Published in final edited form as:

Br J Haematol. 2009 February ; 144(3): 434–445. doi:10.1111/j.1365-2141.2008.07473.x.

Ca²⁺-CaM activation of AMP deaminase contributes to adenine nucleotide dysregulation and phosphatidylserine externalization in human sickle erythrocytes

Richard L. Sabina¹, Nancy J. Wandersee^{2,3}, and Cheryl A. Hillery^{2,3}

¹Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI, USA

²Department of Pediatrics and Children's Research Institute, Medical College of Wisconsin, Milwaukee, WI, USA

³Blood Research Institute, Blood Center of Wisconsin, Milwaukee, WI, USA

Summary

Ca²⁺-calmodulin (Ca²⁺-CaM) activates erythrocyte adenosine monophosphate deaminase (AMPD) in conditions of disturbed calcium homeostasis, prompting us to investigate adenine nucleotide metabolic dysregulation in sickle cell disease (SCD). However, higher ATP concentrations in reticulocytes, compared to erythrocytes, confound a comparative evaluation of SCD and normal RBCs. Therefore, a combination of centrifugation and antiCD71-labelled magnetic bead selection was used to prepare reticulocyte-poor fractions (reticulocytes <4% of total RBCs) of SCD RBCs. ATP and total adenine nucleotide concentrations were 12% lower in sickle erythrocytes compared to normal erythrocytes and inosine monophosphate (IMP) concentrations were threefold elevated (all *P* < 0.05). Furthermore, preincubation with a diffusible CaM antagonist slowed IMP accumulation in sickle erythrocytes during an experimental period of energy imbalance, thus showing that Ca²⁺-CaM activates AMPD in SCD. Finally, adenine treatment (100 μmol/l) of *ex vivo* SCD RBCs significantly expanded ATP levels (16% higher) and reduced phosphatidylserine (PS)-exposure, specifically those cells with the highest levels of PS externalization (46% fewer events) (both *P*-values < 0.05 compared to untreated samples). We conclude that Ca²⁺-CaM activation of AMPD contributes to increased turnover of the adenine nucleotide pool in sickle erythrocytes and that this metabolic dysregulation promotes PS exposure that may contribute to the pathogenesis of SCD.

Keywords

red cells; sickle cell anaemia; calmodulin; AMP deaminase; adenosine triphosphate; phosphatidylserine

Erythrocytes are unique among human tissues and cells because they do not contain enough adenylosuccinate synthetase activity to sustain measurable anabolic flow through the inosine monophosphate (IMP) to adenosine monophosphate (AMP) branch point of purine nucleotide biosynthesis (Bishop, 1960; Lowy & Dorfman, 1970). The immediate metabolic consequence of this non-functional IMP to AMP branch point pathway is an inability to synthesize adenine

nucleotides from either the *de novo* pathway or from the salvage pathway for hypoxanthine, the most abundant purine compound in the circulation (see Fig S1). Although enzymes are present for the salvage synthesis of AMP directly from adenosine and adenine, the circulating levels of both compounds are normally quite low, i.e., $<1 \mu\text{mol/l}$ (Ericson *et al*, 1980; Moser *et al*, 1989). Consequently, erythrocytes have a severely limited capacity for maintaining their adenine nucleotide pool and the associated cellular energy reserves. This is particularly evident under conditions of energy imbalance that result in a net turnover of ATP, which can lead to activation of catabolic enzymes and an accelerated loss of adenine nucleotides.

AMP deaminase (AMPD) converts AMP to IMP, a catabolite that has no anabolic route back into the erythrocyte adenine nucleotide pool (Fig S1). Thus, it has long been recognized that erythrocytes attempt to preserve their adenine nucleotide pool by maintaining an intracellular environment that minimizes AMPD catalytic activity (Askari, 1966). Several small molecule inhibitors of erythrocyte AMPD have been identified, including inorganic phosphate (Askari, 1966; Lian & Harkness, 1974; Yun & Suelter, 1978), 2,3-diphosphoglycerate (Askari & Rao, 1968; Lian & Harkness, 1974; Yun & Suelter, 1978), and phosphatidylinositol 4,5-bisphosphate (Sims *et al*, 1999), with measured K_i values that are similar to estimated intracellular concentrations in erythrocytes (Bontemps *et al*, 1986). The inherent capacity of normal erythrocytes to maintain adenine nucleotides during an energy crisis is reflected by the accumulation of AMP, rather than IMP, during short periods of accelerated ATP turnover in response to glucose deprivation (Bontemps *et al*, 1986; Mahnke & Sabina, 2005).

Conversely, IMP, rather than AMP, accumulates in calcium-permeabilized erythrocytes (Almarez *et al*, 1988; Engström *et al*, 1996). The underlying mechanism responsible for rapid IMP accumulation under these conditions was not immediately evident because calcium activation of AMPD was lost upon dilution of the haemolysate or after the enzyme was partially purified (Almarez & Garcia-Sancho, 1989). This confounding issue was subsequently clarified by the discovery that calcium-calmodulin (Ca^{2+} -CaM) activates erythrocyte AMPD and overcomes the inhibitory mechanisms inherent to these cells (Mahnke & Sabina, 2005). Similar adenine nucleotide metabolic dysregulation exists in clinical disorders of disturbed erythrocyte calcium homeostasis. For example, familial phosphofructokinase deficiency (FPD) is accompanied by a mild compensated anaemia and erythrocytes isolated from these individuals contain increased intracellular calcium (Waldenström *et al*, 2001) and a smaller adenine nucleotide pool concomitant with elevated levels of IMP (Ronquist *et al*, 2001; Sabina *et al*, 2006). The slowing of additional IMP accumulation during an experimental period of energy imbalance when FPD erythrocytes were pre-incubated with a diffusible CaM antagonist (Sabina *et al*, 2006) provided evidence that the protein-protein interaction between erythrocyte AMPD and Ca^{2+} -CaM contributes to this metabolic dysregulation.

Intracellular calcium is also elevated in sickle erythrocytes (Eaton *et al*, 1973; Palek, 1977; Bookchin & Lew, 1980), where it is primarily sequestered into intracellular vesicles by an inside-out ATPase pump (reviewed in Lew & Bookchin, 2005). Calcium uptake and the associated endocytotic process are both stimulated by deoxygenation-induced sickling (Murphy *et al*, 1987; Rhoda *et al*, 1990). We hypothesized that increased intracellular calcium in sickle erythrocytes may also cause a chronic energy deficit by simultaneously increasing the demand for ATP and activating AMPD. Furthermore, this adenine nucleotide metabolic dysregulation could impact on the clinical manifestations of this disorder. However, ATP concentrations are greater in reticulocyte-enriched RBC fractions (Brok *et al*, 1966; Cohen *et al*, 1976; Clark *et al*, 1978), indicating that the higher circulating levels of reticulocytes in SCD would confound the results of a comparative analysis of adenine nucleotide and IMP concentrations in RBCs isolated from these individuals *versus* normal subjects. The present study addressed this issue by using a combination of centrifugation and immunomagnetic

separation techniques to reduce reticulocyte levels in RBC samples, which allowed for a more accurate comparison of adenine nucleotide and IMP levels in sickle and normal erythrocytes.

Materials and methods

Reagents

Sheep antimouse IgG beads (Dynabeads) were purchased from Invitrogen (Carlsbad, CA, USA). Mouse-antihuman CD71 conjugated with and without phycoerythrin (PE), annexin V conjugated with PE, and ReticCount Reagent were obtained from BD Biosciences (San Jose, CA, USA). A Partisil-10 anionexchange high performance liquid chromatography (HPLC) column was available from Whatman Inc. (Clifton, NJ, USA). A haemoglobin (Hb) determination kit was purchased from Pointe Scientific Inc. (Canton, MI, USA). Compound 48/80 was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of the highest quality commercially available.

Human subject recruitment and blood collection

Use of human subjects was approved by the local Institutional Review Board. Informed consent was obtained from normal healthy adult donors ($n = 10$; including six African-Americans, three Caucasians, and one subject with mixed African-American/Caucasian heritage) and individuals with SCD (or guardians with assent when appropriate for minor children). All individuals with SCD in this study attended the Wisconsin Sickle Cell Center and had Hb SS disease as documented by quantitative HPLC. SCD individuals who received blood transfusions within the previous four months were excluded. The SS Group (see text and figure legends for n numbers) consisted of individuals with homozygous SCD who were not receiving hydroxycarbamide (HC, previously termed hydroxyurea) therapy. The SS-HC Group (see text and figure legends for n numbers) consisted of individuals with homozygous SCD who had been receiving approximately 20 mg/kg/d of HC for a minimum of 4 months. The Hb F level, Hb level, mean cell volume (MCV), and reticulocyte counts were also determined from patients in both groups. Blood samples were collected in acid citrate dextrose (ACD) tubes and stored overnight at 4°C.

Blood processing and sample preparation (reticulocyte depletion protocol)

All procedures were performed at room temperature. RBCs were separated from platelet-rich plasma by centrifugation of whole blood (4–8 ml) at 200 g in a clinical tabletop centrifuge for 20 min, the buffy coat was carefully removed, then reticulocyte-rich and reticulocyte-poor fractions were prepared as follows:

Reticulocyte-rich fractions—A 5% volume equivalent (0.2–0.4 ml) of RBCs was removed from the top fraction of the red blood cell (RBC) pellet and resuspended in 2 ml of freshly prepared buffer (PBS, pH 7.4, containing 5 mmol/l glucose and 1 mmol/l calcium chloride). This resuspended RBC sample was centrifuged at 400 g for 10 min in a clinical tabletop centrifuge, washed once more with buffer and centrifuged as before. Forty microlitres of RBCs were recovered from the middle of the final pellet, resuspended in 2 ml of fresh buffer, and reserved until later.

Reticulocyte-poor fractions—A 5% volume equivalent (0.2–0.4 ml) of RBCs was carefully removed from the bottom fraction of the original cell pellet and resuspended in 2 ml of buffer. This resuspended RBC sample was centrifuged at 400 g for 10 min in a clinical tabletop centrifuge, washed once more with buffer and centrifuged as before. Forty microlitres of RBCs were carefully removed from the bottom of the final cell pellet and resuspended in 2 ml of fresh buffer. Twenty microlitres of CD71-labelled magnetic beads (sheep anti-mouse IgG beads conjugated with mouse antihuman CD71 antibody) were added to the RBC

suspension, and the mixture was incubated for 90 min with rotation. The tube was then placed on a magnet for 2 min, which sequestered the beads and bound CD71-positive reticulocytes. The unadsorbed reticulocyte-poor RBCs were recovered for further analysis. Reticulocyte-rich and reticulocyte-poor fractions were then centrifuged at 400 g for 10 min in a clinical tabletop centrifuge, then each cell pellet was resuspended in 100 µl of fresh buffer. Aliquots (5 µl) were removed for Hb determination and for flow cytometry, and the remaining 90 µl of each sample were reserved for acid-soluble metabolite analysis.

CD71-positive reticulocytes—These cells were removed by immunomagnetic separation during the preparation of reticulocyte-poor fractions. The magnetic beads with attached CD71-positive reticulocytes were washed in 1 ml of buffer, sequestered by placing the tube on the magnet, and the wash buffer was removed. The beads with attached CD71-positive reticulocytes were then resuspended in 55 µl of fresh buffer. Five microlitres were removed for Hb determination and the remaining 50 µl were reserved for acid-soluble metabolite analysis. Only SCD samples were used for CD71-positive reticulocytes because control RBCs did not have enough selected cells for further analysis. The selection criterion for inclusion of a sample in the comparative metabolite pool analysis described below was <4% reticulocytes in the final resuspended reticulocyte-poor RBC pellet. If reticulocytes were >4% of RBCs, the sample was excluded from further analysis. After the reticulocyte depletion protocol, six samples from the SS group, 10 samples from the SS-HC group, and all 10 of the control samples met the inclusion criteria and were used for a comparative evaluation of intracellular metabolites.

Autoincubation

Autoincubation (erythrocytes incubated in their own plasma) at 37°C was used as an experimental condition of energy imbalance (Ronquist *et al*, 2001; Sabina *et al*, 2006) to determine if Ca²⁺-CaM activation of isoform E contributes to adenine nucleotide metabolic dysregulation in SCD erythrocytes. Reticulocyte-poor RBC fractions from three SCD individuals (not on HC) and from three similarly treated control samples were suspended at a 40% haematocrit (200 µl total volume) in their own plasma and preincubated overnight at 4°C with rotation in the presence of a diffusible CaM antagonist (10 mg/ml, compound 48/80 suspended in phosphate-buffered saline, PBS) or an equivalent volume of PBS. Because plasma components may adsorb compound 48/80, it was necessary to use a higher concentration than what is typically used to inhibit intracellular CaM functions in other buffer-based experimental systems (Romero *et al*, 1997; Vazquez *et al*, 2000; Yingst *et al*, 2001; Mahnke & Sabina, 2005). RBC suspensions were then incubated at 37°C for 9 h and aliquots (60 µl) were removed at 0, 4 and 9 h.

Determination of the effect of adenine treatment on sickle RBC ATP and phosphatidylserine externalization

Sickle RBCs from 15 SCD patients (including eight patients taking HC) were incubated with adenine (100 µmol/l), then analysed for ATP and phosphatidylserine (PS) exposure. Sickle RBCs were washed twice in CGS buffer (15 mmol/l sodium citrate, pH 7.0, containing 3.3 mmol/l glucose, and 125 mmol/l sodium chloride) and resuspended at a 40% haematocrit (hct) in their own plasma, which was rendered platelet-poor by centrifugation at 25 000 g for 10 min. Parallel aliquots of each sample received a 2% volume equivalent of either 5 mmol/l adenine (100 µmol/l final concentration) or water (as the control) and incubated overnight at room temperature. The following morning, the suspensions were gently mixed and aliquots were removed for nucleotide pool analysis, determination of Hb content, and flow cytometry (see below).

Preparation of acid-soluble extracts

Acid-soluble extracts were prepared from paired reticulocyte-rich fractions, reticulocyte-poor fractions, CD71-positive reticulocytes, and total RBCs (adenine incubation study) by adding ice-cold trichloroacetic acid to a final concentration of 10% (w/v), incubating on ice for 30 min, then centrifuging at 14 000 g for 2 min at 4°C. Supernatants were recovered and neutralized by vortexing for 45 s at room temperature with an equal volume of 0.5 mol/l tri-*N*-octylamine in Freon. Neutralized extracts were centrifuged at 14 000 g for 2 min and the upper phase was recovered and stored at –80°C until further analysis.

Separation and quantification of intracellular metabolites

Adenine nucleotides and IMP were separated by anion-exchange HPLC, as previously described (Swain *et al*, 1982) with slight modification. Briefly, 30 µl of sample was injected onto a Partisil-10 SAX anion-exchange column (Whatman Inc.) and developed with a 40-min linear gradient of 5 mmol/l NH₄H₂PO₄, pH 5.6 to 750 mmol/l NH₄H₂PO₄, pH 4.0. The column was run at a flow rate of 2 ml/min and eluate was monitored at 254 nm. Each metabolite was quantified by comparison to an external standard and normalized to Hb, which was measured in a separate aliquot taken from each sample prior to metabolite extraction (see above) according to a commercially available kit (Pointe Scientific Inc.).

Flow cytometry analysis

To determine CD71-positive (immature reticulocytes) or thiazole orange-positive (total reticulocytes) cells, washed RBCs were resuspended at a 5% hct in PBS, pH 7.4 containing 4 nmol/l PE-labelled CD71 and stored in the dark for 20 min at room temperature, then diluted to 0.04% hct in PBS, pH 7.4 or thiazole orange (ReticCount Reagent; BD Biosciences) and stored in the dark for an additional 30 min. To measure phosphatidylserine (PS) exposure, RBCs were resuspended at a 5% hct with buffer (32 mmol/l HEPES, pH 7.4, containing 5 mmol/l glucose, 125 mmol/l sodium chloride, 5 mmol/l potassium chloride, 1 mmol/l calcium chloride, and 1 mmol/l magnesium sulphate) containing 2 µl of annexin V-PE and stored in the dark for 20 min, then diluted to 0.04% hct in buffer. Flow cytometry, data acquisition, and analyses were performed using a Becton, Dickinson and Company LSR flow cytometer and associated software. RBCs were identified and gated by their characteristic log forward by log side scatter profiles. Reticulocyte levels were expressed as the percentage of RBCs labelled with thiazole orange. PS+ RBCs were expressed as the number of annexin V-positive cells per 100 000 events.

Computer-assisted statistical analysis

Normality tests and statistical analyses were performed using Prism5 software version 5.0a (GraphPad Software, Inc., La Jolla, CA, USA). For normal data, statistical significance was determined using two-tailed student's *t*-tests. For nonparametric data, statistical significance was determined using Mann—Whitney (two group comparison) or Kruskal—Wallace (three group comparison) tests. In all cases, a *P*-value <0.05 was considered statistically significant.

Results

Evaluation of the reticulocyte depletion protocol

A reticulocyte depletion protocol was developed based on the relative higher density of erythrocytes compared to reticulocytes, and the presence of transferrin receptor (CD71) on the extracellular surface of immature reticulocytes, but not mature erythrocytes (Frazier *et al*, 1982). The combination of centrifugation with sampling of the bottom fraction of RBCs followed by negative selection with antiCD71-labelled magnetic beads was evaluated as a means to reduce reticulocyte levels. Thiazole orange-positive cells were quantified in aliquots

of all RBC samples by fluorescence-activated cell sorting analysis as a measure of all reticulocytes present in each sample.

This two-step protocol lowered the percentage of reticulocytes (denoted by being positive for thiazole orange) in all RBC samples, but the degree to which this was achieved was somewhat variable (Fig 1). Consequently, only samples with reticulocyte levels <4% of total RBCs (control, 10/10; SS-HC, 10/15; SS, 6/16) were selected for a comparative analysis of erythrocyte nucleotide pools. In these selected samples, reticulocyte percentages in reticulocyte-rich fractions were: control, $0.8 \pm 0.2\%$; SS-HC, $5.8 \pm 2.4\%$; SS, $11.5 \pm 5.7\%$, and in reticulocyte-poor fractions were: control, $0.6 \pm 0.2\%$; SS-HC, $2.5 \pm 0.9\%$; SS, $2.6 \pm 1.3\%$ of total RBCs. SCD subjects receiving HC whose samples were selected for intracellular metabolite analysis exhibited the expected increases in Hb content, mean cell volume, and HbF, and decreases in the haemolytic markers lactate dehydrogenase and reticulocytes, compared to their pre-therapy levels and compared to SCD individuals not on this therapy (Table SI).

Nucleotide pool quantification

Adenine nucleotides (ATP, ADP and AMP) and IMP were quantified in acid-soluble extracts prepared from reticulocyte-rich fractions, reticulocyte-poor fractions, and CD71-positive reticulocytes (the latter in SS and SS-HC samples only). These data were also used to estimate the *in vivo* activity of AMPD (IMP [product]/AMP [substrate] ratio), and to determine the cellular energy state of the RBCs at the time of metabolite extraction (ATP/ADP ratio). Data in the left panels of Fig 2 showed that reticulocyte-rich fractions of RBCs from the SS group contain significantly higher levels of ATP, total adenine nucleotides (TAN = [ATP] + [ADP] + [AMP]), and IMP, compared to controls. However, data in the right panels of Fig 2 revealed opposite results when these metabolites are quantified in reticulocyte-poor fractions. Compared to controls, RBCs in reticulocyte-poor fractions prepared from the SS group contained significantly lower levels (12%) of ATP and TAN, whereas IMP levels remained significantly higher (threefold).

Regarding metabolite levels in the SS-HC group, IMP levels in these samples were significantly elevated compared to controls in both reticulocyte-rich (lower left panel) and reticulocyte-poor (lower right panel) fractions (Fig 2). However, unlike the SS group, adenine nucleotide concentrations (TAN, ATP, ADP and AMP) in the SS-HC group were not significantly different from control values in either fraction.

Reticulocyte-rich fractions of SS and SS-HC samples contained significantly higher concentrations of all adenine nucleotides compared to the corresponding paired reticulocyte-poor fractions (compare left to right upper panels in Fig 2). The reason for this difference can be explained by data presented in Table I, which demonstrated two- to threefold higher concentrations of all adenine nucleotides in CD71-positive reticulocytes removed by immunomagnetic bead selection from both groups of SCD individuals, compared to the corresponding paired reticulocyte-poor fractions. Therefore, the higher adenine nucleotide concentrations in reticulocyte-rich fractions of SCD individuals are probably predominantly due to the immature reticulocytes in these samples.

The relative concentrations of IMP (product) and AMP (substrate) in reticulocyte-poor fractions can be used as an index of erythrocyte AMPD activity in sickle and control erythrocytes. IMP/AMP ratios in reticulocyte-poor fractions of SS and SS-HC RBCs were both significantly higher than those in the corresponding control samples (Fig 3). These data indicated a more active AMPD in sickle erythrocytes compared to normal erythrocytes.

It is also important to validate this comparative data for adenine nucleotide and IMP concentrations by showing that RBCs in all groups of analysed samples were in a good cellular energy state at the time of metabolite extraction. This was demonstrated by high ATP/ADP ratios, e.g. ≥ 10 . In reticulocyte-rich fractions, the mean ATP/ADP ratios $\pm$ SD were 12.2 ± 1.8 (Control), 12.3 ± 4.0 (SS-HC) and 14.0 ± 2.4 (SS). In reticulocyte-poor fractions, these values were 14.1 ± 1.9 (Control), 13.7 ± 2.6 (SS-HC) and 12.5 ± 1.4 (SS). Thus, all groups of RBC samples were in a good metabolic state at the time of this comparative intracellular metabolite study.

Effect of a diffusible calmodulin antagonist on IMP accumulation in reticulocyte-poor fractions of SCD and control RBCs during an experimental period of energy imbalance

Autoincubation (erythrocytes incubated in their own plasma) at 37°C resulted in net turnover of ATP (Ronquist *et al*, 2001; Sabina *et al*, 2006). This experimental condition of energy imbalance was used to evaluate the effect of a diffusible CaM antagonist (compound 48/80) on the rate of additional IMP accumulation in reticulocyte-poor fractions of SCD and control RBCs. Reticulocyte-poor fractions isolated from SCD individuals accumulated additional IMP at a faster rate under these conditions than corresponding fractions prepared from control samples (Fig 4, upper panel). However, preincubation with the CaM antagonist, compound 48/80 (Fig 4, lower panel), slowed the additional accumulation of IMP in SCD RBCs to levels that were comparable to those in untreated control RBCs. These data strongly suggest that Ca^{2+} -CaM activation of erythrocyte AMPD contributes to the adenine nucleotide metabolic dysregulation in sickle erythrocytes.

Effect of adenine treatment on ATP levels and phosphatidylserine externalization in SCD RBCs

In order to investigate whether the observed adenine nucleotide dysregulation in sickle erythrocytes has physiological significance, a study was conducted using adenine to expand the ATP pool in order to determine whether this metabolite replacement strategy impacts on phosphatidylserine (PS) externalization, which is elevated in SCD RBCs (Chiu *et al*, 1979; Franck *et al*, 1985; Zwaal *et al*, 1989; Tait & Gibson, 1994; Kuypers *et al*, 1996; Wood *et al*, 1996). Washed RBCs from 15 SCD individuals (7 SS and 8 SS-HC) were resuspended in their own platelet-poor plasma containing 100 $\mu\text{mol/l}$ adenine (final concentration) and incubated overnight at room temperature; aliquots were then removed from each paired sample (adenine-treated and untreated) for HPLC analysis of intracellular ATP and flow cytometry analysis of PS exposure. As previously reported (de Jong *et al*, 2001), the percentage of PS⁺ cells in SCD RBCs was quite variable, and in our samples averaged $1.0 \pm 0.1\%$ (SEM; range 0.4–1.9%). Adenine-treated SCD RBCs had 16% higher levels of ATP compared to untreated SCD RBCs ($P < 0.001$) (Fig 5A). PS exposure was examined by relative fluorescence intensity following incubation with annexin V-PE, as shown in Fig 5B; representative flow cytometry dot plots from two separate SCD RBC samples with and without adenine treatment are shown in Fig S2. Although the number of total PS⁺ cells (quadrant 'Q2' in Fig 5B) was not significantly different between the two groups of SCD RBCs (Fig 5C), adenine-treated samples exhibited 9% lower mean fluorescence intensity compared to untreated samples (682 ± 123 vs. 615 ± 62 , mean $\pm$ SD; $P < 0.05$ in a paired two-tailed *t*-test). The significant decrease in mean fluorescence intensity in adenine-treated SCD RBCs prompted us to examine the subpopulation of PS⁺ SCD RBCs with higher PS exposure, termed Type 2 (Yasin *et al*, 2003; see Fig 5B). The median number of Type 2 PS⁺ cells was 46% lower in adenine-treated samples compared to untreated samples (Fig 5D). As Type 2 PS⁺ cells are more likely to be involved in adverse pathology (Yasin *et al*, 2003), these results suggest that adenine nucleotide pool maintenance may provide a therapeutic benefit for SCD.

Discussion

This study explored the hypothesis that sickle erythrocytes suffer from adenine nucleotide metabolic dysregulation, due in part to Ca^{2+} -calmodulin (Ca^{2+} -CaM) activation of AMP deaminase (AMPD), which converts AMP to IMP. Adenine nucleotide and IMP concentrations were quantified in sickle and normal erythrocytes as a means to test this hypothesis. However, ATP concentrations are greater in RBC fractions enriched with reticulocytes (Brok *et al*, 1966; Cohen *et al*, 1976; Clark *et al*, 1978). Thus, the reticulocytosis that accompanies SCD presented an obstacle to the accurate quantification of sickle erythrocyte adenine nucleotides and IMP in RBC samples prepared from these individuals. Centrifugation and immunomagnetic separation techniques were combined to develop a protocol for reducing reticulocyte levels in RBC samples. This afforded a more accurate comparison of adenine nucleotide and IMP concentrations in sickle and control erythrocytes by minimizing the contribution of reticulocytes to this quantitative analysis. Data generated from reticulocyte-poor samples has provided the first complete adenine nucleotide profile of sickle erythrocytes. The results of the comparative analysis with normal erythrocytes showed abnormal purine nucleotide metabolism in sickle erythrocytes that is characterized by lower levels of ATP and TAN, and higher levels of IMP. Sickle erythrocytes also contained higher IMP/AMP ratios, indicative of an activated AMPD. In addition, preincubation with a diffusible CaM antagonist slowed the additional accumulation of IMP in SCD erythrocytes during an experimental period of energy imbalance. These combined observations validated the hypothesis for adenine nucleotide metabolic dysregulation in sickle erythrocytes and demonstrated a contributing role for Ca^{2+} -calmodulin (Ca^{2+} -CaM) activation of AMPD. Notably, similar metabolic abnormalities have been documented in another anaemic disorder of disturbed erythrocyte calcium homeostasis, i.e. familial phosphofructokinase deficiency (Ronquist *et al*, 2001; Sabina *et al*, 2006).

Previous work related to this hypothesis in SCD examined irreversibly sickled cells (ISCs) in RBC fractions prepared by density gradient centrifugation, with one study reporting lower levels of ATP than in normal erythrocytes (Clark *et al*, 1978) and another unable to substantiate this observation (Glader *et al*, 1978). These conflicting results are difficult to resolve because neither study reported the concentrations of other adenine nucleotides, i.e. ADP and AMP, which raises the possibility that the ISCs may have had variable cellular energy states at the time of metabolite extraction. This uncertainty underscores the need to document a good cellular energy state when quantifying ATP because the level of this high-energy phosphate compound can fall rapidly, causing ADP and AMP to accumulate if erythrocytes are exposed to conditions that adversely affect energy metabolism during *ex vivo* manipulation, such as an inadequate supply of glucose or an alkaline pH (Bontemps *et al*, 1986). A high ATP/ADP ratio (≥ 10) or adenylate energy charge ($[\text{ATP}] + \frac{1}{2} [\text{ADP}] / \sum [\text{ATP} + \text{AD-P} + \text{AMP}]$) (Atkinson, 1968) indicates a good erythrocyte energy state and serves to validate measured ATP concentrations. The total adenine nucleotide pool was quantified in the current study and high ATP/ADP ratios were observed in all analysed samples. Therefore, these data further strengthen the observation of aberrant adenine nucleotide metabolism in sickle erythrocytes compared to normal erythrocytes.

As expected from previous studies using density-gradient fractions of sickle and normal RBCs (Brok *et al*, 1966; Cohen *et al*, 1976; Clark *et al*, 1978), higher levels of ATP were observed in reticulocyte-rich fractions of RBCs from SCD individuals compared to the corresponding paired reticulocyte-poor fractions. This difference can be explained by the 2.5-fold higher levels of all adenine nucleotides in CD71-positive reticulocytes compared to the paired reticulocyte-poor fractions. To our knowledge, these data also represent the first report of an adenine nucleotide profile generated directly from isolated SCD reticulocytes. Regardless, it is not surprising that CD71-positive sickle reticulocytes have a larger adenine nucleotide pool

than do their mature erythrocyte counterparts because they contain mitochondria and are able to take advantage of oxidative phosphorylation to help maintain ATP. In addition, capacities for both transcription and translation make it likely that these cells maintain a functional branch point pathway for readily replacing adenine nucleotides lost through catabolism to IMP.

The metabolic basis for adenine nucleotide dysregulation in sickle erythrocytes can be explained by existing information. These cells have elevated levels of calcium (Eaton *et al*, 1973; Palek, 1977; Bookchin & Lew, 1980), which is sequestered into intracellular vesicles by an inside-out pump (reviewed in Lew & Bookchin, 2005). Deoxygenation-induced sickling leads to a transient import of calcium into the cytoplasm (Murphy *et al*, 1987; Rhoda *et al*, 1990), which activates the Gardos channel and contributes to cell dehydration. Since calcium pumping is an ATP-dependent process, the disturbed calcium homeostasis in sickle erythrocytes may also cause a chronic energy crisis, i.e. demand exceeding supply, thus leading to a net increase in ADP that transiently displaces adenylate kinase equilibrium, i.e. $2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$. Adenylate kinase equilibrium would be quickly re-established in a normal erythrocyte environment that maintains AMPD in an inactive state, i.e. $2\text{ADP} \leftrightarrow \text{ATP} + \text{AMP}$. Here again, increased intracellular calcium plays a key role in the adenine nucleotide metabolic dysregulation in sickle erythrocytes by promoting a proteinprotein interaction between Ca^{2+} -CaM and erythrocyte AMPD that results in a more robust enzyme at low (physiological) concentrations of substrate (Mahnke & Sabina, 2005). The higher levels of IMP in sickle erythrocytes reflect this regulatory mechanism, which was confirmed by using a diffusible CaM antagonist to slow additional IMP accumulation during an experimental period of energy imbalance. Consequently, Ca^{2+} -CaM activation of AMPD maintains the displaced equilibrium of adenylate kinase and accelerates the essentially irreversible depletion of adenine nucleotides through the production of IMP, a catabolite that has no anabolic route back into this metabolite pool in erythrocytes (see Fig S1).

In light of the data presented in this study, it is warranted to consider what impact adenine nucleotide metabolic dysregulation in sickle erythrocytes may have on clinical manifestations of SCD. ATP supplies energy for Na^+ , K^+ -ATPase and Ca^{2+} -ATPase pumps, and for protein phosphorylation (Mohandas *et al*, 1980). Therefore, lower levels of this high-energy phosphate compound in sickle erythrocytes may affect these basic processes and lead to several inter-related and clinically important pathological complications of SCD, such as erythrocyte dehydration and modulation of integral membrane components that may affect the adhesive phenotype of the sickle erythrocyte. A significant percentage of sickle RBCs are dehydrated, which is thought to result primarily from loss of intracellular K^+ and the accompanying efflux of osmotic water. Potassium exits from the cell via both a Ca^{2+} -dependent K^+ (Gardos) channel (Brugnara *et al*, 1986) and the co-efflux of K^+ and Cl^- via the K/Cl cotransporter (Canessa *et al*, 1986). The loss of water enhances polymerization of deoxygenated Hb S, hastening RBC sickling and the attendant pathologies. Furthermore, the hydration status of an erythrocyte may directly affect its adhesive phenotype, possibly by exposing or altering potential adhesive components of the membrane (Stone *et al*, 1996; Wandersee *et al*, 2005). For example, dehydration of sickle and normal RBCs causes a dose-dependent increase in adhesion to endothelial cells under static conditions *in vitro* (Hebbel *et al*, 1989). In agreement, dehydration of normal erythrocytes with hypertonic buffer increases adhesion to immobilized thrombospondin, while rehydration of sickle erythrocytes with hypotonic buffer reduces their adhesion to thrombospondin (Wandersee *et al*, 2005).

Dehydrated sickle erythrocytes also exhibit increased extracellular membrane exposure of phosphatidylserine (PS) (Chiu *et al*, 1979; Franck *et al*, 1985; Zwaal *et al*, 1989; Tait & Gibson, 1994; Kuypers *et al*, 1996; Wood *et al*, 1996), which makes them more susceptible to being engulfed by macrophages (Schwartz *et al*, 1985), increases their procoagulant activity (Franck *et al*, 1985), and contributes to adhesion (Schwartz *et al*, 1985). Phospholipid asymmetry is

normally maintained by the balanced activities of a non-specific floppase, which flops phospholipids from the inner to the outer membrane surface (Bitbol & Devaux, 1988), and an ATP-dependent aminophospholipid translocase, or flippase, which transports PS from the outer to the inner membrane surface (Seigneuret and Devaux, 1984). However, increased intracellular calcium alters this balance by activating a scramblase that can rapidly result in greater PS exposure (Zwaal *et al*, 1993; Devaux and Zachowski, 1994). There is also evidence to suggest that a limitation in adenine nucleotides might play a role in the disruption of erythrocyte phospholipid asymmetry, as elevated PS exposure during an *ex vivo* period of energy crisis in normal erythrocytes (Martin & Jesty, 1995; Niemoeller *et al*, 2007) is partially reversed by adenosine treatment (Niemoeller *et al*, 2007).

Additional data presented in this study show that adenine treatment expands the intracellular ATP pool and reduces PS externalization in *ex vivo* SCD RBCs, with the latter effect being specific to the subpopulation of cells with the highest levels of phospholipid exposure. High PS-exposing cells are termed Type 2, and they appear to be more specific for SCD and more likely to be involved in pathological sequelae (Yasin *et al*, 2003). The selective reduction of Type 2 PS+ RBCs in adenine-treated samples strongly suggests that adenine nucleotide dysregulation contributes to increased PS exposure in SCD, possibly due to a limitation in ATP-dependent flippase. Moreover, based on their significant response to adenine treatment, Type 2 PS+ cells may represent a subpopulation of SCD RBCs with more profound adenine nucleotide dysregulation. Finally, these data suggest that adenine may be beneficial in reversing clinically important behaviors of SCD RBCs, such as increased adhesion and a shorter life span.

Another potentially important finding of this study is that adenine nucleotide metabolic dysregulation in sickle erythrocytes is not as severe in individuals undergoing HC therapy. The higher adenine nucleotide levels are despite the fact that HC typically improves the haemolytic rate and presumably lengthens the erythrocyte lifespan, which would tend to lower the average adenine nucleotide level in circulating RBCs due to fewer reticulocytes and young erythrocytes. This observation could also be due to increased levels of HbF, which are probably associated with a significant decrease in Hb polymerization and erythrocyte sickling (Eaton & Hofrichter, 1990). HC therapy is also associated with reduced PS exposure on the surface of sickle erythrocytes (Covas *et al*, 2004), reduced cell adhesion molecule expression (Styles *et al*, 1997), and decreased adhesion of sickle erythrocytes to the adhesive plasma and extracellular matrix proteins thrombospondin and laminin (Hillery *et al*, 2000). Consequently, these combined effects of HC could reduce the demand for ATP and partially correct the energy deficit in sickle erythrocytes. Further studies that advance our understanding of the apparent beneficial effect of HC on adenine nucleotide metabolic dysregulation in sickle erythrocytes might provide useful information for this and other therapeutic strategies aimed at SCD.

In summary, this study demonstrated that sickle erythrocytes suffer from adenine nucleotide metabolic dysregulation that is accelerated by Ca^{2+} -CaM activation of AMPD. AMP deamination to IMP in erythrocytes, unlike all other tissues and cells, generates a catabolic product of ATP that has no avenue for anabolic conversion back into the metabolite pool that holds nearly the entire cellular energy reserves. The resulting loss of adenine nucleotides cannot be ameliorated by *de novo* synthesis from small molecule precursors or by salvage synthesis from hypoxanthine, the most abundant purine compound in the circulation, because IMP is an intermediate in both pathways. Furthermore, we found that the *ex vivo* treatment of sickle blood with adenine reduces the subpopulation of sickle RBCs with the highest PS exposure. As sickle RBC PS exposure probably contributes to pathological sequelae, such as adhesion, activation of the coagulation cascade, and shortened erythrocyte life span, a reduction in PS exposure should be beneficial in SCD. Thus, therapeutic strategies targeted at maintaining an optimal

metabolic status of sickle erythrocytes provides a novel approach for treating human and murine SCD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by an Advancing a Healthier Wisconsin Program grant (R.L.S.), NIH grants HL44612 (C.A.H.) and HL90503 (C.A.H. and N.J.W.), AHA grant 0530073N (N.J.W.), a General Clinical Research Center grant M01 RR00058, and support to C.A.H. and N.J.W. from the Midwest Athletes Against Childhood Cancer (MACC) Fund.

References

- Almarez L, Garcia-Sancho J. Activation by calcium of AMP deaminase from the human red cell. *FEBS Letters* 1989;244:417–420. [PubMed: 2920838]
- Almarez L, Garcia-Sancho J, Lew VL. Calcium-induced conversion of adenine nucleotides to inosine monophosphate in human red cells. *Journal of Physiology* 1988;407:557–567. [PubMed: 3151496]
- Askari A. Modifying effects of anions on the alkali-cation-activated AMP deaminase of human erythrocytes. *Molecular Pharmacology* 1966;2:518–525. [PubMed: 5966650]
- Askari A, Rao SN. Regulation of AMP deaminase by 2,3-diphosphoglyceric acid: a possible mechanism for the control of adenine nucleotide metabolism in human erythrocytes. *Biochimica et Biophysica Acta* 1968;151:198–203. [PubMed: 5640153]
- Atkinson DE. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry* 1968;7:4030–4034. [PubMed: 4972613]
- Bishop C. Purine metabolism in human and chicken blood in vitro. *Journal of Biological Chemistry* 1960;235:3228–3232.
- Bitbol M, Devaux PF. Measurement of outward translocation of phospholipids across human erythrocyte membranes. *Proceedings of the National Academy of Sciences of the United States of America* 1988;85:6783–6787. [PubMed: 3137572]
- Bontemps F, Van den Berghe G, Hers HG. Pathways of adenine nucleotide catabolism in erythrocytes. *Journal of Clinical Investigation* 1986;77:824–830. [PubMed: 3949980]
- Bookchin RM, Lew VL. Progressive inhibition of the Ca-pump and Ca:Ca exchange with normal cytoplasmic Ca buffering in sickle red cells. *Nature* 1980;284:561–562. [PubMed: 6445042]
- Brok F, Ramot B, Zwang E, Danon D. Enzyme activities in human red blood cells of different age groups. *Israel Journal of Medical Science* 1966;2:291–296.
- Brugnara G, Bunn HF, Tosteson DC. Regulation of erythrocyte cation and water content in sickle cell anemia. *Science* 1986;232:388–390. [PubMed: 3961486]
- Canessa M, Spalvins A, Nagel RL. Volume-dependent and NEM-stimulated K^+ , Cl^- transport is elevated in oxygenated SS, SC and CC human red cells. *FEBS Letters* 1986;200:197–202. [PubMed: 3699160]
- Chiu D, Lubin B, Shohet SB. Erythrocyte membrane lipid reorganization during the sickling process. *British Journal of Haematology* 1979;41:223–234. [PubMed: 427032]
- Clark MR, Unger RC, Shohet SB. Monovalent cation composition and ATP and lipid content of irreversibly sickled cells. *Blood* 1978;51:1169–1178. [PubMed: 647122]
- Cohen NS, Ekholm JE, Luthra MG, Hanahan DJ. Biochemical characterization of density-separated human erythrocytes. *Biochimica et Biophysica Acta* 1976;419:229–242. [PubMed: 129156]
- Covas DT, Angulo IL, Vianna P, Palma B, Zago MA. Effects of hydroxyurea on the membrane of erythrocytes and platelets in sickle cell anemia. *Haematologica* 2004;89:273–280. [PubMed: 15020264]
- de Jong K, Larkin SK, Styles LA, Bookchin RM, Kuypers FA. Characterization of the phosphatidylserine-exposing sub-population of sickle cells. *Blood* 2001;98:860–867. [PubMed: 11468189]

- Devaux PF, Zachowski A. Maintenance and consequences of membrane phospholipid asymmetry. *Chemistry and Physics of Lipids* 1994;73:107–120.
- Eaton JW, Skelton TD, Swofford HS, Kolpin CE. Elevated erythrocyte calcium in sickle cell disease. *Nature* 1973;246:105–106. [PubMed: 4585849]
- Eaton WA, Hofrichter J. Sickle cell hemoglobin polymerization. *Advances in Protein Chemistry* 1990;40:263–279.
- Engström I, Waldenström A, Ronquist G. Activation of AMP deaminase in human erythrocytes by calcium ions. *Scandinavian Journal of Clinical and Laboratory Investigation* 1996;56:345–350. [PubMed: 8837241]
- Ericson A, Groth T, Niklasson F, De Verdier C-H. Plasma concentration and renal excretion of adenine and 2,8-dihydroxyadenine after administration of adenine in man. *Scandinavian Journal of Clinical and Laboratory Investigation* 1980;40:1–8. [PubMed: 7367806]
- Franck PFH, Bevers EM, Lubin BH, Comfurius P, Chiu DT, Op den Kamp JA, Zwaal RF, van Deenen LL, Roelofsen B. Uncoupling of the membrane skeleton from the lipid bilayer: the cause of accelerated phospholipid flip-flop leading to an enhanced procoagulant activity of sickled cells. *Journal of Clinical Investigation* 1985;75:183–190. [PubMed: 3965502]
- Frazier JL, Caskey JH, Yoffe M, Seligman PA. Studies of the transferrin receptor on both human reticulocytes and nucleated human cells in culture: comparison of factors regulating receptor density. *Journal of Clinical Investigation* 1982;69:853–865. [PubMed: 6804495]
- Glader BE, Lux SE, Muller-Soyano A, Piatt OS, Propper RD, Nathan DG. Energy reserve and cation composition of irreversibly sickled cells in vivo. *British Journal of Haematology* 1978;40:527–532. [PubMed: 728369]
- Hebbel RP, Ney PA, Foker W. Autoxidation, dehydration, and adhesivity may be related abnormalities of sickle erythrocytes. *American Journal of Physiology* 1989;256:C579–C583. [PubMed: 2923193]
- Hillery CA, Du MC, Wang WC, Scott JP. Hydroxyurea therapy decreases the in vitro adhesion of sickle erythrocytes to thrombospondin and laminin. *British Journal of Haematology* 2000;109:322–327. [PubMed: 10848818]
- Kuypers FA, Lewis RA, Hua M, Schott MA, Discher D, Ernst JD, Lubin BH. Detection of altered membrane phospholipid asymmetry in subpopulations of human red blood cells using fluorescently labeled annexin V. *Blood* 1996;87:1179–1187. [PubMed: 8562945]
- Lew VL, Bookchin RM. Ion transport pathology in the mechanism of sickle cell dehydration. *Physiology Reviews* 2005;85:179–200.
- Lian C-Y, Harkness DR. The kinetic properties of adenylate deaminase from human erythrocytes. *Biochimica et Biophysica Acta* 1974;341:27–40. [PubMed: 4828847]
- Lowy BA, Dorfman B-Z. Adenylosuccinase activity in human and rabbit reticulocyte lysates. *Journal of Biological Chemistry* 1970;245:3043–3046. [PubMed: 5432795]
- Mahnke D, Sabina RL. Calcium activates erythrocyte AMP deaminase (isoform E) through a protein-protein interaction between calmodulin and the N-terminal domain of the AMPD3 polypeptide. *Biochemistry* 2005;44:5551–5559. [PubMed: 15807549]
- Martin DW, Jesty J. Calcium stimulation of procoagulant activity in human erythrocytes - ATP dependence and the effects of modifiers of stimulation and recovery. *Journal of Biological Chemistry* 1995;270:10468–10474. [PubMed: 7737981]
- Mohandas N, Clark MR, Jacobs MS, Shohet SB. Analysis of factors regulating erythrocyte deformability. *Journal of Clinical Investigation* 1980;66:563–570. [PubMed: 6156955]
- Moser GH, Schrader J, Deussen A. Turnover of adenosine in plasma of human and dog blood. *American Journal of Physiology* 1989;256:C799–C806. [PubMed: 2539728]
- Murphy E, Berkowitz LR, Orringer E, Levy L, Gabel SA, London RE. Cytosolic free calcium in sickle red blood cells. *Blood* 1987;69:1469–1474. [PubMed: 3105623]
- Niemoeller OM, Bentzen PJ, Lang E, Lang F. Adenosine protects against suicidal erythrocyte death. *Pflügers Archives - European Journal of Physiology* 2007;454:427–439. [PubMed: 17285297]
- Palek J. Red cell calcium content and transmembrane calcium movements in sickle cell anemia. *Journal of Laboratory and Clinical Medicine* 1977;89:1365–1374. [PubMed: 864308]
- Rhoda MD, Apovo M, Beuzard Y, Giraud F. Ca²⁺ permeability in deoxygenated sickle cells. *Blood* 1990;75:2453–2458. [PubMed: 2161689]

- Romero I, Maldonado AM, Eraso P. Glucose-independent inhibition of yeast plasma-membrane H⁺-ATPase by calmodulin antagonists. *Biochemical Journal* 1997;322:823–828. [PubMed: 9148755]
- Ronquist G, Rudolph O, Engström I, Waldenström A. Familial phosphofructokinase deficiency is associated with a disturbed calcium homeostasis in erythrocytes. *Journal of Internal Medicine* 2001;249:85–95. [PubMed: 11168788]
- Sabina RL, Waldenström A, Ronquist G. The contribution of Ca²⁺-calmodulin activation of human erythrocyte AMP deaminase (isoform E) to the erythrocyte metabolic dysregulation of familial phosphofructokinase deficiency. *Haematologica* 2006;91:652–655. [PubMed: 16670071]
- Schwartz RS, Tanaka Y, Fidler IJ, Chiu D, Lubin B, Schroit AJ. Increased adherence of sickled and phosphatidylserine enriched human erythrocytes to cultured human peripheral blood monocytes. *Journal of Clinical Investigation* 1985;75:1965–1972. [PubMed: 4008648]
- Seigneuret M, Devaux PF. ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. *Proceedings of the National Academy of Sciences of the United States of America* 1984;81:3751–3755. [PubMed: 6587389]
- Sims B, Mahnke-Zizelman DK, Profit AA, Prestwich GD, Sabina RL, Theibert AB. Regulation of AMP deaminase by phosphoinositides. *Journal of Biological Chemistry* 1999;274:25701–25707. [PubMed: 10464307]
- Stone PC, Stuart J, Nash GB. Effects of density and of dehydration of sickle cells on their adhesion to cultured endothelial cells. *American Journal of Hematology* 1996;52:135–143. [PubMed: 8756077]
- Styles LA, Lubin B, Vichinsky E, Lawrence S, Hua M, Test S, Kuypers F. Decrease of very late activation antigen-4 and CD36 on reticulocytes in sickle cell patients treated with hydroxyurea. *Blood* 1997;89:2554–2559. [PubMed: 9116302]
- Swain JL, Sabina RL, McHale PA, Greenfield JC Jr, Holmes EW. Prolonged myocardial nucleotide depletion after brief ischemia in the open-chest dog. *American Journal of Physiology* 1982;242:H818–H826. [PubMed: 7081454]
- Tait JF, Gibson D. Measurement of membrane phospholipid asymmetry in normal and sickle erythrocytes by means of annexin V binding. *Journal of Laboratory and Clinical Medicine* 1994;123:741–748. [PubMed: 8195679]
- Vazquez G, de Boland AR, Boland RL. Involvement of calmodulin in $\alpha,25$ -dihydroxyvitamin D₃ stimulation of store-operated Ca²⁺influx in skeletal muscle cells. *Journal of Biological Chemistry* 2000;275:16134–16138. [PubMed: 10747862]
- Waldenström A, Engström I, Ronquist G. Increased erythrocyte content of Ca²⁺ in patients with Tarui's disease. *Journal of Internal Medicine* 2001;249:97–102. [PubMed: 11168789]
- Wandersee NJ, Punzalan RC, Rettig MP, Kennedy MD, Pajewski NM, Sabina RL, Scott JP, Low PS, Hillery CA. Erythrocyte adhesion is modified by alterations in cellular tonicity and volume. *British Journal of Haematology* 2005;131:366–377. [PubMed: 16225657]
- Wood BL, Gibson DF, Tait JF. Increased erythrocyte phosphatidylserine exposure in sickle cell disease: flow-cytometric measurement and clinical associations. *Blood* 1996;88:1873–1880. [PubMed: 8781447]
- Yasin Z, Witting S, Palascak MB, Joiner CH, Rucknagel DL, Franco RS. Phosphatidylserine externalization in sickle red blood cells: associations with cell age, density, and hemoglobin F. *Blood* 2003;102:365–370. [PubMed: 12609840]
- Yingst DR, Davis J, Schiebinger R. Effects of extracellular calcium and potassium on the sodium pump of rat adrenal glomerulosa cells. *American Journal of Physiology* 2001;280:C119–C125. [PubMed: 11121383]
- Yun S-L, Suelter CH. Human erythrocyte 5'-AMP aminohydrolase: purification and characterization. *Journal of Biological Chemistry* 1978;253:404–408. [PubMed: 618880]
- Zwaal RFA, Bevers EM, Comfurius P, Rosing J, Tilly RHJ, Verhallen PFJ. Loss of membrane phospholipid asymmetry during activation of blood platelets and sickled red cells: mechanism and physiologic significance. *Molecular and Cellular Biochemistry* 1989;91:23–31. [PubMed: 2695831]
- Zwaal RFA, Comfurius P, Bevers EM. Mechanism and function of changes in membrane phospholipid asymmetry in platelets and erythrocytes. *Biochemical Society Transactions* 1993;21:248–253. [PubMed: 8359475]

Fig 1.

Reticulocyte levels in RBC fractions from normal and SCD individuals. Data are presented as the percentage of reticulocytes in aliquots of RBCs before (reticulocyte-rich) and after (reticulocyte-poor) the depletion protocol described in Design and Methods. Reticulocytes were quantified in all samples by flow cytometry using thiazole orange. Dotted lines in the SCD groups (SS-HC and SS) represent a reticulocyte level of 4%, which was selected as the cut-off for inclusion of these samples in a comparative metabolite analysis. Note the different range of values for the y-axis in the normal (control) and SCD (SS-HC and SS) graphs. Paired two-tailed student's *t*-tests between reticulocyte-rich and reticulocyte-poor fractions generated *P*-values of <0.001 for all groups (control, *n* = 10; SS-HC, *n* = 15; SS, *n* = 16).

Fig 2.

Metabolite levels in SCD and control RBCs. Upper panels: Total adenine nucleotides (TAN; down cross-hatched bars) and ATP (black bars) concentrations in reticulocyte-rich (left panel) and reticulocyte-poor (right panel) RBC fractions from normal subjects (Co; $n = 10$), SCD individuals on HC therapy (SS-HC; $n = 10$), and SCD individuals not on HC (SS; $n = 6$). Lower panels: ADP (grey bars), AMP (up cross-hatched bars) and IMP (white bars) concentrations in reticulocyte-rich (left panel) and reticulocyte-poor (right panel) RBC fractions. Data are reported as $\mu\text{mol/g Hb}$ and are presented as the mean $\pm$ SD. * $P < 0.05$ when compared to the corresponding value in the control group using an unpaired two-tailed student's t -test. † $P < 0.05$ when compared to the corresponding value in the SS-HC group using an unpaired two-tailed student's t -test. ‡ $P < 0.05$ when compared to the corresponding value in the reticulocyte-rich fraction using a paired two-tailed student's t -test.

Fig 3.

IMP/AMP ratios in reticulocyte-poor fractions of SCD and control RBCs. The ratio of product (IMP) to substrate (AMP) can be used as an index of AMPD activity *in vivo*. Other abbreviations are the same as described in the legend to Fig 2. Boxed data represent medians (horizontal lines), 25th percentiles (lower edges), and 75th percentiles (upper edges). Whiskers depict minimum and maximum values. * $P < 0.05$ when compared to the corresponding values in the control group using a non-parametric analysis (Kruskal–Wallace test).

Fig 4.

Effect of a diffusible CaM antagonist on IMP accumulation in reticulocyte-poor fractions of SCD and control RBCs during an experimental period of energy imbalance. Reticulocyte-poor fractions of RBCs suspended in their own plasma were incubated at 37°C (autoincubation) for 9 h following preincubation overnight at 4°C in the absence (upper graph) or presence (lower graph) of compound 48/80 (10 mg/ml). Aliquots were removed at 0, 4 and 9 h. Data presented as the increase in IMP concentration over time (mean $\pm$ SEM, $n = 3$) for control (closed symbols) and SCD (open symbols) RBCs. * $P < 0.05$ when compared to the corresponding control value using an unpaired two-tailed student's t -test.

Fig 5.

Effect of adenine treatment on intracellular ATP and phosphatidylserine exposure in sickle RBCs. Paired aliquots of washed sickle RBCs from 15 SCD individuals (eight receiving HC) were resuspended in their own platelet-poor plasma with (white bars) and without (black bars) 100 $\mu\text{mol/l}$ adenine and incubated overnight at room temperature, then analysed for intracellular ATP content (panel A) and phosphatidylserine (PS) exposure by flow cytometry. Panel B, representative untreated SCD RBC sample using annexin V-PE fluorescence to quantify PS exposure. Gates for total PS+ events ($>4 \times 10^2$ relative fluorescence intensity denoted by quadrant 'Q2') and relative high fluorescence PS+ events ($>4 \times 10^3$ relative fluorescence intensity denoted by the 'Type 2' region within quadrant 'Q2') are indicated. Panel C, all PS+ events; Panel D, Type 2 PS+ events. Bar data in panel A expressed as the mean $\pm$ SEM. Boxed data in panels C and D represent medians (horizontal lines), 25th percentiles (lower edges), and 75th percentiles (upper edges). Whiskers depict minimum and maximum values. * $P < 0.05$ compared to untreated samples using a paired two-tailed student's t -test (panel A) or a Mann-Whitney test (panel D), respectively.

Table I
Metabolite levels in CD71-positive reticulocytes *versus* reticulocyte-poor RBC fractions from SCD patient blood.

Group	n	Fraction	TAN	ATP	ADP	AMP	IMP
SS	5	CD71-positive	15.00 ± 2.12	13.00 ± 1.70	1.62 ± 0.54	0.46 ± 0.16	0.58 ± 0.20
	5	Reticulocyte -poor	5.91 ± 0.34	5.42 ± 0.37	0.43 ± 0.04	0.06 ± 0.03	0.26 ± 0.03
P-value			0.001	0.001	0.008	0.007	0.030
SS-HC	10	CD71-positive	16.30 ± 4.43	14.70 ± 4.28	1.48 ± 0.53	0.47 ± 0.17	0.30 ± 0.23
	10	Reticulocyte -poor	6.31 ± 0.51	5.83 ± 0.46	0.44 ± 0.10	0.04 ± 0.02	0.21 ± 0.14
P-value			<0.001	<0.001	<0.001	<0.001	NS

Data are expressed in µmol/g Hb and are presented as the mean ± SD.

The P-values were generated in paired two-tailed student's t-tests between the corresponding CD71-positive and reticulocyte-poor fractions within each group.

Data were not obtained for CD71-positive reticulocytes in one of the six SS samples.

NS, not significant.