Hydroxycarbamide Decreases Sickle Reticulocyte Adhesion to Resting Endothelium by Inhibiting Endothelial Lutheran/Basal Cell Adhesion Molecule (Lu/BCAM) through Phosphodiesterase 4A Activation*

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Vicky Chaar F-08-1104, Sandrine Laurance F-08-1104, Claudine Lapoumeroulie F-11512, Sylvie Cochet F-11512, Maria De Grandis F-11512, Yves Colin F-11512, Jacques Elion F-11512, Caroline Le Van Kim F-11512, and Wassim El Nemer F-11512

From the INSERM, U1134, F-75739 Paris, France, the Université Paris Diderot, Sorbonne Paris Cité, UMR_S 1134, F-75739 Paris, France, the Institut National de la Transfusion Sanguine, F-75739 Paris, France, the Laboratoire d’Excellence GR-Ex, F-75238 Paris, France, and the Assistance Publique-Hôpitaux de Paris, Département de Génétique, Hôpital Robert Debré, Paris F-75019, France.

Background: Hydroxycarbamide treatment may inhibit the proadhesive features of vascular endothelium in sickle cell disease.

Results: Hydroxycarbamide treatment of endothelial cells inhibits the interaction between erythroid integrin $\alpha_4\beta_1$ and endothelial Lu/BCAM.

Conclusion: Hydroxycarbamide increases the expression of phosphodiesterase 4A, which decreases cAMP levels leading to less Lu/BCAM phosphorylation and less cell adhesion.

Significance: This is the first example of a phosphodiesterase being regulated by hydroxycarbamide.

Vaso-occlusive crises are the main acute complication in sickle cell disease. They are initiated by abnormal adhesion of circulating blood cells to vascular endothelium of the microcirculation. Several interactions involving an intricate network of adhesion molecules have been described between sickle red blood cells and the endothelial vascular wall. We have shown previously that young sickle reticulocytes adhere to resting endothelial cells through the interaction of $\alpha_4\beta_1$ integrin with endothelial Lutheran/basal cell adhesion molecule (Lu/BCAM). In the present work, we investigated the functional impact of endothelial exposure to hydroxycarbamide (HC) on this interaction using transformed human bone marrow endothelial cells and primary human pulmonary microvascular endothelial cells. Adhesion of sickle reticulocytes to HC-treated endothelial cells was decreased despite the HC-derived increase of Lu/BCAM expression. This was associated with decreased phosphorylation of Lu/BCAM and up-regulation of the cAMP-specific phosphodiesterase 4A expression. Our study reveals a novel mechanism for HC in endothelial cells where it could modulate the function of membrane proteins through the regulation of phosphodiesterase expression and cAMP-dependent signaling pathways.

Sickle cell disease (SCD) is a monogenic red blood cell disorder characterized by chronic hemolytic anemia, painful vaso-occlusive crises (VOC), and increased susceptibility to infection. The SCD classical physiological scheme involves hemoglobin S polymerization and less deformable sickle red blood cell (SS RBC) formation under hypoxic conditions. In addition to their propensity to sickle, SS RBCs can abnormally adhere to the vascular endothelium, contributing to microvascular occlusions (1, 2) and thus to the initiation and progression of VOC which represent the main SCD acute complication (3). Several interactions involving an intricate network of adhesion molecules have been described between SS RBCs and the endothelial vascular wall. CD36, expressed on reticulocytes and endothelial cells, could contribute to SS reticulocyte adhesion to the endothelium through a bridge of plasma thrombospondin-1. Two members of the immunoglobulin superfamily expressed both on reticulocytes and mature RBCs, Lutheran/basal cell adhesion molecule (Lu/BCAM) and Landsteiner-Wiener/intercellular adhesion molecule-4 (LW/ICAM-4), are also involved in abnormal SS RBC adhesion to the endothelium through their interaction with extracellular matrix laminin 511/521 and endothelial integrin $\alpha_v\beta_3$, respectively (4–11). Moreover, Lu/BCAM- and ICAM-4-mediated SS RBC adhesion is regulated by phosphorylation events involving the physiologic stress mediator epinephrine through the up-regulation

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1 Both authors contributed equally to this work.

2 Recipient of a doctoral fellowship from the French Ministère de l’Enseignement Supérieur et de la Recherche at the Ecole Doctorale B3MI.

3 To whom correspondence should be addressed: INSERM, UMR_S 1134, INTS, 6, rue Alexandre Cabanel, 75015 Paris, France. Tel.: 33-1-44-49-30-71; Fax: 33-1-43-06-50-19; E-mail: wassim.el-nemer@inserm.fr.

4 The abbreviations used are: SCD, sickle cell disease; AI, adhesion index; FSK, forskolin; HC, hydroxycarbamide; HPMEC, human pulmonary microvascular endothelial cell; IBMX, 3-isobutyl-1-methylxanthine; Lu/BCAM, Lutheran/basal cell adhesion molecule; LW/ICAM-4, Landsteiner-Wiener/intercellular adhesion molecule-4; PDE, phosphodiesterase; PECAM, platelet endothelial cell adhesion molecule; PI, phosphorylation index; RQ-PCR, real-time quantitative PCR; SS RBC, sickle red blood cell; TrHBMEC, transformed human bone marrow endothelial cell; VCA-1, vascular cell adhesion molecule-1; VOC, vaso-occlusive crises.
of intracellular cyclic adenosine monophosphate (cAMP) and the activation of the protein kinase A (PKA) signaling pathway (5, 12, 13). Whereas LW/ICAM-4 is an erythroid-specific protein, Lu/BCAM exhibits a broad expression pattern (14, 15). Lu/BCAM is expressed on resting endothelial cells where it contributes to abnormal SS reticulocyte adhesion by interacting with erythroid integrin α₄β₁ (16). Hydroxycarbamide (HC, the recommended international nonproprietary name of hydroxyurea) is the only drug that has demonstrated clinical benefits for SCD patients by reducing VOC and hospitalization frequencies (17). HC was initially administered to induce fetal hemoglobin expression to interfere with and decrease hemoglobin S polymerization. However, HC-associated clinical benefits appear shortly upon starting the treatment before the elevation of fetal hemoglobin expression, suggesting that HC could act through other targets and mechanisms. Several studies investigated the effects of HC treatment on blood cells and gave insightful clues regarding its associated clinical improvements, mainly in relation with adhesion molecules. Recently, we showed that HC could reduce the abnormal RBC adhesion to laminin by inhibiting the adhesion function of erythroid Lu/BCAM independently of its expression level (18). The effects of HC on the endothelium are much less explored because of the difficulties in obtaining endothelial cells from SCD patients. However, few groups investigated these effects on human endothelial cell lines and human primary endothelial cells. Cokic et al. (19) showed that in vitro short exposure of endothelial cells to HC increases cAMP and cGMP (cyclic guanosine monophosphate) and induces nitric oxide (NO) production in a NO synthase-dependent manner. We showed that in vitro treatment of endothelial cells with HC leads to a decreased expression of vascular cell adhesion molecule-1 (VCAM-1) (20) and of the vasoconstrictor peptide endothelin-1, which is in accordance with the decreased levels of plasma endothelin-1 in HC-treated children (21, 22).

In this study, we investigated the effect of HC on the interaction between erythroid α₄β₁ integrin and endothelial Lu/BCAM using a transformed human endothelial cell line from the bone marrow microcirculation (TrHBMECs) and primary human pulmonary microvascular endothelial cells (HPMECs). We found a decreased SS reticulocyte adhesion to HC-treated endothelial cells and revealed a new mechanism where HC upregulates the expression of the cAMP-specific phosphodiesterase 4A leading to decreased levels of cAMP and Lu/BCAM phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Cells**—TrHBMECs were grown as described previously (20) and used between passages 20 and 25. Primary HPMECs were purchased from PromoCell (Heidelberg, Germany), grown in MV2 medium, and used between passages 4 and 6. Wild type (WT) K562 cells (human erythroleukemic cells) and transfected K562 cells expressing α₄β₁ (16,000 sites/cell) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Invitrogen). TrHBMECs and HPMECs were treated by HC (Sigma-Aldrich) at 250 μM for 24 h before undergoing adhesion, phosphorylation, flow cytometry, or Western blot analyses. When required, TrHBMECs and HPMECs were pretreated with forskolin (200 μM, 30 min), 3-isobutyl-1-methylxanthine (IBMX; 200 μM, 30 min), and/or rolipram (100 μM, 30 min) (Sigma-Aldrich).

**Plasmid Construction and K562 Cell Transfection**—A NotI/XhoI 3.1-kb fragment, carrying the entire coding DNA sequence of the α₄ integrin human gene, was inserted in the pcDNA3.1 vector (Invitrogen). K562 cells were transfected with 4 μg of plasmid DNA using the Amaza® Cell Line Nucleofector® Kit V (Lonza, Basel, Switzerland) according to the manufacturer’s instructions. Transfected cells were maintained in culture medium supplemented with 0.8 g/liter Geneticin® (Invitrogen). Stably transfected clones were then isolated, and their level of expression of α₄β₁ integrin was estimated by flow cytometry.

**RNA Isolation, Retrotranscription, and Real-time Quantitative PCR Experiments (RQ-PCR)**—Total RNA was extracted from cultured cells using a commercial kit following the manufacturer’s instructions (NucleospinRNA II; Macherey-Nagel, Düren, Germany). The RNA samples underwent retrotranscription; the volume of the final reaction mix was 20 μl and was composed of 2 μg of total RNA, 2 μl of 10× PCR buffer, 0.8 μl of dNTP (25 mM), 2 μl of random hexamers (50 μM), 4 units of reverse transcriptase, and 2 units of RNase inhibitor (PE Applied Biosystems). The mix was incubated for 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C. The synthesized cDNA was kept at −20°C until real-time PCR experiments.

RQ-PCR experiments were carried out on ABI 7300 (PE Applied Biosystems). The SYBR Green intercalant was used for the amplification detection, and the SYBR Green Core Reagent was used following the manufacturer’s instructions (PE Applied Biosystems). The final concentration of cDNA was 5 ng/μl. The primers were used at 300 nM and designed by Primer Express Software (PE Applied Biosystems). A fragment of 67 bp corresponding to the immunogenic region of PDE4A gene was amplified using the following primers: forward PDE4-F, 5'-TCTGCCCTGCGCTTTAAG-3' and reverse PDE4-R, 5'-GGG-CATGCTCTGAAACAGACA-3'. Results are expressed as mean of the percentage of gene expression ± S.D. of the control value of five independent experiments. Cells nontreated with HC were used as control.

**cAMP Quantification**—Total cAMP was measured in cell lysates of TrHBMECs treated or not by HC for 24 h as described in the Cayman Chemical cAMP enzyme immunoassay kit (Ann Arbor, MI). Briefly, 5 × 10⁶ cells were lysed in 1 ml of 0.1 M HCl supplemented with the PDE general inhibitor IBMX at 200 μM. After centrifugation, supernatants were acetylated, and 50 μl of each was added per well in a 96-well ELISA plate provided in the kit. After 18 h of incubation at 4°C, wells were washed, and 200 μl of revelation buffer was added to each well. After 2 h of incubation at room temperature, total cAMP levels were measured according to the manufacturer’s instructions.

**Reticulocyte Enrichment**—Reticulocyte-rich fractions were prepared from freshly drawn heparin-anticoagulated venous blood from six homozygote SCD patients. Patients were at steady state, defined by at least 3 months after blood transfusion or 1 month after an acute clinical event. The six patients were at least 18 years old and able to give their informed consent. The
study was conducted in accordance with the Declaration of Helsinki and local laws.

Reticulocytes were enriched from whole blood using Percoll double density separation (densities used: 1.090 and 1.076), as described previously (18). Reticulocyte enrichments for the three blood samples used in the adhesion assays with TrHBMECs were from 10 to 48\%, 9 to 31\%, and 6 to 57\%; the enrichments for those used in the adhesion assays with HPMECs were from 9 to 17\%, 5 to 22.5\%, and 12 to 51\%.

Flow Cytometry—The percentage of reticulocytes in whole or fractionated blood samples was determined using thiazole orange dye (Retic-CountTM; BD Biosciences) and a BD FACSCanto II flow cytometer (BD Biosciences) with FACSDiva software (v6.1.2) for acquisition and analysis. The percentage was determined by gating the red cell population based on size (forward scatter) and granularity (side scatter). Expression of cell surface Lu/BCAM on TrHBMECs and α4 integrin on transduced K562-αβ1 cells was evaluated with the mouse anti-Lu/BCAM F241 monoclonal antibody (13) (mAb) and a mouse anti-human α4-integrin mAb (BD Biosciences), respectively. Mean fluorescence intensity was determined under the same conditions for all samples.

Immunofluorescence Staining—TrHBMECs were grown in ibiTreat μL Lucetm2 microslides (ibidi GmbH, Munich, Germany), then fixed for 20 min with 4% paraformaldehyde/PBS and washed three times with PBS-BSA 0.2%. Cells were incubated with purified monoclonal mouse anti-human CD106 (VCAM-1) (BD Biosciences) or anti-human Lu/BCAM (F241) (13) for 1 h at room temperature. After three washes with PBS cells were incubated with a goat anti-mouse Alexa Fluor 488 secondary antibody for 1 h at room temperature. Prolong gold antifade reagent (Invitrogen) was deposited in the microslides, and cells were examined by confocal microscopy using a Nikon EC-1 system equipped with 60× NA 1.4 and 100× 1.30 objectives (Nikon Corp, Tokyo, Japan).

Cell Adhesion Assays—Cell adhesion to TrHBMEC and HPMEC monolayers or to immobilized VCAM-1-Fc (R&D Systems) was determined under physiological flow conditions using ibidi microslides. TrHBMECs and HPMECs were seeded in ibiTreat μL Lucetm2 microslides (internal channel dimensions: length 50 mm, width 5 mm, height 0.2 mm) and grown for 24 h to form monolayers. VCAM-1-Fc was immobilized (1 μg/cm²) in uncoated μL Lucetm2 microslides at 4°C overnight. SS reticulocytes (0.5% hematocrit) or K562 cells (5 × 10⁶ cells/ml) were perfused at a shear stress of 0.2 dyn/cm² for 10 min and washouts used Hank’s buffer at 0.5, 1, 1.5, 2, and 3 dynes/cm² for 5 min each. After each wash, adherent cells were counted in 11 representative areas along the centerline of the microslide using the AxioObserver Z1 microscope and AxioVision 4 analysis software (Carl Zeiss, Le Pecq, France). Images of the same 11 areas were obtained throughout each experiment using the Mark and Find module of AxioVision analysis software.

For inhibition assays, TrHBMECs were incubated with 50 μg/ml anti-Lu MAB1481 (R&D Systems) or anti-PECAM-1 (Immunotech SAS, Marseille, France) antibodies for 30 min at 37°C prior to the adhesion assay.

Phosphorylation Assays—Phosphorylation of Lu/BCAM was assessed in TrHBMECs and HPMECs as described (13, 18). Phosphorylated Lu/BCAM was immunopurified from 2 × 10⁶ cells treated or not with HC (250 μM, 24 h), forskolin (200 μM, 30 min), IBMX (200 μM, 30 min), and/or rolipram (100 μM, 30 min) (Sigma-Aldrich).

Western Blot Analyses—TrHBMECs were washed twice with cold PBS and lysed with 250 μl of ice-cold radioimmunoprecipitation assay buffer (Sigma-Aldrich), containing 1× (final concentration) protease inhibitor mixture (Roche Diagnostics), phosphatase inhibitor mixture (Sigma-Aldrich), 10 μg/ml sodium orthovanadate. Cell lysates were centrifuged (14,000 g, 15 min, 4°C). Ten μg of proteins from the supernatant was diluted (5:1) in Laemmli sample buffer containing 1% β-mercaptoethanol and boiled for 5 min. After SDS 10% polyacrylamide gel electrophoresis and protein transfer on nitrocellulose membrane (Whatman-Protan, Dassel, Germany), PDE4A was detected by using a rabbit anti-PDE4A antibody (Abcam, Cambridge, UK) as primary labeling and a secondary peroxi-dase-conjugated goat anti-rabbit antibody (SouthernBiothech, Birmingham, AL). PDE4A was revealed using enhanced chemiluminescence (ECL) (GE Healthcare) with a Molecular Imager Gel Doc XR system (Bio-Rad) and quantified by using Quantity One software (Bio-Rad). The signal obtained for PDE4A when 10 μg of total proteins was loaded fell in the linear range of the signals obtained from 5 to 30 μg of proteins. The PDE4A:actin ratio was calculated to determine the variation of the expression of PDE4A.

Statistical Analyses—Results are presented as means ± S.D. Statistical significance was determined using unpaired or paired t test, as indicated in the figure legends; a difference between two groups was considered statistically significant when p < 0.05.

RESULTS

Endothelial Lu/BCAM Sustains SS Reticulocyte Adhesion to TrHBMECs—First, we addressed the endothelial characteristics of the TrHBMEC cell line by analyzing the expression of VCAM-1 at the cell surface. VCAM-1 is normally absent on resting endothelial cells but expressed under inflammatory conditions. As expected, using immunofluorescence and confocal microscopy we found that VCAM-1 was not expressed at the surface of resting TrHBMECs and was induced after incubating the cells with TNF-α (Fig. 1A). Resting TrHBMEC monolayers were then used in adhesion assays under flow conditions together with SS reticulocyte-enriched blood fractions (termed SS reticulocytes). High numbers of SS reticulocytes adhered to TrHBMECs and resisted high shear forces (Fig. 1B). SS reticulocytes express integrin αβ1, that is known to bind to VCAM-1 on activated endothelial cells (23, 24). In the absence of VCAM-1 on resting TrHBMECs, we investigated the role of another ligand, Lu/BCAM, in sustaining this adhesion. This is supported by our previous data showing that endothelial Lu/BCAM interacts with integrin αβ1, on SS reticulocytes (16). SS reticulocyte adhesion was significantly inhibited by incubating TrHBMECs with a blocking anti-Lu/BCAM antibody, but not with an anti-PECAM-1 antibody, indicating that it was mediated at least partially by endothelial Lu/BCAM (Fig. 1B). This result was confirmed by immunofluorescence staining showing a uniform expression of Lu/BCAM on the surface of resting TrHBMECs (Fig. 1C).
To analyze selectively the effect of HC on the Lu/BCAM-\(\alpha_4\beta_1\) interaction in our model, we established a cell line expressing recombinant \(\alpha_4\beta_1\) integrin. Erythroleukemic K562 cells, which express endogenous integrin \(\alpha_5\beta_1\) but not \(\alpha_4\beta_1\), were transfected with a plasmid encoding the human integrin \(\alpha_4\beta_1\) chain. Transfecting K562 cells with \(\alpha_4\beta_1\) chain only was sufficient to express \(\alpha_4\beta_1\) heterodimer at the cell surface because recombinant \(\alpha_4\beta_1\) recruited endogenous \(\beta_1\) chain and was addressed to the cell membrane. Indeed, as indicated by flow cytometry, appropriate expression of \(\alpha_4\beta_1\) integrin at the cell surface was observed: 73.33% of \(\alpha_4\beta_1\)-positive cells versus 7.53% for K562-WT, with a mean fluorescence intensity of 662. Next, we examined the adhesion function of the recombinant \(\alpha_4\beta_1\) dimer by performing adhesion assays with K562-\(\alpha_4\beta_1\) cells on a VCAM-1-coated surface. K562-\(\alpha_4\beta_1\) cells exhibited important and significant adhesion to VCAM-1 compared with K562-WT, with a 7-fold increase of the adhesion level at all shear stresses tested (Fig. 1D). All of these data indicated that expression of recombinant \(\alpha_4\beta_1\) chain led to the expression of a functional \(\alpha_4\beta_1\) dimer at the cell surface. Next, K562-\(\alpha_4\beta_1\) cells were used in adhesion assays on TrHBMEC monolayers and showed significant adhesion and resistance to shear forces compared with K562-WT (Fig. 1E). The adhesion of K562-\(\alpha_4\beta_1\) cells to TrHBMECs was mediated at least by endothelial Lu/BCAM as it was significantly inhibited by the blocking anti-Lu/BCAM antibody but not by the anti-PECAM-1 antibody (Fig. 1F).

**Treating TrHBMECs with HC Inhibits SS Reticulocyte Adhesion**—Next, we examined the effects of HC on Lu/BCAM-mediated SS reticulocyte adhesion to TrHBMECs. Treating the cell monolayers with HC for 24 h prior to the adhesion assays significantly inhibited SS reticulocyte adhesion (Fig. 2A).
HC Activates Endothelial PDE4A Inhibiting Sickle RBC Adhesion

HC Decreases cAMP Levels and Increases PDE4A Expression—In our previous work we have shown that decreased Lu/BCAM phosphorylation in SS RBCs from HC-treated patients was associated with decreased cAMP levels (18). To determine whether HC could have a similar effect on endothelial cells we measured the intracellular concentrations of cAMP in TrHBMECs and found a 1.75-fold decrease (p < 0.02) after 24 h of HC treatment (Fig. 3A). Intracellular cAMP levels are tightly regulated by adenylate cyclase, which converts ATP into cAMP, and PDEs, which hydrolyze it into AMP. In our published microarray data we found that HC did not modulate adenylate cyclase expression but did increase the mRNA levels of the cAMP-specific PDE4A and PDE7A by 2.6- and 3.2-fold, respectively (25). We investigated PDE4A and PDE7A expression on the protein level by Western blotting. We found a significant 1.36-fold increase of PDE4A in HC-treated TrHBMECs (1.36 ± 0.07, p < 0.02, n = 4) (Fig. 4B) together with a slight, but not significant increase of PDE7A (data not shown). The experiments quantifying PDE4A were performed in the linear range of the Western blotting as shown in Fig. 4C. The Western blotting results were in accordance with the data obtained by RQ-PCR with the same cells, which showed increased levels of PDE4A mRNA in the presence of HC (1.45-fold ± 0.28 compared with nontreated cells, p < 0.05, n = 5). RQ-PCR assays were performed also with HPMECs and showed increased PDE4A mRNA levels in HC-treated cells (1.2-fold ± 0.1, p < 0.02, n = 5), indicating that HC had similar effects in primary endothelial cells.

Inhibition of PDE4A Abolishes the Inhibitory Effect of HC—To determine whether the HC-induced overexpression of PDE4A was involved in the inhibition of Lu/BCAM phosphorylation and cell adhesion we performed a series of phosphorylation and adhesion experiments in the presence of the adenylate cyclase activator forskolin (FSK), the PDE general inhibitor IBMX, and the PDE4-specific inhibitor rolipram. The effect of FSK alone, or together with IBMX or rolipram, on Lu/BCAM phosphorylation was determined for both HC-treated and untreated TrHBMEC by quantifying the phosphorylated fraction of Lu/BCAM in all conditions. As already shown in Fig. 3C, incubating the cells with HC inhibited Lu/BCAM phosphorylation (Fig. 5A). The inhibitory effect of HC was evaluated by calculating the phosphorylation index (Pi) of Lu/BCAM that reflects the phosphorylation level of reticulocyte adhesion to resting TrHBMECs this suggested that HC inhibited its interactions with erythroid adhesion molecules. To explore the selective effect of HC on the interaction between Lu/BCAM and integrin αβ1, adhesion assays were performed by perfusing K562-αβ1 cells on TrHBMECs treated or not with HC. K562-αβ1 cell adhesion to HC-treated TrHBMECs was significantly inhibited at all shear forces indicating that the interaction between endothelial Lu/BCAM and erythroid αβ1 integrin was negatively targeted by HC (Fig. 2B).

HC Increases Endothelial Lu/BCAM Expression and Inhibits its Phosphorylation—To address the mechanism by which HC inhibited SS reticulocyte adhesion to endothelial cells, we investigated its effects on Lu/BCAM expression in TrHBMECs, both on the mRNA and the surface protein levels. RQ-PCR experiments showed a slight but significant increase of Lu/BCAM mRNA after 24 h of HC treatment (116.3 ± 2.5% of the control level, p < 0.05) (Fig. 3A). Flow cytometry analysis revealed a 1.77-fold increase (p < 0.05) of Lu/BCAM expression at the surface of HC-treated TrHBMECs compared with untreated cells (Fig. 3B). The overexpression of Lu/BCAM at the cell surface of HC-treated TrHBMECs seemed contradictory with the observed decrease of SS reticulocyte adhesion to these cells. However, we have shown previously that HC had similar effects on erythroid Lu/BCAM expression in SS RBCs, increasing its expression but decreasing its mediated RBC adhesion to its specific ligand laminin 511/521. This decrease was associated with a significant inhibition of Lu/BCAM phosphorylation (18). Therefore, we tested for Lu/BCAM phosphorylation in TrHBMECs in the presence or absence of HC. Lu/BCAM was phosphorylated in resting TrHBMECs and HC treatment led to a 2-fold decrease of this phosphorylation (Fig. 3C), indicating that HC negatively regulated Lu/BCAM phosphorylation in these cells.

We then investigated the same parameters in the primary HPMECs and found comparable results. After 24 h of HC treatment Lu/BCAM mRNA was significantly increased (148 ± 18% of the control level, p < 0.05) (Fig. 3D). This was associated with a 1.2-fold increase in the protein expression level at the cell surface (p < 0.02, Fig. 3E). Similarly to the results obtained in the TrHBMEC model Lu/BCAM phosphorylation was significantly decreased in the presence of HC (p < 0.05, Fig. 3F).

FIGURE 2. Treatment of TrHBMECs with HC inhibits the adhesion of SS reticulocytes and K562-αβ1 cells. Adhesion of reticulocyte-enriched SS RBCs (A) or K562-αβ1 cells (B) on TrHBMECs treated or not with HC for 24 h is shown. Cell suspensions were perfused at 0.2 dyne/cm², and sequential washes were performed from 0.5 to 3 dynes/cm². Adherent cells were counted after each wash. Results are expressed as the mean number of adherent cells/mm² ± S.D. (error bars) from triplicate assays. Unpaired t test; *, p < 0.05; **, p < 0.01, versus not treated (NT).
Lu/BCAM in HC-treated cells compared with nontreated cells (Pi = Lu phosphorylated fraction with HC/without HC). This Pi was of 0.5 ± 0.22 indicating a 2-fold inhibition of Lu/BCAM phosphorylation by HC (Fig. 5B). When HC-treated cells were incubated with FSK there was no significant increase in the phosphorylated fraction of Lu/BCAM (Pi = 0.63 ± 0.21, Fig. 5, A and B), suggesting that HC inhibited adenylate cyclase or its downstream signaling pathways. The latter possibility was consistent with the elevated levels of PDE4A that we measured in HC-treated TrHBMECs. Adding IBMX or rolipram restored the activating effect of FSK (Pi = 0.98 ± 0.19, p < 0.05 and 1.14 ± 0.26, p < 0.05, respectively, Fig. 5, A and B), strongly suggesting that adenylate cyclase was not inhibited in these cells and that HC induced an elevated PDE activity. Adhesion assays were performed with K562-α4β1 cells and TrHBMECs under the same incubation conditions. Similarly to the phosphorylation index, an adhesion index (Ai) for K562-α4β1 adhering to TrHBMECs was calculated (Ai = number of K562-α4β1 adhering to HC-treated/untreated TrHBMECs). Consistent with the phosphorylation results, FSK alone did not significantly activate K562-α4β1 adhesion to HC-treated TrHBMECs (Ai = 0.78 ± 0.038, Fig. 5C). The activating effect of FSK was restored when the cells were co-incubated with rolipram (Ai = 1.17 ± 0.044, p < 0.001, Fig. 5C). These results indicated that the diminished cell adhesion on HC-treated TrHBMECs was most probably because of elevated PDE4A activity.

We further confirmed these results using the primary human endothelial cells and SS reticulocytes. When HC-treated HPMECs were incubated with FSK there was no significant activation of Lu/BCAM phosphorylation (Pi = 0.63 ± 0.27, p = 0.9, Fig. 5D). Next, we analyzed adhesion of SS reticulocytes to HPMECs and found that it was inhibited by HC (Ai = 0.61 ± 0.1, p < 0.02, Fig. 5E). This adhesion was not significantly activated by FSK in HC-treated cells (Ai = 0.95 ± 0.3, p = 0.2, Fig. 5E), which was consistent with the phosphorylation results. Finally, incubating HPMECs with FSK and IBMX and/or rolipram provoked unexpected cell detachment from the culture dish preventing us from determining the phosphorylation index of Lu/BCAM and the adhesion index of SS reticulocytes in the presence of these components.

**DISCUSSION**

Although the primary defect in SCD is the mutated hemoglobin S that drives RBC sickling at low oxygen levels, clinical
manifestations in this disease are not restricted to the erythroid tissue. The pathophysiology of SCD is complex and includes hemolysis, activation of cell adhesion, chronic inflammation, leukocytosis, increased oxidative stress and endothelial dysfunction (26). The majority of these manifestations can be investigated in vitro or ex vivo using cells and soluble factors from patients’ blood samples. One of the major challenges in SCD is the study of endothelial cells because they are not accessible for ex vivo investigations. Therefore, endothelial cells used in such studies are either primary cells or cell lines derived from the macro- or the microcirculation of healthy individuals. Another alternative is the use of SCD mouse models that enable investigations under physiological conditions but do not offer the complete panel of the interactions taking place in SCD because of the differences between mice and human protein expression patterns in the vascular compartment. In this study we used primary human endothelial cells from the lung microcirculation and a human endothelial cell line derived from the bone marrow microcirculation because VOC are particularly common in both tissues, contributing to pulmonary artery hypertension (27) and bone marrow infarction (28, 29). This latter may be caused by marrow hypercellularity that impairs blood flow leading to regional hypoxia (30). In addition, blood hyperviscosity has been suggested as a factor involved in the genesis of osteonecrosis (31), and recently increased RBC deformability was shown to be associated with this clinical manifestation (32).

In our study we analyzed the adhesion of the deformable RBC population by fractionating the blood samples and selecting the RBCs of the low density fraction. We showed that these RBCs had a significant adhesion to TrHBMECs that was mediates by endothelial Lu/BCAM because adhesion was inhibited by anti-Lu/BCAM blocking antibody, although not totally abrogated. The residual adhesion measured in the presence of the blocking antibody might be because of the interaction between other proteins such as erythroid LW/ICAM-4 and endothelial integrin $\alpha_v\beta_3$ (5–7). HC inhibited reticulocyte adhesion to TrHBMECs and HPMECs, and our results with the K562-$\alpha_v\beta_1$ cellular model indicated that the Lu/BCAM-$\alpha_v\beta_1$ interaction was negatively targeted by HC. We showed previously that the Lu/BCAM-$\alpha_v\beta_1$ interaction takes place between SS RBCs and peripheral blood mononuclear cells and is also partially inhibited by HC (33). Erythroid Lu/BCAM was first described in SCD to mediate RBC adhesion to laminin in a cAMP-dependent manner in response to the activation of the $\beta_2$-adrenergic receptor (13). Recently, we studied the effects of HC on SS RBC adhesion and signaling by analyzing blood samples from patients before and at regular intervals during HC treatment. We found a time-dependent decrease of intracellular cAMP levels together with increased Lu/BCAM expression but decreased phosphorylation associated with decreased SS RBC adhesion to laminin (18). The similarities with the data obtained in this study strongly suggest that HC might act through a similar mechanism in SS RBCs, up-regulating PDE4A. This is supported by a strong evidence for the presence of PDE4 in human RBCs because rolipram potentiates the up-regulation of cAMP in response to the $\beta$-adrenergic receptor activation in these cells (for review, see Ref. 34).

HC is a cytostatic agent that inhibits DNA synthesis by inactivating ribonucleotide reductase. HC was primarily administered to SCD patients because it increases fetal hemoglobin production in erythrocytes, thereby inhibiting hemoglobin S polymerization. However, several studies indicate that HC might also act independently from its fetal hemoglobin-inducing property by targeting adhesion molecules on blood cells (18, 35–37) and endothelial cells (22, 25) and by modulating NO production (38). Recently, Almeida et al. (39) investigated the effects of short time exposure to HC (3 h) on VOC in a SCD mouse model under inflammatory conditions. They showed that administration of HC altered leukocyte recruitment to the microvasculature, abrogated endothelial cell activation, and prolonged animal survival. These benefits were mediated by cGMP-dependent mechanisms and were potentiated by the PDE9 inhibitor BAY73-6691 (39, 40). Another study investi-
gated the effects of short-time exposure to HC (up to 3 h) on primary HUVECs and the TrHBMEC cell line used in our study (19). The authors found a dose- and time-dependent activation of endothelial NO synthase by HC through phosphorylation of its serine 1177 in a PKA-dependent manner. Increased levels of cAMP and cGMP were measured within the first 30 min of exposure to HC which were no longer detected after 2 h of incubation (19). In contrast to these high levels measured at 30 min, we showed that long time exposure to HC was associated with decreased cAMP concentrations. Our data suggest that decreased cAMP most probably results from the combined effects of the feedback loop triggered by the cAMP-dependent PKA/Akt activation, leading to PDE phosphorylation and cAMP hydrolysis, and of the enhanced expression of PDE4A (see Fig. 6). PDEs form a large multigenic family of enzymes that hydrolyze cAMP and/or cGMP into their 5’-monophosphate derivatives (41, 42). Eleven families of PDEs have been classified, and among these, enzymes of the PDE4 family are particular by their specificity for cAMP, their sequence homology, and their unique sensitivity to inhibition by rolipram (43). PDE4s are the most highly expressed cAMP-specific PDEs in the endothelium (42, 44). The PDE4 family comprises four members, PDE4A, 4B, 4C, and 4D, encoded by four different genes and each represented by several isoforms that arise from alternative mRNA splicing (43). Gene expression studies performed in human and rodent cells showed that some PDE4 genes are positively regulated in response to an increase of cAMP (43). Consequently, the increased cAMP levels reported by Cokic et al. after a short exposure to HC could be at the origin of the increased PDE4A mRNA levels that we measured after incubating the cells with HC for 24 h (see Fig. 6).

Moreover, the subcellular localization of PDEs is known to be a key mechanism for compartmentalization of cyclic nucleotide signaling which enables to produce highly selective cellular responses to adenylylate cyclase stimulation by G protein-coupled receptors (45, 46). Our experiments measuring cAMP concentrations showed a global 2-fold decrease of cAMP in

FIGURE 5. The activating effect of FSK on Lu/BCAM phosphorylation and mediated adhesion is abrogated by HC and restored by rolipram. A, Lu/BCAM phosphorylation was analyzed in TrHBMECs treated (HC) or not (NT) by HC in the absence (–) or presence of FSK, IBMX, and rolipram (Rol). The top (P) and bottom (WB) panels show the phosphorylation and the total amounts of the immunopurified proteins, respectively. The effects of HC on Lu/BCAM phosphorylation (B and D) and mediated cell adhesion (C and E) were measured in TrHBMECs and HPMECs in the absence (–) or presence of FSK, IBMX, and rolipram. Results are presented as the Pi of Lu/BCAM for each condition (Pi = Lu phosphorylated fraction with HC/without HC) and as the Ai of K562-a2B1 unto TrHBMECs (C) or of SS reticulocytes unto HPMECs (E) (Ai = number of K562-a2B1 or SS reticulocytes adhering to HC-treated/untreated TrHBMECs or HPMECs, at 2 dynes/cm²). Histograms represent mean values ± S.D. (error bars) from triplicate assays. Paired t test; *, p < 0.05; **, p < 0.01 versus FSK alone.

FIGURE 6. Adenylate cyclase-cAMP pathway. Adenylate cyclase is activated by G protein-coupled receptors in response to extracellular stimuli. Adenylate cyclase converts ATP to cAMP which activates serine/threonine kinases PKA and Akt leading to activation of effector proteins such as adhesion proteins at the cell surface. cAMP is hydrolyzed to AMP by phosphodiesterases such as PDE4A whose function is activated by PKA-dependent phosphorylation (a feedback loop controlling cAMP levels). Our results indicate that hydroxy-carbamide treatment is associated with an increase in PDE4A expression.
HC Activates Endothelial PDE4A Inhibiting Sickle RBC Adhesion

TrHBMECs treated with HC. The 1.2-fold increase of PDE4A might not account for this global decrease but seems to be strongly associated with decreased signaling at the cell membrane as revealed by the phosphorylation and adhesion experiments. Indeed, signaling at the cell membrane in response to adenylyl cyclase activation by FSK was abrogated by HC and totally restored by inhibiting PDE4 by rolipram, indicating increased PDE4 activity in this compartment in HC-treated TrHBMECs.

Finally, our study reveals a novel role for HC in resting endothelial cells where it could modulate the function of membrane adhesion proteins through the regulation of PDE expression and cAMP-dependent signaling pathways. It opens new perspectives for future investigations to finely characterize the role of HC in PDE regulation on both the transcriptional and the compartment distribution levels, in erythroid and endothelial cells. Such studies may give new insights into the regulation of PDE expression and activity as therapeutic targets in sickle cell disease.

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