of the patients. Hemoglobin levels of the patients were between 9.9 and 12.7 g/dL with an average of 11.3 g/dL. Reticulocyte count (325 ± 10^9/L, range 269-445 ± 10^9/L; 10.8%, range 7.5-15.8%) and the mean red cell volume of the patients had increased (134 fl, range 122-144 fl). Written informed consent was obtained from all patients and controls in accordance with the Declaration of Helsinki.

Preparation of RBC lysates, metabolite extraction from RBCs, parameters for liquid chromatography coupled to electrospray-LTQ-Orbitrap mass spectrometry, and data processing were performed as previously described. Control RBCs were fractioned according to the age of the RBCs. The percentage of leukocytes in RBC purified samples was determined using an automated cell counter (Abacus Junior Hematology Analyzer, Diatron). For all samples, the percentage of leukocytes was less than 0.01%.

**Results and Discussion**

Red blood cell metabolites from OHS patients and controls were analyzed by LC/MS with detection in both ion modes: negative and positive. Detected biomolecules are amino acids, organic acids, amines, lipids, sugars, hormones, peptides, and proteins. The selectivity of metabolite detection is obtained from the chromatographic retention time and to the accuracy of mass measurements. Processing of the metabolic fingerprints led to 1524 and 785 relevant ions in the negative and positive ion modes, respectively. The hundreds of thousands of signals contained in the data sets make statistical analysis problematic. The resulting data matrices were subjected to multivariate statistical analyses using Principal Component Analysis (PCA), an unsupervised method that can summarize the information content of the original data set and visualize it in a 2-dimensional space. As shown in Figure 1A, the PCA score plot discriminated the healthy subjects (green diamonds) from the OHS patients (blue squares) on the basis of the information obtained from the metabolic fingerprints.

Eighty-nine metabolites were identified from the metabolic fingerprints. Twenty-nine displayed significantly decreased (Figure 1B) or increased (Figure 1C) concentrations between controls and OHS patients are shown. Values are ratios of individual levels observed for OHS patients (blue point) and controls (green, mean with 95% CI) to mean levels obtained for healthy subjects. (B) Metabolites whose levels are decreased in OHS patients. (C) Metabolites whose levels are increased in OHS patients.
tions in OH St patients compared with controls. No significant metabolite concentration differences were found between RBC extracts from OH St patients with low (7.5%) and high (15.8%) reticulocyte count (data not shown) indicating that the differences observed were not directly related to the number of reticulocytes present in the analyzed samples. A similar absence of effect of reticulocyte concentration on the RBC metabolome was found in a previous study on sickle cell disease.8

To ensure that this metabolic signature was directly related to OH St and not to the diminished half-life of OH St RBCs (data not shown), we compared the metabolomes of young RBCs, i.e. less than 20-day old cells, containing 1.6% reticulocytes, from healthy subjects to those of OH St patients’ RBCs. To this end, control’s RBCs were fractioned according to their ages8 and the metabolomes of the different fractions were analyzed by LC/MS. Then, for each discriminating metabolite previously identified, we compared the metabolite concentration ratio of OH St patients’ RBCs on controls’ RBCs with the ratio of young fraction on all fractions in normal RBCs. Metabolites exhibiting comparable concentration ratios were considered to have been impacted by the age of RBCs. This analysis showed that the concentration of metabolites, such as GSH, desoxy-ribose-5-phosphate, aspartate, leucine, spermine or threonate, was related to the youth of OH St RBCs and not to intrinsic metabolic disorders of OH St patients’ RBCs (Figure 2A and B).

In the glycolytic and pentose phosphate pathways, we detected decreased concentrations of many metabolic intermediates, such as hexose-1,6-bisphosphate, phosphoglycerate, i.e. 2-phospho-glycerate or 3-phospho-glycerate, or desoxy-ribose-5-phosphate (Figures 1B and 3A), while others, like hexose-6-phosphate, glyceraldehyde-3-phosphate, bisphospho-glycerate, phosphoenolpyruvic acid and disphosphoglyceric acid, did not vary (Figure 3A). All final products of glycolysis, i.e. pyruvate, lactate and malate (which can be produced from pyruvate), displayed higher concentrations in OH St patients’ RBCs (Figures 1C, and 3A) than in controls’ RBCs. As RBCs of the OH St patients display a very high activity of the Na⁺K⁺-ATPase, thought to compensate for the monovalent cation leakage associated with the mutated RhAG transporter, these results are consistent with the high levels of ATP required for the Na⁺K⁺-ATPase activity;1 ATP production only depending on glycolysis in RBCs. Interestingly, we did not observe any variations in concentration of glycolysis end-products, such as pyruvate or lactate, in sickle cell disease RBCs,8 strongly suggesting that

Figure 2. A metabolic signature of OH St patients and of young normal RBCs can be obtained from LC/MS analysis of RBC extracts. Metabolites showing significant concentration variations between controls and OH St patients are shown. (A) Metabolites whose levels had decreased in OH St patients were analyzed in young normal RBCs. (B) Metabolites whose levels had increased in OH St patients were analyzed in young normal RBCs. Values are ratios of mean levels observed for OH St patients and controls (gray panel), compared to ratios of mean levels found for young RBCs and all aged RBCs (hatched panel).
the alteration in the concentrations of these glycolysis end-products was related to specific alterations of OHSt patients’ RBCs. Because of the reduction or absence of stomatin in OHSt patients’ RBC membranes, the DHA transport by the Glut1 transporter decreases. Although DHA could not be detected by the method used, the ascorbate concentration underwent a 1.7-fold decrease in OHSt patients’ RBCs together with an increase in the concentrations of threonate and threonolactone; two metabolites originating from ascorbate degradation products (Figures 1B and C, and 3B). The same decrease in threonate concentration was found in young erythrocytes compared to whole RBC population, thus indicating the relative impacts of OHSt and age of RBCs on the increase or decrease of the metabolite concentrations detected. Finally, the variations in the concentration of metabolites that are a part of the ascorbate pathway were moderate explaining why, despite the decreased uptake of DHA by OHSt patients’ RBCs, no clinical manifestation of an ascorbate deficit in OHSt patients was reported.

The moderate decrease in reduced glutathione (GSH) concentration found in OHSt patients’ RBCs is in line with decreased RBC GSH concentration in 3 cases of hemolytic anemia and is consistent with the decrease in GSH concentration probably being linked to the young age of OHSt patients’ RBCs (Figure 2A). We also found a 12-fold decrease of oxydized glutathione (GSSG) concentration (Figures 1B and 3A and B). This variation might be due to an increased GSSG efflux from RBCs, given that the membrane GSSG efflux transporter MRPI is ATP-dependent and has a higher affinity for GSSG than for GSH. As OHSt RBCs are known to produce more ATP than normal RBCs, the efflux of GSSG through MRPI might increase.

Only two amino acids (glutamine and tryptophan) showed increased concentrations in OHSt patients’ RBCs (Figures 1C and 3C), whereas the concentrations of glycine, serine, glutamate, alanine, choline, valine, tyrosine, phenylalanine, histidine, asparagine, and lysine remained unchanged. Carnitine and acetyl-carnitine concentrations had increased in OHSt cells (Figures 1C and 3C) which might indicate increased membrane turnover.
A decreased concentration of creatine (Figures 1B, 2A and 3C) was detected in OHS patients’ RBCs. Creatine has been reported to be a youth marker in RBCs\textsuperscript{13} and in hemolytic anemia. Creatine concentration is expected to increase due to the short RBC lifespan (see also in sickle cell anemia\textsuperscript{3}). This discrepancy might be the consequence of a lower intake of creatine dependent on the sodium gradient\textsuperscript{14}, itself altered in OHS RBCs\textsuperscript{15}.

Finally, we found a decreased concentration of ergothioneine in OHS patients’ RBCs. Ergothioneine is synthesized from histidine in organisms such as actinobacteria or filamentous fungi\textsuperscript{16} but not in humans in whom its presence is due to dietary intake.\textsuperscript{17} Ergothioneine has anti-oxidant properties and is imported into human cells like erythrocytes by a specific transporter (OCTN1).\textsuperscript{18} The decreased concentrations of ergothioneine observed in OHS patients’ RBCs may be linked to a decreased OCTN1 activity or to an increased consumption compensating for the decreased concentration of GSSG in OHS patients’ RBCs.

In conclusion, the metabolic signature of OHS patients’ RBCs provided new insights into their molecular alterations, including increased GSSG efflux, decreased creatine transport, accumulation of glutamine and tryptophan, and decreased concentration of ergothioneine. Some of the metabolomic alterations found in the OHS patients’ RBCs, such as GSH or deoxyribose 5P variations, are also found both in young normal RBCs and in sickle cell disease RBCs. This strongly suggests that these alterations are related to the young age of RBCs in these 2 hemolytic anemias. Conversely other alterations, such as pyruvate or lactate variations, were found only in the metabolic signature of OHS patients’ RBCs, and not in sickle cell disease patients’ RBCs, suggesting that these alterations are linked to OHS patients’ RBCs phenotype. Finally, this study demonstrates that RBC metabolomics could be an appropriate and useful method to assess large cohorts of patients with frequent pathologies, like sickle cell disease,\textsuperscript{7} as well as patients with rare diseases such as OHS, for which specific metabolomic signatures were obtained from 4 patients.

**Authorship and Disclosures**

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.