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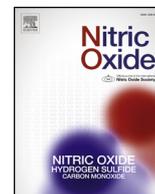
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## Hydroxyurea therapy modulates sickle cell anemia red blood cell physiology: Impact on RBC deformability, oxidative stress, nitrite levels and nitric oxide synthase signalling pathway



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### ABSTRACT

Hydroxyurea (HU) has been suggested to act as a nitric oxide (NO) donor in sickle cell anemia (SCA). However, little is known about the HU NO-related effects on red blood cell (RBC) physiology and NO signalling pathway.

Thirty-four patients with SCA (22 under HU treatment (HU+) and 12 without (HU-)) and 17 healthy subjects (AA) were included. RBC nitrite content, deformability and reactive oxygen species (ROS) levels were measured. RBC NO-synthase (RBC-NOS) signalling pathway was assessed by the measurement of RBC-NOS serine<sup>1177</sup> and RBC-AKT serine<sup>473</sup> phosphorylation. We also investigated the *in vitro* effects of Sodium Nitroprusside (SNP), a NO donor, on the same parameters in SCA RBC.

RBC nitrite content was higher in HU+ than in HU- and AA. RBC deformability was decreased in SCA patients compared to AA but the decrease was more pronounced in HU-. RBC ROS level was increased in SCA compared to AA but the level was higher in HU- than in HU+. RBC-NOS serine<sup>1177</sup> and RBC-AKT serine<sup>473</sup> phosphorylation were decreased in HU+ compared to HU- and AA. SCA RBC treated with SNP showed increased deformability, reduced ROS content and a decrease in AKT and RBC-NOS phosphorylation.

Our study suggests that HU, through its effects on foetal hemoglobin and possibly on NO delivery, would modulate RBC NO signalling pathway, RBC rheology and oxidative stress.

### 1. Introduction

Sickle cell anemia (SCA) is caused by a single mutation in the  $\beta$ -globin gene responsible for the production of an abnormal hemoglobin

called hemoglobin S (HbS). Under deoxygenation, HbS polymerizes leading red blood cells (RBCs) to sickle. Sickle RBCs have reduced deformability and are more fragile than healthy RBCs [1,2]. Consequently, SCA patients suffer from chronic hemolytic anemia and

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repeated painful vaso-occlusive crisis. Enhanced hemolysis is also at the origin of chronic vascular dysfunction and organ damages in patients [3]. Hydroxyurea (HU) is currently the main drug used to effectively treat SCA patients. HU improves the clinical course of SCA patients by progressively raising foetal hemoglobin (HbF) levels and therefore reducing HbS concentration thus limiting its deleterious effects [4]. However, this mechanism cannot explain alone all the observed beneficial impacts of HU in SCA [5]. Indeed, the drug has been shown to decrease RBC adhesion to the endothelium, limit endothelial activation, decrease leukocytes and platelets count, which also participate to improve the clinical status of the patients [6,7]. Moreover, HU has also been suggested to act as a nitric oxide (NO) donor [8,9].

NO plays a major role in several biological functions such as endothelial dependant vasodilation, platelets activation inhibition and decreased leukocytes adhesion [10–12]. Several observations also support a key role of NO on the rheology and the physiology of RBCs. Bor-Kucukatay et al. [13] demonstrated that NO donors (sodium nitroprusside (SNP) and DETA-NONOate) increased RBC deformability in healthy RBCs while the use of NO Synthase (NOS) inhibitor (L-NAME) decreased it. Kleinbongard et al. [14] reported the presence of a functional endothelial Nitric-Oxide-Synthase (NOS) like enzyme in healthy RBCs (RBC-NOS) and showed that modulation of its activity affected the deformability of the cells. Finally, NO has been shown to protect healthy RBCs from pharmacologically induced eryptosis [15,16].

Whether HU modulates RBC rheology and physiology in SCA through its putative NO-related effects is currently unknown. The aim of this work was to investigate the potential NO donor specific effect of HU therapy on RBC physiology and RBC-NOS signalling pathway in SCA patients. RBC deformability, reactive oxygen species (ROS) content, nitrite levels and NO-signalling pathway were analysed in healthy subjects and SCA patients with or without HU treatment. SCA RBCs were also incubated with SNP to evaluate the NO-related specific effects on RBC rheology and physiology.

## 2. Material and methods

### 2.1. Subjects and blood sampling

Thirty-four SCA (HbSS) subjects under HU treatment (HU+, n = 22) or not (HU-, n = 12) and 17 healthy subjects (AA) were included in the study. SCA patients were recruited from the Edouard Herriot Hospital from Lyon (France) the day of their annual follow-up between March 2016 and December 2017. They were in clinical steady-state at the time of the study i.e. without vaso-occlusive crisis or other acute medical complication within the last 2 months and without blood transfusion for at least 3 months before inclusion. The study was conducted in accordance with the guidelines set by the Declaration of Helsinki and the subjects gave informed written consent to participate. The study was approved by the “CPP Sud-Est IV” Ethics Committees (L16-47).

Venous blood was taken from the antecubital vein and collected into lithium heparin tubes for hemorheological and NO pathway assessments and into citrate tubes for RBC-ROS analysis (BD Vacutainer, Plymouth, UK).

### 2.2. Sample preparation for hemorheological and NO pathway assessment

Whole blood collected in heparin tube was immediately centrifuged at 800 g and 4 °C for 10 min to separate RBCs from plasma. The buffy coat containing platelets and leucocytes was removed and the plasma was stored on ice. RBCs were washed with Phosphate Buffered Saline 1X (PBS, pH 7.4) and then mixed with autologous plasma to achieve a hematocrit (Hct) of 20%. Two aliquots were prepared: one for control condition and the second one with SNP (100 µM) as a NO donor. The chosen SNP concentration was determined upon preliminary experiments and previous studies [13,17,18]. After incubation for 40 min at

37 °C, the samples were centrifuged at 800 g and 4 °C for 10 min and the plasma supernatant was aliquoted and stored at –80 °C until analysis. The RBCs were divided into three fractions stored at –80 °C: (i) the first one was mixed with a preservation solution (0.8 M ferricyanide, 0.1 M N-ethylmaleimide and 10% Igepal) to stabilize RBC nitrite until measurement, (ii) the second one was transferred into tubes for the analysis of RBC RxNO (the sum of all nitrosated species which includes RSNO – mercury labile S-nitrosated species – and RNNO – mercury resistant nitroso adducts) and (iii) the third one was dedicated to the analysis of RBC protein S-nitrosylation. A last RBC fraction dedicated to immunohistochemical assessment was incubated for 20 min with 4% paraformaldehyde for fixation.

### 2.3. Measurement of RBC and plasma nitrite and RBC RxNO

Previous studies have shown that nitrite levels reflect NO content in both physiological and pathophysiological conditions [19,20]. Measurement of nitrite and RxNO content of the samples was performed according to previous studies [21,22].

Plasma nitrite content was analysed directly after thawing the samples on ice. For nitrite measurement in RBCs, methanol (VWR international, Darmstadt, Germany) was added to the frozen samples in a 1:2-ratio to remove proteins and the suspensions were centrifuged at 21 000 g, at 4 °C for 15 min to collect the supernatants. For RBC RxNO measurement, RBC samples were mixed with an acidified sulfanilamide solution in a 1:10 ratio (Sigma Aldrich, St. Louis, Missouri, USA) resulting in 10% sulfanilamide and 0.1 N HCl final concentrations. The samples were incubated for 15 min at 4 °C in the dark. Sulfanilamide reacts with nitrite to form a diazo complex that is stable in triiodide [23].

Nitrite and RxNO levels were determined using an ozone-based chemiluminescence NO detector (CLD 88e, EcoPhysics, Switzerland). Samples were injected into an acidified tri-iodide solution that reduces nitrite but also iron-nitrosylheme, and S-nitrosothiols to NO gas. NO is transported by a helium gas stream to a NaOH trap (1N) and finally transported to the CLD device where it can be measured by its gas-phase chemiluminescent reaction with ozone [24]. The tri-iodide solution stoichiometrically releases NO from nitrite. For nitrite measurement, 100 µl of plasma or supernatant was injected into the tri-iodide solution. Samples were measured in triplicate. For RxNO measurement, total sample volume was injected into the reaction chamber and measured as described above. A calibration curve with solution containing growing known concentrations of nitrite was realized to calculate nitrite and RxNO concentration in the samples.

Nitrite content of methanol and nitrite preservation solution were also determined and nitrite concentrations measured in RBC samples were adjusted accordingly. As mentioned above, the resulting CLD signal might not only be specific for nitrite. Thus, the RxNO concentration was subtracted from the calculated nitrite concentration to receive the “net” nitrite levels of the sample. Indeed, the data we present refer to Total NOx, RxNO and nitrite levels. Data analysis was done with the Chart FIA software (Ecophysics, Switzerland) to integrate the area under the curve.

### 2.4. Immunohistochemical analysis

Fixed RBCs were centrifuged (125 g, 3min, 20 °C) and the supernatant was removed. Then, the RBC pellet was washed two times with PBS 1X and suspended in PBS 1X (v/v). RBC smears were prepared for each condition and heat fixed prior immunohistochemical staining [25].

A control and a test area were marked on each slide using a grease pencil. Both areas were washed with 0.1 M Tris-Buffered Saline (TBS) and RBCs were permeabilized for 30 min with a 0.1% trypsin solution at 37 °C. Then, endogenous peroxidase activity was blocked by using a solution containing 2% hydrogen peroxide and 80% methanol for

30 min at room temperature (RT). After washing with TBS, RBCs were treated with 3% non-fat milk in 0.1 M TBS for 30 min at RT for blocking unspecific binding sites. The test area of each slide was incubated with rabbit primary antibody against either RBC-NOS-Ser<sup>1177</sup> (dilution 1:150, phospho-eNOS-Ser<sup>1177</sup>; Millipore, Schwalbach, Germany) for 24 h at 4 °C or Akt-Ser<sup>473</sup> (dilution 1:500, Cell Signalling, Danvers, MA, USA) for 1 h at RT. The control area was treated similarly, except for the absence of primary antibodies. After rinsing with TBS, the sections were incubated 30 min at RT with 3% Normal Goat Serum and then with the secondary goat-anti-rabbit antibody (dilution 1:400, Dako, Glostrup, Denmark) for 30 min at RT. A streptavidin-horseradish-peroxidase solution (dilution 1:400, Sigma-Aldrich, St. Louis, MO, USA) was applied for 30 min at RT, and the staining was developed using a 3,3-diaminobenzidine-tetrahydro-chloride solution (Sigma-Aldrich) in 0.1 M TBS. RBC slides were then dehydrated with alcohol solutions and xylol and covered with cover slides using Entellan.

Immunostaining of RBCs was quantified according to previous published protocols [26,27]. Pictures of the slides were taken by using a Leica microscope coupled to a CCD Camera with a 400-fold magnification (DXC-1850P, Sony, Berlin, Germany). The staining intensity was analysed by the semi-quantitative measurement of the grey values conducted with “Image J” software (National Institutes of Health, Bethesda, MD, USA). RBC were edged and the grey values were calculated in arbitrary units (au). For each slide, the grey values of a total of 50 RBC from at least four field images were determined in the test area and subtracted from the background value, which was measured in a cell-free area of test area. Then, the grey values of a total of 10 RBCs from at least two images were determined in the control area and also subtracted from the background value to obtain the grey values of unstained RBCs. Grey values of RBCs from test and control areas were subtracted to obtain actual staining intensities.

## 2.5. Red blood cell deformability

RBC deformability was assessed at 37 °C at 3 and 30 Pa (Pa) by laser diffraction analysis (ektacytometry), using the Laser-assisted Optical Rotational Cell Analyzer (LORCA MaxSis, RR Mechatronics, Hoorn, The Netherlands). The system has been described in detail elsewhere [28]. Briefly, 4 µl of RBC were mixed with 1 ml polyvinylpyrrolidone (PVP; viscosity = 30 cP) and sheared into the Couette system. The diffraction pattern was analysed by computer and an elongation index (EI) was calculated. An increase of the EI indicates greater RBC deformability. The procedure was realized according to the recent methodological recommendations for RBC deformability measurement in SCA [28,29].

## 2.6. RBC S-Nitrosylation

### 2.6.1. RBC β-spectrin

RBC-NOS dependent NO production leads to S-nitrosylation of cytoskeletal protein β-spectrin [20]. S-nitrosylation of the β-spectrin in AA and SCA patients was assessed using the S-Nitrosylated Protein Detection Assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturers' instructions. The protocol consists of three steps: 1) blocking of free SH groups; 2) cleavage of potential S-NO bonds and 3) biotinylation and avidin labelling of the newly formed SH groups. Additionally, the protein concentration of the samples was determined using the DC-Protein Assay Kit (BioRad, Munich, Germany) to ensure that equal amounts of protein were analysed. A total of 20 µg protein was loaded into each lane of a 3–8% Tris-acetate gel (BioRad) and separated for 1 h under constant 90 mA current in a 1 × XT Tricine running buffer (BioRad). Proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (0.45 mm pore size). Background was blocked in 2% bovine serum albumin (in 1 × TBS with 0.1% Tween 20) overnight at room temperature with gentle shaking. Horseradish peroxidase (dilution 1:2000) was added which binds to the biotin-avidin complex, and the reaction was developed using an enhanced

chemiluminescence kit containing peroxidase substrate (Thermo Fischer Scientific, Darmstadt, Germany). S-nitrosylated protein band of 220 kDa, previously identified as β-spectrin [20], was examined for differing “Integrated densities” between SCA and AA samples using “Image J” software.

### 2.6.2. RBC-NOS S-nitrosylation

In order to identify the proteins isolated with the S-nitrosylation kit, SDS-PAGE separation and bottom-up protein identification were employed. In brief, 100 µg of the protein extract were separated on a 3–8% Tris-acetate gel (BioRad) for 1 h under constant 90 mA current in a 1 × XT Tricine running buffer (BioRad) and subsequently stained with Page Blue Protein Staining solution (Thermo Fisher Scientific). Protein bands potentially containing RBC-NOS (theoretical MW: 140 kDa [14]) were excised and subjected to in-gel tryptic digestion and nano-liquid chromatography high resolution/high accuracy Orbitrap mass spectrometry as previously described [20,30]. MS data were evaluated by using Proteome Discoverer Software (Thermo Fisher, Version 1.4, 2012) in combination with the SEQUEST algorithm and uniprot\_sprot\_human.fasta database (2014). The identification of a protein was considered successful if at least two peptides fulfilling a high level of confidence were detected or the sequence coverage was above 10% [31].

## 2.7. Red blood cell reactive oxygen species

Blood collected in citrate tube was centrifuged (800 g, 10 min at 20 °C) and plasma and buffy coat were discarded. RBCs were washed in PBS 1X and RBC pellets were suspended at 0.4% Hct in PBS 1X. For SNP modulation experiments, RBC suspensions were incubated for 40 min at 37 °C with 100 µM SNP or without for control condition. Intracellular RBC oxidative stress levels were determined using 2',7'-dichlorofluorescein diacetate (DCFDA, Sigma-Aldrich, Saint-Quentin-Fallavier, France). RBC suspensions at 0.4% Hct were incubated for 30 min at 37 °C in the dark with 10 µM of DCFDA (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and analysed by FACS according to manufacturer's instructions. Median Fluorescence Intensity (MFI) of the 50 000 gated events was recorded to quantify ROS levels.

## 2.8. Statistical analysis

Data are expressed as mean ± SEM. Comparisons between groups were achieved by using ANOVA (followed by Tukey post-hoc test) or student test when appropriate. Comparison of gender repartition between groups was achieved by using chi-square test. The effects of SNP treatment on SCA RBC were analysed by using paired t-tests. Statistical analyses were performed with GraphPad Prism 6 software (La Jolla, CA, USA). A p-value < 0.05 was considered as significant.

## 3. Results

### 3.1. SCA patient clinical characteristics

Among the 34 SCA patients included, 22 were on HU treatment since at least 6 months. The mean daily dose of HU was 18.3 ± 0.8 mg/kg. As expected, patients under HU therapy had higher HbF levels (19.0 ± 2.2% vs 4.9 ± 1.14%, respectively, p < 0.001) and higher mean cellular volume (96.6 ± 2.7 fL vs 78.1 ± 3.2 fL, respectively, p < 0.001) than HU- patients (Table 1). No statistical difference was observed for gender and age between all groups.

### 3.2. Comparisons between HU+, HU- and AA groups

#### 3.2.1. RBC deformability

RBC deformability from AA measured at 3 Pa (Fig. 1A) and 30 Pa (Fig. 1B) was higher compared to SCA groups (p < 0.001). RBC

**Table 1**

Clinical characteristics of the subjects. Difference between HU+ and HU- \*\*\* $p < 0,001$ . No difference between all groups NS. fl: femtoliter.

	AA (n = 17)	HU+ (n = 22)	HU- (n = 12)	
Gender (n,%)	10 (59) ♂/7 (41) ♀	13 (59) ♂/9 (41) ♀	6 (50) ♂/6 (50) ♀	NS
Age (years)	29.3 ± 1.6	33.3 ± 2.1	30.8 ± 2.6	NS
HbF (%)	–	19.0 ± 2.2	4.9 ± 1.14	***
MCV (fl)	–	96.6 ± 2.7	78.1 ± 3.2	***

deformability of HU+ group was significantly higher at 3 Pa ( $p < 0.01$ ) and 30 Pa ( $p < 0.05$ ) compared to HU- group.

### 3.2.2. Plasma and RBC total NOx, RxNO and nitrite levels

RBC total NOx (Fig. 2A), RxNO (Fig. 2B) and nitrite (Fig. 2C) levels were higher in HU+ compared to HU- ( $p < 0.05$  for all) and AA ( $p < 0.05$  for all). No difference was found between AA and HU- groups. Examples of RBC Total NOx chemiluminescence peaks obtained for AA, HU+ and HU- subjects are shown in Fig. 5A. Although plasma total NOx levels comparisons between groups did not reach statistical significance, plasma from HU+ patients tended to contain more NOx than HU- ( $p = 0.06$ ) and AA ( $p = 0.09$ ) subjects (Fig. 2D). HU+ group had higher plasma RxNO levels than AA group ( $p < 0.05$ , Fig. 2E) and higher plasma nitrite levels than both AA and HU- groups ( $p < 0.05$  for both, Fig. 2F).

### 3.2.3. AKT and RBC NOS activation

Immunohistochemical staining revealed lower RBC Akt Serine<sup>473</sup> phosphorylation in HU+ compared to AA ( $p < 0.001$ ) and HU- ( $p < 0.05$ ) (Fig. 2G). RBC-NOS Serine<sup>1177</sup> phosphorylation was lower in HU+ compared to AA and HU- patients ( $p < 0.05$  for both; Fig. 2H). Examples of RBC-NOS<sup>Ser1177</sup> immunostaining for AA, HU+ and HU- subjects are shown in Fig. 5B.

### 3.2.4. RBC S-Nitrosylation

RBC  $\beta$ -spectrin S-nitrosylation of HU+ group was significantly higher compared to both AA and HU- groups ( $p < 0.05$ ). S-nitrosylation of  $\beta$ -spectrin did not differ between AA and HU- (Fig. 2I). Proteins isolated with the S-nitrosylation kit were analysed by using bottom up proteomic approaches, however, RBC-NOS was not among the identified RBC proteins (data not shown).

### 3.2.5. RBC ROS content

Flow cytometry analysis showed that AA RBC contained lower level of ROS compared to SCA RBC ( $p < 0.01$  and  $p < 0.05$  for HU- and HU+, respectively). HU+ RBC ROS content was lower in comparison to HU- patients ( $p < 0.05$ ; Fig. 3).

### 3.3. Effects of SNP on SCA RBC

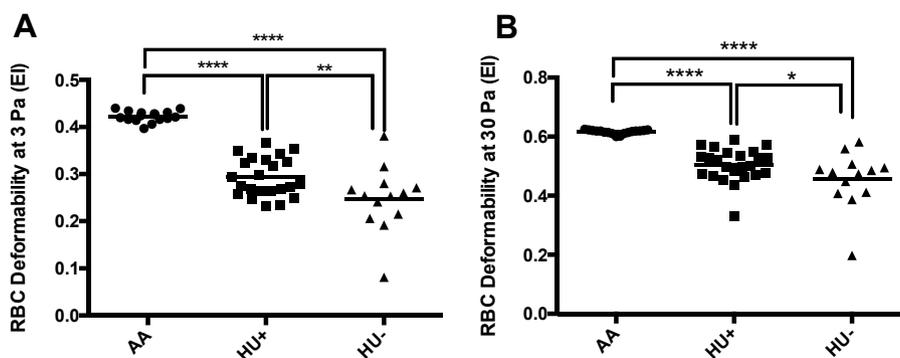
As shown in Fig. 4A, B and C, incubation with SNP increased SCA RBC deformability at both 3 and 30 Pa ( $p < 0.05$ ) and enhanced RBC total NOx concentration ( $p < 0.01$ ). Incubation of RBC with SNP decreased Akt Serine<sup>473</sup> phosphorylation ( $p < 0.05$ ; Fig. 4D) and RBC-NOS Serine<sup>1177</sup> phosphorylation ( $p < 0.05$ ; Fig. 4E). RBC ROS levels were decreased after SNP incubation ( $p < 0.05$ ; Fig. 4F).

## 4. Discussion

The results of the present study indicate that SCA patients under HU therapy show reduced activation of Akt and RBC-NOS proteins but higher RBC NO levels, increased S-nitrosylation of cytoskeletal spectrin and higher RBC deformability compared to patients without HU treatment. In addition, the *in vitro* use of the NO donor SNP induced the same RBC responses than those found in HU treated patients compared to those without. The findings suggest that HU could modulate RBC physiology and RBC-NOS signalling pathway.

Several studies previously suggested that HU would act as a NO donor. Nahavandi et al. [32] were the first to report higher NO metabolites in plasma from SCA patients under HU therapy. Both plasma and RBC nitrite levels, as well as other NO related metabolites, have been shown to increase shortly after HU ingestion [8,32–34]. Conran et al. [35] and Nahavandi et al. [36] also reported higher soluble guanylate cyclase (sGC) activity and higher cGMP content in SCA patients under HU treatment. Since NO activates sGC, which leads to increased cGMP production, the authors suggested that HU would be able to increase NO concentration [35,36]. Interestingly, the NO-dependant sGC activity was also reported to be involved in the production of HbF in SCA patients under HU therapy [37]. Food intake may affect blood nitrite content in humans [38]. Nevertheless, although it was not possible to control or normalize food intake before blood drawing in the present report, our results are in agreement with these previous studies since we observed higher RBC NOx, nitrite, RXNO and plasma nitrite content in HU+ compared to HU- patients and healthy individuals.

RBC deformability is severely reduced in SCA and plays a major role in the pathophysiology of SCA [1,2,39]. Our HU+ patients had higher RBC deformability than HU- individuals. This is in agreement with several previous studies showing that HU treatment improves RBC deformability in SCA, which participates to the clinical benefits observed with this drug [40–44]. The improvement of RBC deformability in HU treated patients has been mainly attributed to the increased production of HbF and a better RBC hydration status (higher mean cellular volume). Huang et al. [45] demonstrated that *in vitro* exposure of SCA RBC with HU resulted in a decrease of deformability, which was mainly attributed to oxidative stress mechanisms [45]. This study strongly suggests that the *in vivo* improvement of RBC deformability observed in patients treated with HU is secondary to the stimulation of HbF



**Fig. 1.** Comparison of RBC deformability at 3 Pa (1A) and 30 Pa (1B) in AA (n = 17), HU+ (n = 22) and HU- subjects (n = 12). Differences between group \* $p < 0.05$ , \*\*\*\* $p < 0.0001$ . EI, elongation index.

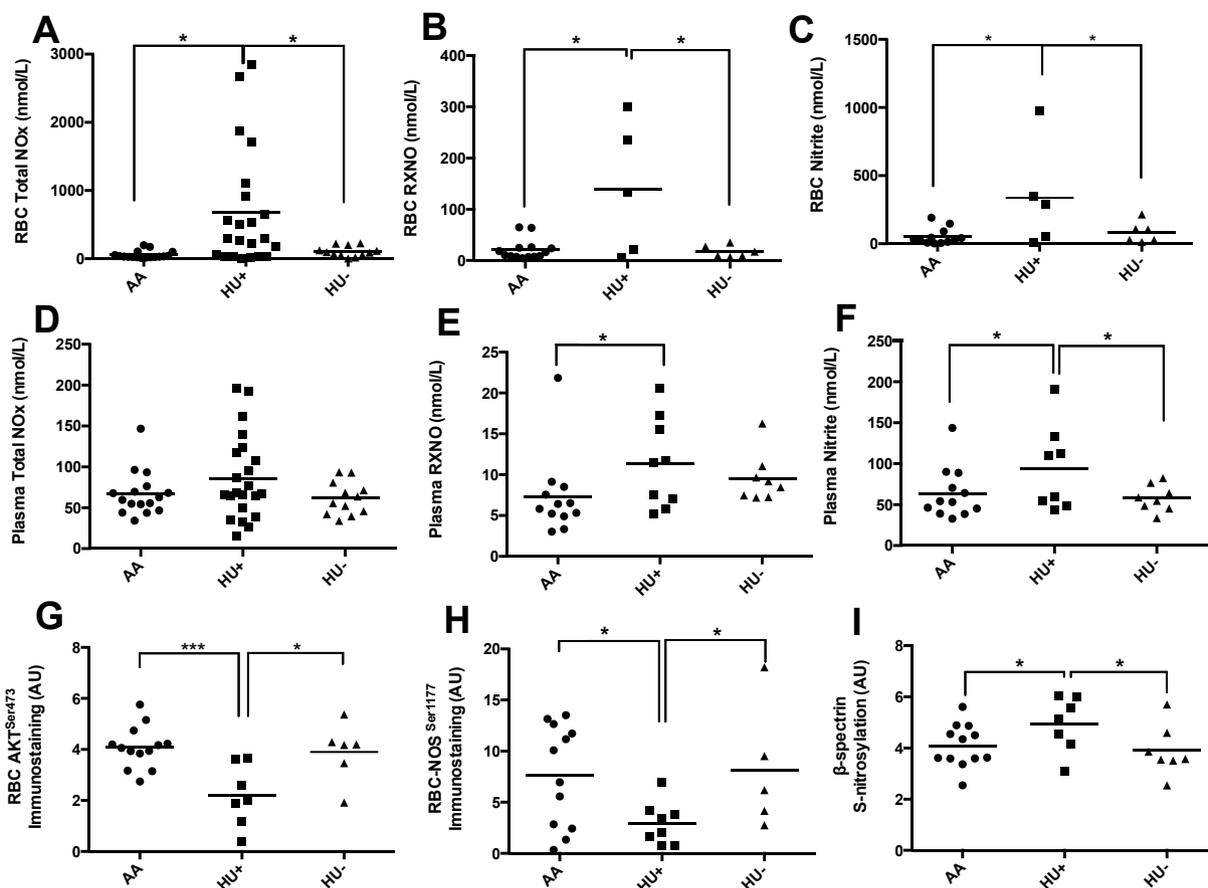


Fig. 2. RBC Total NOx (2A, AA n = 17, HU+ n = 22, HU- n = 12), RxNO (2B, AA n = 14, HU+ n = 5, HU- n = 6) and Nitrite content (2C, AA n = 12, HU+ n = 5, HU- n = 6) and plasma Total NOx (2D, AA n = 17, HU+ n = 22, HU- n = 12), RxNO (2E, AA n = 12, HU+ n = 9, HU- n = 8) and nitrite (2D, AA n = 12, HU+ n = 9, HU- n = 8) content. 2G) RBC AKT phosphorylated Serine<sup>473</sup> immunostaining AA (n = 13), HU+ (n = 7) and HU- (n = 6) and 2H) RBC-NOS phosphorylated Serine<sup>1177</sup> immunostaining in AA (n = 12), HU+ (n = 8) and HU- (n = 5) groups. 2I) S-nitrosylation of  $\beta$ -spectrin in AA (n = 12), HU+ (n = 7) and HU- (n = 7). Differences between groups: \*p < 0.05, \*\*\*p < 0.001. AU, Arbitrary Units.

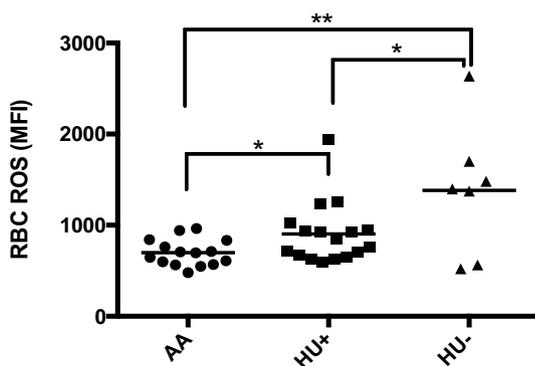


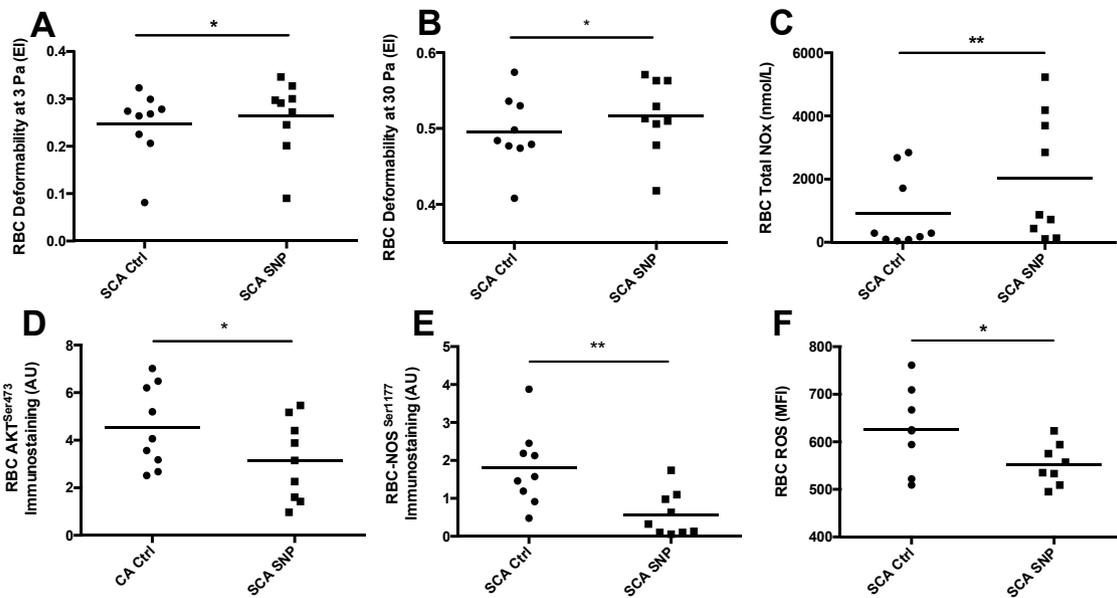
Fig. 3. Comparison of RBC ROS content in RBC from AA (n = 15), HU+ (n = 17) and HU- (n = 7) subjects. Differences between group \*p < 0.05, \*\*p < 0.01. MFI, Median Fluorescence Intensity.

production and not to the NO donor property of the drug. However, *in vitro* incubation of healthy RBC with NO donors or pharmacological agents known to modulate RBC-NOS activity has been shown to increase healthy RBC deformability [13,14,20]. While the exact mechanisms have been poorly explored, the improvement of RBC deformability caused by NO-related molecules is suspected to be related to the increased S-nitrosylation status of cytoskeletal proteins  $\alpha$ - and  $\beta$ -spectrin [20]. S-nitrosylation is a posttranslational protein modification induced by NO through its binding to active cysteine thiol groups resulting in the formation of s-nitrosothiol, which in turn can affect

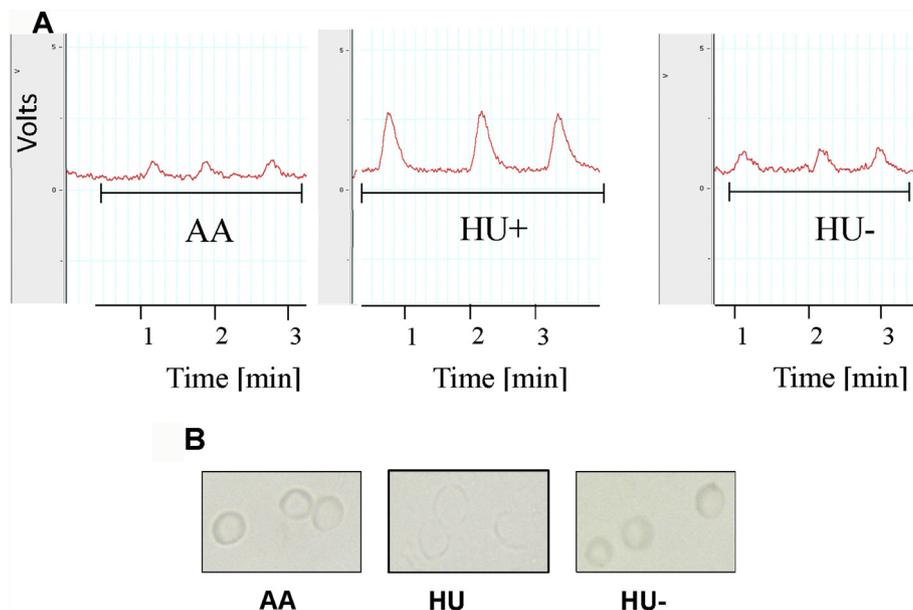
protein activity, localization or molecular interaction [46]. S-nitrosylation of proteins has also been suggested to prevent SH groups from being oxidized [47]. We observed a small but significant increase of deformability in SCA RBC incubated with SNP. In contrast, Belanger et al. [17] and Barodka et al. [48] did not observe any improvement of sickle RBC deformability with SNP. Nevertheless, the drug was shown to protect RBC from the deleterious effect of calcium influx on deformability, which suggests that NO may have some rheological impacts [17,48].

Besides, the higher RBC nitrite level found in HU+ patients was associated with a higher level of  $\beta$ -spectrin S-nitrosylation, suggesting that HU could also impact RBC deformability through its NO-related effects. Because of the *in vitro* deleterious effect of HU on RBC deformability [45], we hypothesize that, in certain patients with low levels of oxidative stress and reduced RBC damage, such as those under HU therapy, the NO donor effect of HU could potentially counterbalance and overcome this deleterious effects by acting synergistically with the predominant *in vivo* effect of HU on HbF to improve RBC deformability. Indeed, Huang et al. [45] reported that HU had no detrimental effects on healthy RBC, which are likely to be less damaged than SCA RBC. Nevertheless, it is particularly difficult to differentiate the various *in vivo* effects of HU independently of its action on HbF production, which is certainly the main effect of this therapy.

Very recently, a study by Diederich reported that NO donors would be able to preserve RBC deformability in situations where oxidative stress would be enhanced [49]. Oxidative stress plays a critical role in the pathophysiology of SCA [50]. Systemic and RBC ROS levels are



**Fig. 4.** Effect of SNP on RBC deformability measured at 3 Pa (4A) and 30 Pa (4B) and on RBC total NOx content (4C). Effect of SNP on RBC RBC AKT Serine<sup>473</sup> phosphorylation (4D), RBC-NOS Serine<sup>1177</sup> phosphorylation (4E) in SCA patients and on RBC ROS content in SCA patients (4F), SNP concentration = 100  $\mu$ M, n = 9. Differences between groups: \*p < 0.05, \*\*p < 0.01. AU, arbitrary units; EI, Elongation Index; MFI, Median Fluorescence Intensity.



**Fig. 5.** Representative graphs of RBC Total NOx chemiluminescence peaks obtained for AA, HU+ and HU- subjects (5A) and representative pictures of RBC-NOS<sup>Ser1177</sup> immunostaining for AA, HU+ and HU- subjects (5B).

particularly increased in SCA patients mainly because of the high HbS auto-oxidation rate, repeated sickling and unsickling cycles, repeated ischaemia and reperfusion injuries and intravascular hemolysis [51,52]. This particularly harmful pro-oxidant state participates to the reduction in RBC deformability in SCA [1,53]. Although the use of DCFDA to determine oxidative stress level presents several limitations and more particularly into RBC, which contains heme and iron that can interfere with this probe [54], our results suggest that ROS content is higher in RBC from SCA patients compared to RBC from healthy subjects. However, patients under HU therapy had lower RBC ROS level than SCA patients without HU. Few authors previously examined the relationship between HU treatment and oxidative stress. Torres et al. found a reduction in systemic oxidative stress in SCA patients taking hydroxyurea [55]. The reduction in oxidative stress in HU treated patients could

result from the increased production in HbF, which is accompanied by a reduction in HbS level and limits Rbc sickling. However, *in vitro* treatment with SNP also reduced ROS level within SCA RBC. The potential mechanisms by which NO may exert a direct anti-oxidant effect in RBC is currently unknown. Nevertheless, it has been shown that NADPH oxidase plays a critical role in ROS generation in RBC from SCA patients [56]. Interestingly, Selemidis et al. suggested that the treatment of endothelial cells with NO donors, such as SNP, was able to reduce NADPH oxidase superoxide production through S-nitrosylation dependant mechanisms [57]. Although S-nitrosylation of NADPH oxidase was not examined, the higher S-nitrosylation of  $\beta$ -spectrin found in HU+ patients also suggests that other proteins might be S-nitrosylated and that the reduction of ROS generation in these patients and with SNP treatment could be attributed to this mechanism. Moreover, NO and HU

are known to have strong interaction with heme-protein and particularly with hemoglobin, a property that could modulate HbS polymerisation or/and oxidation rate. The effect of NO on HbS polymerisation and oxygen affinity has been strongly debated. Head et al. [58] first suggested that NO could improve HbS oxygen affinity and therefore potentially reduce HbS polymerisation and subsequent generation of ROS [58]. However, the method used by the authors was not validated and their conclusions could not have been confirmed by other groups. Indeed, using the gold standard method to determine HbS solubility, Xu et al. [59] concluded that NO therapy did not have any solubilizing effect [59]. Furthermore, Gladwin et al. [60] did not find any consequences of inhaled NO on HbS oxygen affinity [60]. Whatever the exact mechanism at the origin of the reduction in ROS level inside the RBC of SCA patient under HU therapy, the decrease in oxidative stress probably participates to the increase of RBC deformability observed in these patients [1,53].

The results of the present investigation revealed decreased activation levels of RBC-NOS enzyme but also of Akt kinase in HU treated patients and in SNP experiments. Phosphorylation of Akt kinase was shown to affect phosphorylation and thus activation of RBC-NOS [25]. Reduction of both activation states in combination with increased NO levels might suggest that external application of NO through NO donors acts as negative feedback finally reducing RBC-NOS activation and eventually limiting RBC NO endogenous production. The mechanism by which NO may limit Akt activation in RBC is unknown but it has been proposed that Akt could be inactivated in response to a NO donor through S-nitrosylation of the kinase [61]. A study performed on endothelial cells reported that NO was able to inhibit eNOS activation through S-nitrosylation of the protein [62]. The present results failed to detect S-nitrosylation of RBC-NOS or differences between HU+ and HU- patients and it is therefore difficult to conclude whether the difference found in this study is attributable to this mechanism.

Overall, our results shown that besides its predominant effect on HbF level, HU would impact SCA RBC physiology and more particularly RBC NO signalling pathway. This study opens new perspectives on the consequences of HU and NO focused therapies in SCA and on the regulation of RBC NO production.

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EN, RF, CR, BC, NG, KW, MT and PC performed the research. GC, AG, SP and AH recruited the patients. EN, MG, CM, WB, MR, NG and PC designed the research study. EN, MG, RF, BC, NG and PC analysed the data. EN, MG and PC wrote the first draft of the paper. All authors carefully read and revised the manuscript before submission.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.niox.2018.10.003>.

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