SYMPOSIUM

Pharmaco-Proteomic Study of Hydroxyurea-Induced Modifications in the Sickle Red Blood Cell Membrane Proteome

SWATI S. GHATPANDE,* PANKAJ K. CHOUDHARY,† CHARLES T. QUINN,‡ AND STEVEN R. GOODMAN*,§,[1]

Department of Molecular and Cell Biology, University of Texas at Dallas, Richardson, Texas 75083-0688; †Department of Mathematical Sciences and Statistics, University of Texas at Dallas, Richardson, Texas 75083-0688; ‡Department of Pediatrics, Division of Pediatric Hematology-Oncology, University of Texas Southwestern Medical Center at Dallas and Children's Medical Center of Dallas, Dallas, Texas 75390-9063; and §Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390

Hydroxyurea (HU) is an effective oral drug for the management of homozygous sickle cell anemia (SS) in part because it increases fetal hemoglobin (HbF) levels within sickle red blood cells (RBCs) and thus reduces sickling. However, results from the Multicenter Study of HU suggested that clinical symptoms often improved before a significant increase in HbF levels occurred. This indicated that HU may be acting through the modification of additional cellular mechanisms that are yet to be identified. Hence, in this study, we focused on the analysis of the sickle RBC membrane proteome +/- HU treatment. 2D-DIGE (Two Dimensional Difference In-Gel Electrophoresis) technology and tandem mass spectrometry has been used to determine quantitative differences between sickle cell membrane proteins in the presence and absence of a clinically relevant concentration of HU. *In vitro* protein profiling of 13 sickle RBC membrane samples +/- 50 μM HU identified 10 statistically significant protein spots. Of these, the most remarkable class of proteins to show a statistically significant increase was the antioxidant enzymes—catalase, thioredoxin peroxidase and biliverdin reductase and the chaperonin containing TCP1 complex assisting in the folding of RBC cytoskeletal proteins. Interestingly, catalase immunoblots showed an increase in the acidic forms of the enzyme within sickle RBC membranes on incubation with 50 μM HU. We further identified this modification in catalase to be phosphorylation and demonstrated that HU exposed SS RBC membranes showed a 2-fold increase in tyrosine phosphorylation of catalase as compared to counterparts not exposed to HU. These results present an attractive model for HU-induced post-translational modification and potential activation of catalase in mature sickle RBCs. These findings also identify protein targets of HU other than fetal hemoglobin and enhance the understanding of the drug mechanism. Exp Biol Med 233:1510–1517, 2008

Key words: sickle cell disease; hydroxyurea; 2D-DIGE; mass spectrometry; catalase; red blood cell

Introduction

Sickle cell anemia (SS) is a recessive genetic disorder caused by the substitution of glutamic acid by valine in the 6th amino acid position of the β-globin subunit of hemoglobin (1). In the deoxygenated state, this abnormal hemoglobin (HbS) forms rigid insoluble polymers that deform RBCs producing their characteristic sickle shape. The formation of sickle RBCs leads to the complex pathophysiology associated with SS that includes vaso-occlusion, chronic hemolysis and ischemic tissue damage (2). HU is an effective drug for the management of SS in part due to its capacity to increase HbF levels within sickle

This work was supported by the NIH Sickle Cell Center grant HL070588 project 1 awarded to SRG.

[1] To whom correspondence should be addressed at SUNY Upstate Medical University, 1258 Weiskotten Hall, 750 E. Adams Street, Syracuse, NY 13210-2311. E-mail: goodmans@upstate.edu

DOI: 10.3181/0805-S-149
1535-3702/08/23312-1510$15.00

RBCs. Increased HbF levels have been shown to inhibit the polymerization of HbS and reduce sickling (2). However, the pivotal Multicenter Study of HU suggested that patients often improved clinically before a significant rise in HbF levels occurred (3). Several published reports demonstrate that an increase in HbF is not the only benefit of HU. Some of the factors involved in ameliorating the pathophysiology of sickle cell disease after HU treatment are increased mean cell volume (MCV) of sickle RBCs (4), reduced adhesion of sickle RBCs to the endothelium (5) and increased deformability of sickle RBCs (6). These findings point towards HU-induced alterations of additional cellular mechanisms that are yet to be identified and that may explain the clinical benefits of hydroxyurea. We believed that some of these alterations that will be reflected as changes in protein modifications and protein content in the HU-treated sickle RBC membranes. Whereas significant changes in HbF concentration within SS RBCs requires the exposure of developing erythroid precursors in the bone marrow to HU over many months, the more rapid clinical changes effected by HU could be explained by HU-induced post-translational modifications of membrane proteins of circulating mature SS RBCs. The erythrocyte membrane is likely to be an important target for HU-induced changes since it is responsible for establishing interactions with the endothelial cells lining the blood vessels and maintaining the biconcave shape of the RBC. The best way to analyze HU-induced quantitative changes is to use a proteomics technology that enables an effective comparative study of the SS RBC membrane proteome in the presence and absence of HU and subsequent identification of the proteins of interest. A combination of 2D-DIGE (Two Dimensional-Fluorescent Difference In-Gel Electrophoresis) and tandem mass spectrometry aided by statistical tests enabled us to produce a list of 10 proteins of interest that showed a significant change in content as a response to 50 μM HU. These proteins mainly represented the anti-oxidant defense system and protein repair and degradation machinery within RBCs. Interestingly, quantitative western blots performed to confirm the identity of these proteins supplied us with interesting information about the nature and state of modifications undergone by these proteins.

Materials and Methods

Subjects. After informed consent, human blood samples (10 ml) were collected from homozygous SS patients by venipuncture using lithium heparin as an anticoagulant at the Southwestern Comprehensive Sickle Cell Center. Patient selection was by convenience sampling, with the criteria being that no patient had received any HU or a blood transfusion within the preceding 4 months.

Incubation of SS Whole Blood with HU. Equal volumes of the same SS blood sample plus or minus 50 μM HU (Sigma-Aldrich, ≥98% (TLC), powder) was incubated at 37°C for 15 hrs with constant shaking. 50 μM HU was prepared by diluting a stock solution of 1 mM HU in PBS (10 mM NaPO4, pH 7.6, 150 mM NaCl). Erythrocyte membranes were prepared from individual samples. A fixed HU concentration and time point was chosen based on data obtained from fixed time point (15 hrs), variable HU concentration (50, 100, 400 and 500 μM) and fixed HU concentration (50 μM), variable time point (5, 10 and 15 hrs) experiments.

Preparation of Erythrocyte Membranes. Erythrocyte membranes were prepared as described (7). The RBCs were sedimented at 1000 *g* for 10 minutes at 4°C and resuspended in PBS (10 mM $NaPO_4$, pH 7.6, 150 mM NaCl). This step was repeated four times. The RBCs were then resuspended in 10 volumes of PBS and sedimented at 2000 *g* for 10 minutes. The washed RBCs were lysed in six volumes of lysis buffer (5 mM $NaPO_4$, 1 mM EDTA, pH 7.6) and were sedimented at 31,000 *g* for 30 minutes. This step was repeated until the pellet becomes white or light pink. Membrane protein concentration was measured by Protein assay reagent (Bio-Rad). The membranes containing ~4–6 mg of protein/ml were vacuum dried and solubilized in lysis buffer (30 mM Tris-HCl, pH 8.5, 7 M urea, 2 M thiourea, and 2% (w/v) nonionic detergent ASB 14).

Minimal Labeling of SS Membrane Proteins. SS membrane proteins corresponding to 100 μg each of (-)HU and (+)HU samples solubilized in lysis buffer were minimally labeled with Cy3 and Cy5 fluorophores respectively according to the manufacturer's protocol (Amersham Biosciences).

Separation of Proteins in First Dimension (IEF). 100 μg each of the control and drug treated samples were mixed together and rehydration buffer (1% Pharmalyte 3–10 NL, 7 M urea, 2 M thiourea, 2% ASB-14, 2 mg/ml DTT) was added to the final protein mixture. The sample, included in the rehydration solution was loaded on the IPG (Immobilized pH Gradient) strip holder. Immobiline DryStrip gel (Amersham Biosciences, pH gradient 3–10 NonLinear, length of strip 13 cm) was placed over the sample and finally IPG cover fluid was added to minimize evaporation and urea crystallization. Rehydration of the IPG strip proceeded on an Ettan IPGphor Isoelectric Focusing System (Amersham Biosciences) for 12 hours at 20°C. Isoelectric focusing was performed in three steps: at 500 V for 1 hr, at 1000 V for 1 hr and at 8000 V for 33,000 Vhr.

Reduction and Alkylation of the IEF Separated Proteins. Prior to running the second dimension, the Immobiline strip with separated proteins were equilibrated and reduced in a solution containing 50 mM Tris-HCl buffer, pH 8.6, 2% SDS, 30% glycerol and 5mg/ml DTT at 90°C for 1 minute. This step was followed by equilibration and alkylation at room temperature in a solution containing 50 mM Tris-HCl buffer, pH 8.6, 6 M urea, 2% SDS, 30% glycerol, and 20 mg/ml iodoacetamide for 10 min.

Separation of Proteins in the Second Dimension (SDS-PAGE). After IEF separation, reduction and

alkylation steps, the proteins were further separated on a 10% SDS-polyacrylamide gel. The IPG strip containing isoelectrically focused proteins was directly loaded on the top of the separating gel.

Gel Imaging and Analysis. The separated proteins labeled with Cy3 and Cy5 fluorophores were detected using 2920 2D-Master Imager (Amersham Biosciences). The Cy3 (primary) and Cy5 (secondary) gel images were then processed using the DeCyder DIA (Differential In-Gel Analysis) software (Amersham Biosciences). This software detects protein spots (spots migrating to the same position on the SDS gel) and quantifies the spot volumes for each image and expresses these volumes as ratios. This ratio can then be used for protein abundance comparisons between the primary Cy3 labeled (-)HU and Cy5 (+)HU SS RBC membrane samples. DeCyder BVA (Biological Variation Analysis) software was used for matching multiple images from different gels for identification of protein spots that are consistently changing in abundance. Statistical analysis was performed on all the protein spots that exhibit $> \pm$ 2.5-fold change in protein content at least in one DIGE experiment. Protein spots with statistically significant change in protein content were chosen for identification by mass spectrometry.

Statistical Analysis. The goal of statistical analysis of these data was to discover protein spots whose mean ratios (HU treated/untreated) differed significantly from one. The data consisted of fold-changes in volumes of protein spots in $n = 13$ patients. However, not every protein spot was observed in every patient. So we only analyzed those spots that were observed in at least six patients. This resulted in data on 37 spots. The fold-changes were converted into ratios and then natural log of the ratios was taken to normalize the data. We used the two-sided Wilcoxon signed rank test (8) to compute *P*-values for testing the null hypothesis that the distribution of log-ratios on each spot had median zero. This test is a nonparametric counterpart of the more familiar *t*-test for means that is used when the distribution of the data can be reasonably approximated using a normal distribution. But this assumption of normality was not appropriate in our case as a few patients exhibited very large changes in protein volumes. The *P*-values for each individual spot were adjusted to account for multiple testing. We used the false discovery rate (FDR) method (9) for this adjustment. A cutoff of 0.10 was used for the FDR to assess significance of results. This means that 10% of the spots declared to be significant are expected to be false positives. To estimate the magnitude of the change in protein volumes, we also computed the estimates of median ratios for each spot. The statistical analysis was performed using the statistical software R (10).

Protein Identification by Tandem Mass Spectrometry and Database Search. The selected protein spots were excised from Sypro Ruby-stained gels using Ettan Spot Picker (Amersham Biosciences). The spots were digested using an in-gel trypsin digestion kit (Pierce). The identity of the tryptic peptides generated out of each excised protein spot was determined using LC/MS/MS (LC-Liquid Chromatography and MS-Mass Spectrometry) and database search. The LC/MS/MS was performed on a HPLC system connected to a LCQ XP ion trap mass spectrometer with a nanospray ionization source (ThermoFinnigan). The parameters for the HPLC system were as described (6). The database used for tryptic peptide search was NCBI non-redundant protein sequence database (human.nr.fasta, 2007) using the Sequest algorithm. The criteria used to confirm the identity of a certain protein was as described in (11).

Immunoblot Analysis. Following 2D-electrophoresis, proteins were electroblotted onto nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM glycine, 0.005% (w/v) SDS, 20% (v/v) methanol) overnight at 4°C and 25 V. Membranes were blocked for 1 hr with 5% milk blocking agent (Amersham Biosciences) for anti-catalase and 5% BSA for anti-pTyr antibody. Working dilutions of rabbit monoclonal catalase antibody was 1:3000 (Calbiochem) and mouse monoclonal primary antibody to phosphotyrosine is 1:2500 (Upstate Biotechnologies). Membranes were incubated in primary antibody (prepared in PBST-milk or 5% BSA) overnight at 4°C followed by 4 washes of PBST (10 mM $NaPO_4$, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 5 min each. Immunoreactive spots were detected by incubating the membranes for 1 hr in goat anti-mouse or donkey anti-rabbit secondary antibody conjugated with horseradish peroxidase. After 4 PBST washes of 5 min each, membranes were developed using ECL plus as the chemiluminescent system (Amersham Biosciences). Densitometric analysis was performed on the spots using an AlphaImager densitometer (Alpha Innotech).

Results

Determination of Inherent Variation in 2D-DIGE Method. We used 2D-DIGE technology coupled with LC MS/MS (tandem mass spectrometry) to identify alterations in the sickle RBC membrane proteome in response to a clinically relevant dose of hydroxyurea. In order to validate drug specific alterations, the inherent variation in the DIGE technique was determined by labeling equal amounts of the same RBC membrane sample with flourescent dyes Cy3 and Cy5. Figure 1A represents the frequency distribution of the Cy5/Cy3 spot volume ratios of the analyzed gel spots from a single experiment. In three independent experiments performed, it was observed that 99.7–99.9% of the total spots detected changed by less than 2.5-fold. Hence a $\geq$ 2.5 times increase or decrease in Cy5/Cy3 spot volume ratios in (+)HU SS RBC membrane samples as compared to their (-) HU counterparts was attributed to drug effect.

Analysis of HU Dependent Modifications Using 2D-DIGE and LC/MS/MS. To analyze HU-induced changes, RBC membrane samples were prepared from peripheral blood of 13 SS patients incubated with plus or minus 50 μM HU for 15 hrs at 37°C. Equal amounts of the

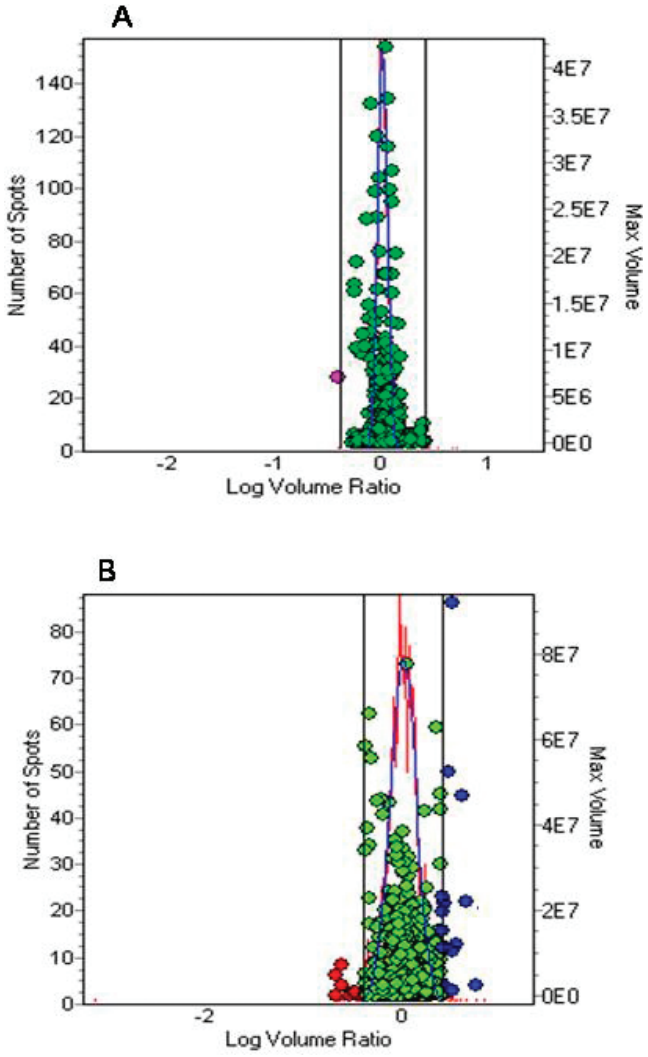


Figure 1. A. Study of inherent variation in the 2D-DIGE method. Equal amounts of the same healthy control RBC membrane proteins were minimally labeled with Cy3 and Cy5 fluorescent dyes and separated by 2D-gel electrophoresis. Gel images were obtained on a 2920 2D-Master fluorescent gel imager. Using DeCyder software, overlapping gel spots from the two images were identified and their volume ratios (Cy5/Cy3) determined. The frequency distribution histogram of all the Cy5/Cy3 ratios of the analyzed gel spots indicated 99.9% of the Cy5/Cy3 volume ratios vary by less than 2.5-fold. Spots showing an increase or decrease in volume ratios by more than 2.5-fold (compared with their AA counterparts) are shown in blue or red respectively. **B.** Determination of HU-induced protein changes in a typical 2D-DIGE experiment. Equal amounts of (-) and (+) HU exposed SS RBC membrane proteins were minimally labeled with Cy3 and Cy5 fluorescent dyes respectively and separated by 2D-gel electrophoresis and analysis is performed as described in Figure 1A. A typical frequency distribution histogram of all the Cy5/Cy3 ratios of the analyzed gel spots in a (-) and (+) HU 2D-DIGE experiment is represented. Spots showing HU-induced increase or decrease in volume ratios by more than 2.5-fold in (+) HU [50 μM] SS RBC membranes (compared with their (-) HU counterparts) are shown in blue or red respectively. For both, A and B, each circle represents a protein spot whose position is determined by its log volume ratio on the X axis and its intensity (max. vol.) on the Y axis and the spots lying within the 2.5-fold threshold (black solid lines) are shown in green.

SS RBC membrane preparations were minimally labeled with Cy3 [(-)HU samples] and Cy 5 [(+) HU samples] fluorescent dyes. Figure 1B gives the frequency distribution of the Cy5/Cy3 spot volume ratios of analyzed gel spots in a representative 2D-DIGE experiment. The blue spots demonstrate an increase in protein content by more than 2.5-fold and the red spots demonstrate a decrease in protein content by more than 2.5-fold (in comparison to their (-) HU counterparts). A protein spot that increased or decreased in content by ≥ 2.5 (Cy5/Cy3 ≥ 2.5) in at least one SS RBC membrane sample out of the 13 analyzed samples and that was present in 6 or more gels was considered for statistical analysis. Out of the 37 spots that met this criterion, 10 spots were statistically significant. Tandem mass spectrometry yielded successful identification of 8 of these 10 statistically significant spots (Table 1). The position of the 8 spots on the two dimensional gel is demonstrated in Figure 2. A total of 10 proteins identified in these 8 spots showed a statistical increase in composition after exposure to 50 μM HU. Three out of the 10 belonged to the class of anti-oxidant enzymes that participate in defense against reactive oxygen species. These are catalase, thioredoxin peroxidase and flavin reductase. T complex protein, delta subunit and chaperonin containing TCP1, subunit eta are protein repair participants. Proteasomal alpha 2 subunit falls into the category of protein turnover. Palmitoylated membrane protein (p55) and β hemoglobin can be classified as structural components within RBCs.

Immunoblot Analysis of Catalase. Catalase is an abundant protein in the RBCs and plays a pivotal role in anti-oxidant defense by converting toxic H_2O_2 to oxygen and water. Catalase showed a statistically significant increase in composition in the (+) HU SS RBC membranes. To confirm our proteomic findings and investigate any HU dependent post-translational modifications of the protein, quantitative western blots were performed. Immunoblot analysis using monoclonal antibodies against catalase identified 9 immunoreactive spots in SS RBC membrane samples in the absence of HU and 8 immunoreactive spots in SS RBC membrane samples exposed to 50 μM HU (Fig. 3A). The pronounced appearance of spot 1 (most acidic spot) and absence of catalase spot 9 (least acidic spot) in the (+) HU SS RBC membranes clearly indicates a shift of the protein forms from the basic side to the acidic side. Densitometric analysis of the catalase spots (Fig. 3B) suggested an increased intensity of the acidic forms and decreased intensity of the basic forms of the protein after exposure to 50 μM HU (in comparison with SS RBC membranes in the absence of HU). Spot 1 showed a 98% increase in spot intensity in the (+) HU SS RBC membranes and spot 9 showed an infinite decrease (1400%) in spot intensity as compared to the (-) HU counterparts.

To investigate the protein modification involved in the acidic shift, immunoreactivity of catalase to a monoclonal phosphotyrosine antibody was tested. Five proteins showed a characteristic immunoreactivity pattern (data not shown),

Table 1. Summary of SS RBC Membrane Proteins of Interest Identified as Statistically Changing in Composition After Exposure to 50 μM HU. HU Dependent Changes in Sickle RBC Membrane Proteins.

Spot no.	Identified protein	Accession no.	Median $SS_{(+)HU}/SS_{(-)HU}$ N=13	Adjusted P value	Experimental M_r/pI on 2D gel
1	Catalase	7245755	1.47	0.04	56,551/6.15
	TCPD_HUMAN T complex protein 1, delta subunit	52001478			57,793/8.13
2	Catalase	7245755	1.45	0.08	56,551/6.95
	Chaperonin containing TCP1,subunit 7 (eta)	62896515			59,340/7.55
3	Aldehyde dehydrogenase	2183299	1.40	0.08	54,731/6.29
	Palmitoylated membrane protein 1 variant (p55)	62898353			52,224/7.22
4	Carbonic anhydrase I	515109	1.34	0.08	28,739/6.63
	Carbonic anhydrase II	6980910			29,115/6.86
5	Proteasome alpha 2 subunit variant	62897513	1.32	0.04	25,840/7.72
	Flavin reductase	32891807			21,988/7.31
6	Peroxiredoxin 2 isoform a	32189392	1.51	0.03	21,988/7.31
7	Thioredoxin peroxidase 1	1617118	1.71	0.06	21,761/5.67
8	Hemoglobin β	3212437	2.02	0.08	15,867/6.81

catalase being one of them (Fig. 4A). Four spots at the position of catalase exhibited immunoreactivity in the (-) HU SS RBC membranes as against three spots in the (+) HU SS RBC membranes. Densitometric analysis of the 4 phosphotyrosine positive catalase spots indicated an approximately two-fold increase (40% increase) in tyrosine phosphorylation of spot 1 (most acidic) after exposure to 50 μM HU as compared to its (-) HU counterparts. It is remarkable that Spot 4 in the (-) HU SS RBC membranes, the least acidic spot completely disappears on HU exposure, suggested by an infinite decrease in % spot intensity (Fig. 4B). It should be noted that 9 spots were identified as being immunoreactive to the catalase antibody and only 4 were identified as immunoreactive to the phosphotyrosine antibody. This can be attributed to diminished phosphorylation of catalase spots 5, 6, 7, 8 and 9 in the (+) HU SS RBC membranes that could not be detected by the phosphotyrosine antibody. Based on ProMoST (Protein Modification Screening Tool) analysis and isoelectric point (pI) calculations, the pIs of catalase positive spots 1, 2, 3 and 4 match the pIs of phosphotyrosine positive spots 1, 2, 3 and 4, ranging from 6.29 to 6.55.

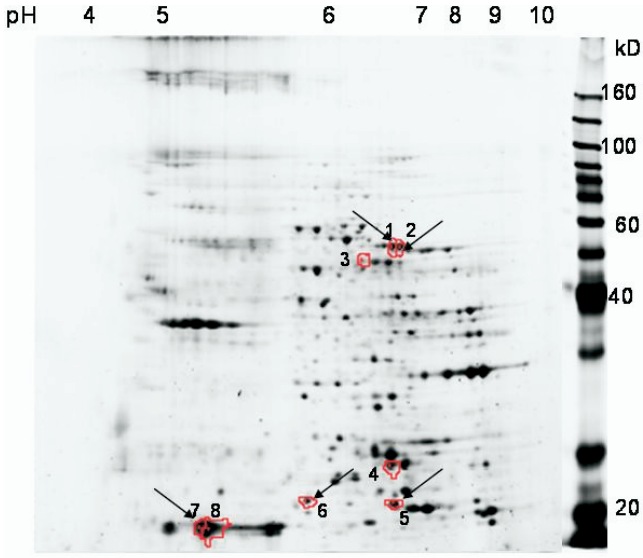
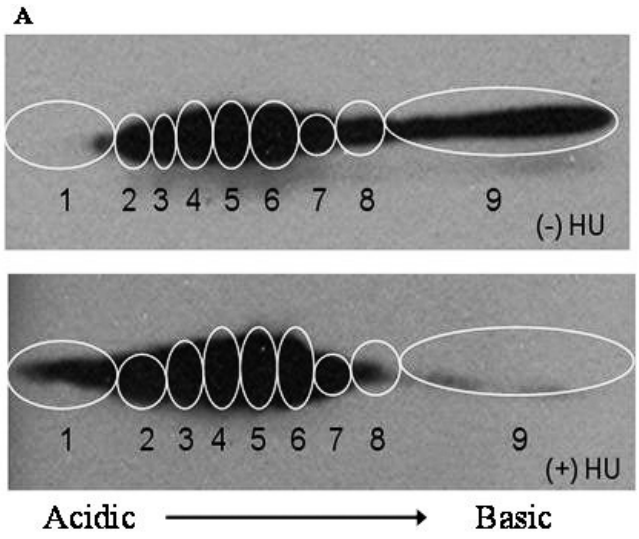


Figure 2. Positions of SS RBC membrane proteins that showed a statistically significant change in their composition on exposure to 50 μM HU. The proteins of interest are marked. The molecular weight marker lane is indicated on the right and the pH gradient used for isoelectric focusing is indicated on the top, determining the 2D position of any given protein spot based on its pI and molecular weight.



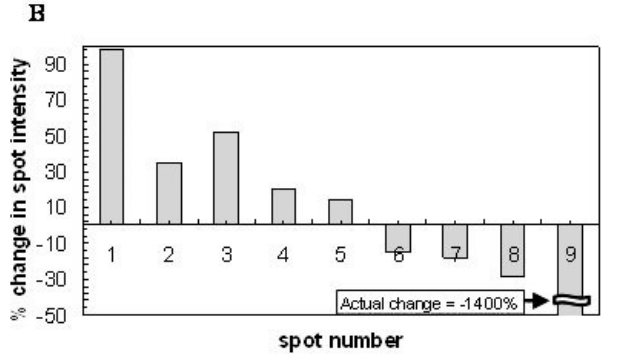
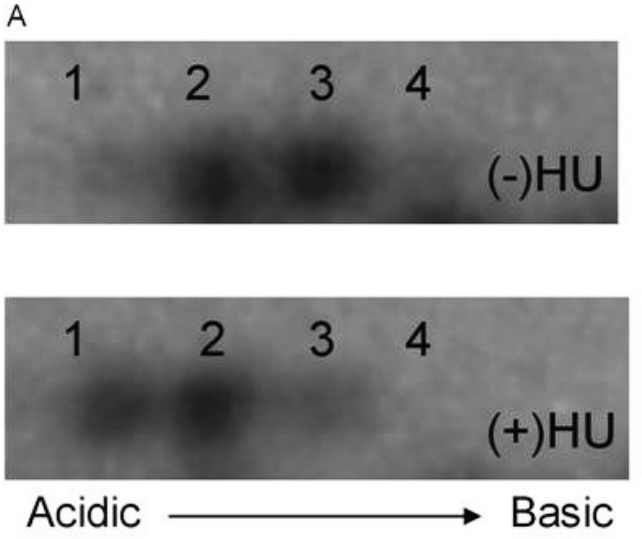


Figure 3. HU increases acidic forms of SS RBC membrane catalase. **A.** 2D western blots of SS RBC membranes without and with 50 μM HU exposure using an anti-catalase monoclonal antibody. Equal volumes of the same SS blood sample without HU and with 50 μM HU was incubated at 37°C for 15 hrs with constant shaking. SS RBC membranes were prepared for immunoblotting (as described in Methods). The acidic (+) and basic (-) side of the blot are noted at the bottom. **B.** Densitometric analysis of the catalase spots in panel A. Results are represented as % change in spot intensity of the (+) HU catalase spots versus the (-) HU catalase spots on the Y-axis and corresponding spot number on the X-axis.



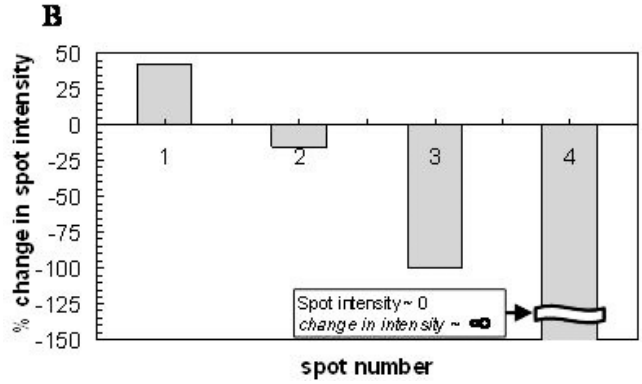


Figure 4. HU increases tyrosine phosphorylation of SS RBC membrane catalase. **A.** 2D western blots of SS RBC membranes without and with 50 μM HU exposure using an anti-phosphotyrosine monoclonal antibody. Equal volumes of the same SS blood sample without HU and with 50 μM HU was incubated at 37°C for 15 hrs with constant shaking. SS RBC membranes were prepared for immunoblotting (as described in Methods). The acidic (+) and basic (-) side of the blot are noted at the bottom. **B.** Densitometric analysis of the catalase spots in panel A. Results are represented as % change in spot intensity of the (+) HU catalase spots versus the (-) HU catalase spots on the Y-axis and corresponding spot number on the X-axis.

Discussion

HU is the first and only drug approved by the FDA for the management of SS. Induction of HbF levels has been thought to be the main therapeutic benefit of HU because it reduces the polymerization of HbS in its deoxygenated state. This effect is mainly due to the tendency of HbF molecules to form mixed hybrids with HbS molecules that are more soluble (12). However, the Multicenter Study of Hydroxyurea showed that patients often improved clinically before achieving significant elevation in HbF levels (3). Various reports published during the last 5 years demonstrate that HbF increase is not the only major beneficial effect of HU (4–6). Some of the factors involved in improving the pathology of SS RBC membrane after HU treatment are increased mean corpuscular volume and hence improved hydration status (4), increased deformability (6) and decreased adhesion to the endothelium (5). These findings strongly indicate the likelihood of HU targeting the membrane and acting through cellular mechanisms that are independent of elevated HbF and are yet to be identified. With this rationale, we used a proteomics approach to identify HU-induced changes in the SS RBC membrane. Since mature RBCs are enucleated cells lacking active protein synthesis, we utilized the powerful technology of 2D-DIGE coupled with LC/MS/MS to quantitate post-translational protein modifications in SS RBC membranes

Table 2. Classification of Proteins of Interest Based on Functionality. Three Proteins out of the Total Nine Identified Belong to the Class of Anti-Oxidant Enzymes (Group 1). Functional Categories of Sickle RBC Proteins Showing Significant HU-Induced Changes.

Identified Proteins	Functional category
Catalase Flavin reductase Peroxiredoxins (thioredoxin peroxidase 1 and peroxiredoxin 2 isoform a)	Anti-oxidant enzymes
Aldehyde dehydrogenase	Oxidoreductases
Human T complex protein, delta subunit Chaperonin containing TCP1,subunit 7 (eta)	Protein repair participants
Proteosome alpha 2 subunit variant	Protein degradation machinery
Carbonic anhydrase I and II	Catalyze the rapid inter-conversion of CO_2 to bicarbonate and protons
Palmitoylated membrane protein (p55)	Structural membrane component
Hemoglobin β	Membrane associated hemoglobin

with and without physiologic concentrations of HU (50 μM).

A list of membrane proteins that increased upon incubation of SS RBCs with 50 μM HU is presented in Table 2. The major category of proteins that showed a statistically significant increase after 50 μM HU exposure was that of the anti-oxidant enzymes—catalase, thioredoxin peroxidase and flavin reductase. An increase in these enzymes suggests an adaptive response of the RBCs to oxidative damage. These findings are in agreement with the findings of Iyamu *et al.* (13) that HU-induced oxidative damage of Hb can be ameliorated by radical scavengers. Similarly, Eskenazi *et al.* (14) demonstrated that resistance to exogenous H_2O_2 after HU treatment in B16 melanoma cells was accompanied by an increase in free radical scavengers like glutathione reductase, glutathione peroxidase and catalase activities. Nagai *et al.* (15) have suggested the involvement of oxidative stress in HU-induced erythroid differentiation. Based on our results and previous literature, we hypothesize that HU induces oxidative stress at a concentration of 50μM. Our hypothesis is further strengthened by the fact that equal amounts of the same SS RBC membrane sample plus or minus HU were subjected to 2D-DIGE such that the levels of oxidative stress before and after HU exposure were comparable. To test this hypothesis, the measurement of reactive oxygen species produced by the (-) HU and (+) HU SS RBC membranes is currently underway.

Two proteins, namely chaperonin TCP1 delta as well as eta subunits and proteasomal subunit alpha 2, participating in protein turn over were also identified. A statistically significant increase in these proteins after HU exposure may also reflect an increased burden on the SS RBCs to remove oxidatively damaged proteins. Functional proteasomes within mature RBCs has recently been demonstrated by our laboratory (Neelam and Goodman, manuscript in preparation).

In order to characterize the post translational modifications involved in HU-induced response, we use 2D quantitative western blots. Cao *et al.* (16) showed that the non-receptor tyrosine kinases c-Abl and Arg are responsible for the phosphorylation of catalase as a response to oxidative stress. Based on this report, we investigated tyrosine phosphorylation of catalase and showed that there is a 2-fold increase in tyrosine phosphorylation of catalase in SS RBC membranes exposed to 50 μM HU as compared to the unexposed counterparts. Interestingly, Eskenazi *et al.* (14) reported that B16 melanoma cells treated with 0.3 mM

HU exhibited a modest increase in catalase activity with no increase in its mRNA, suggestive of post translational modifications regulating enzyme activity. Thus, HU stimulated increased tyrosine phosphorylation of catalase presents an attractive model for potential activation of catalase in mature sickle RBCs that do not contain the necessary machinery for new protein synthesis.

To our knowledge, this work is the first attempt to identify and characterize in vitro HU-induced protein modifications in SS RBC membranes using 2D-DIGE protein profiling and tandem mass spectrometry. A relatively low but physiologic concentration of HU (50 μM) was used to elicit a change in protein content. This concentration is equivalent to plasma HU concentration after 4 hrs of drug administration in SS patients receiving an initial drug dose of 10 mg/kg/day. The maximum HU dose administered to SS patients is ~35 mg/kg/day. Thus the proteins of interest identified in the present study represent a class of initial response factors to HU exposure. Taken together, the current proteomic study is an important step in the identification of HU targets other than fetal hemoglobin and in the understanding of the mechanism of HU action in SS RBCs.

We thank Dr. David Kakhniashvili and Dr. Anita Hryniewicz-Jankowska at the Department of Molecular and Cell Biology, The University of Texas at Dallas for their valuable discussion and assistance in the proteomic techniques.

1. Ingram VM. A specific chemical difference between the globins of normal human and sickle-cell anemia haemoglobin. Nature 178:792–794, 1956.
2. Rodgers GP. Overview of pathophysiology and rationale for treatment of sickle cell anemia. Semin Hematol 34:2–7, 1997.
3. Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, MacMahon RP, Bonds DR. Effect of hydroxyurea on the painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. N Engl J Med 332:1317–1322, 1995.
4. Orringer EP, Blythe DS, Johnson A, Philips G Jr, Dover GJ, Parket JC. Effects of hydroxyurea on hemoglobin F and water content in the red blood cells of dogs and of patients with sickle cell anemia. Blood 78: 212–216, 1991.
5. Hillery CA, Du MC, Wang WC, Scott JP. Hydroxyurea therapy decreases the in vitro adhesion of sickle erythrocytes to thrombospondin and laminin. British J Haematol 109:322–327, 2000.
6. Ballas SK, Dover GJ, Charache S. Effect of hydroxyurea on rheological properties of sickle cell erythrocytes in vivo. Am J Hematol 67:151–156, 2001.
7. Kakhniashvili DG, Griko NB, Bulla LA Jr, Goodman SR. The proteomics of sickle cell disease: profiling of erythrocyte membrane proteins by 2D-DIGE and tandem mass spectrometry. Exp Biol Med 230:787–792, 2005.
8. Hollander M, Wolfe DA. Nonparametric Statistical Methods. New York: John Wiley, 1999.
9. Dudoit S, Shaffer JP, Boldrick JC. Multiple hypotheses testing in microarray experiments. Statistical Science 18:71–103, 2003.
10. R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07–0, URL http://www.R-project.org. 2007.
11. Kakhniashvili DG, Bulla LA Jr, Goodman SR. The human erythrocyte proteome: analysis by ion trap tandem mass spectrometry. Mol Cell Proteomics 3:501–509, 2004.
12. Noguchi CT, Schechter AN, Rodgers GP. Sickle cell disease pathophysiology. Billieres Clin Haematol 6:57–91, 1993.
13. Iyamu EW, Fasold H, Roa D, del Pilar Aguinaga M, Toshio A, Turner E. Hydroxyurea-induced oxidative damage of normal and sickle cell hemoglobins in vitro: amelioration by radical scavengers. J Clin Lab Anal 15:1–7, 2001.
14. Eskenazi AE, Pinkas J, Whitin JC, Arguello F, Cohen HJ, Frantz CN. Role of antioxidant enzymes in the induction of increased experimental metastasis by hydroxyurea. J Natl Cancer Inst 85:711–721, 1993.
15. Nagai T, Tarumoto T, Miyoshi T, Ohmine K, Muroi K, Komatsu N, Sassa S, Ozawa K. Oxidative stress is involved in hydroxyurea-induced erythroid differentiation. British J Haematol 121:657–661, 2003.
16. Cao C, Leng Y, Kufe D. Catalase activity is regulated by c-abl and arg in the oxidative stress response. J Biol Chem 278:29667–29675, 2003.