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Research Paper

Identification of a soluble guanylate cyclase in RBCs: preserved activity in patients with coronary artery disease

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ABSTRACT

Endothelial dysfunction is associated with decreased NO bioavailability and impaired activation of the NO receptor soluble guanylate cyclase (sGC) in the vasculature and in platelets. Red blood cells (RBCs) are known to produce NO under hypoxic and normoxic conditions; however evidence of expression and/or activity of sGC and downstream signaling pathway including phopshodiesterase (PDE)-5 and protein kinase G (PKG) in RBCs is still controversial. In the present study, we aimed to investigate whether RBCs carry a functional sGC signaling pathway and to address whether this pathway is compromised in coronary artery disease (CAD). Using two independent chromatographic procedures, we here demonstrate that human and murine RBCs carry a catalytically active $\alpha_1\beta_1$-sGC (isoform 1), which converts ^{32}P-GTP into ^{32}P-cGMP, as well as PDE5 and PKG. Specific sGC stimulation by NO + BAY 41-2272 increases intracellular cGMP-levels up to 1000-fold with concomitant activation of the canonical PKG/VASP-signaling pathway. This response to NO is blunted in α1-sGC knockout (KO) RBCs, but fully preserved in α2-sGC KO. In patients with stable CAD and endothelial dysfunction red cell eNOS expression is decreased as compared to aged-matched controls; by contrast, red cell sGC expression/activity and responsiveness to NO are fully preserved, although sGC oxidation is increased in both groups. Collectively, our data demonstrate that an intact sGC/PDE5/PKG-dependent signaling pathway exists in RBCs, which remains fully responsive to NO and sGC stimulators/activators in patients with endothelial dysfunction. Targeting this pathway may be helpful in diseases with NO deficiency in the microcirculation like sickle cell anemia, pulmonary hypertension, and heart failure.

1. Introduction

Red blood cells (RBCs) were once considered to be little more than supple bags for transport of hemoglobin and other proteins required for gas exchange. Recent translational studies show that RBCs may play additional non-canonical [1], yet fundamental roles in cardiovascular homeostasis by regulating systemic nitric oxide (NO) metabolism, thereby contributing to vascular function and integrity [2–6] as well as cardioprotection [7,8].

One of the main targets of NO signaling is the NO-sensitive soluble isoform of guanylate cyclase (sGC; GTP-pyrophosphate lyase [cyclizing], E.C. 4.6.1.2), which catalyzes the conversion of GTP into the second messenger cyclic GMP (cGMP). On turn cGMP allow signal transduction to downstream targets [9]; these include cGMP-specific phosphodiesterase (PDE) − 5, breaking down cGMP (i.e. “shutting down” the signal) as well as protein kinase G (PKG), transducing the signal further downstream, leading for example to modulation of vascular tone, cardiac contractility, or inhibition of platelet aggregation.

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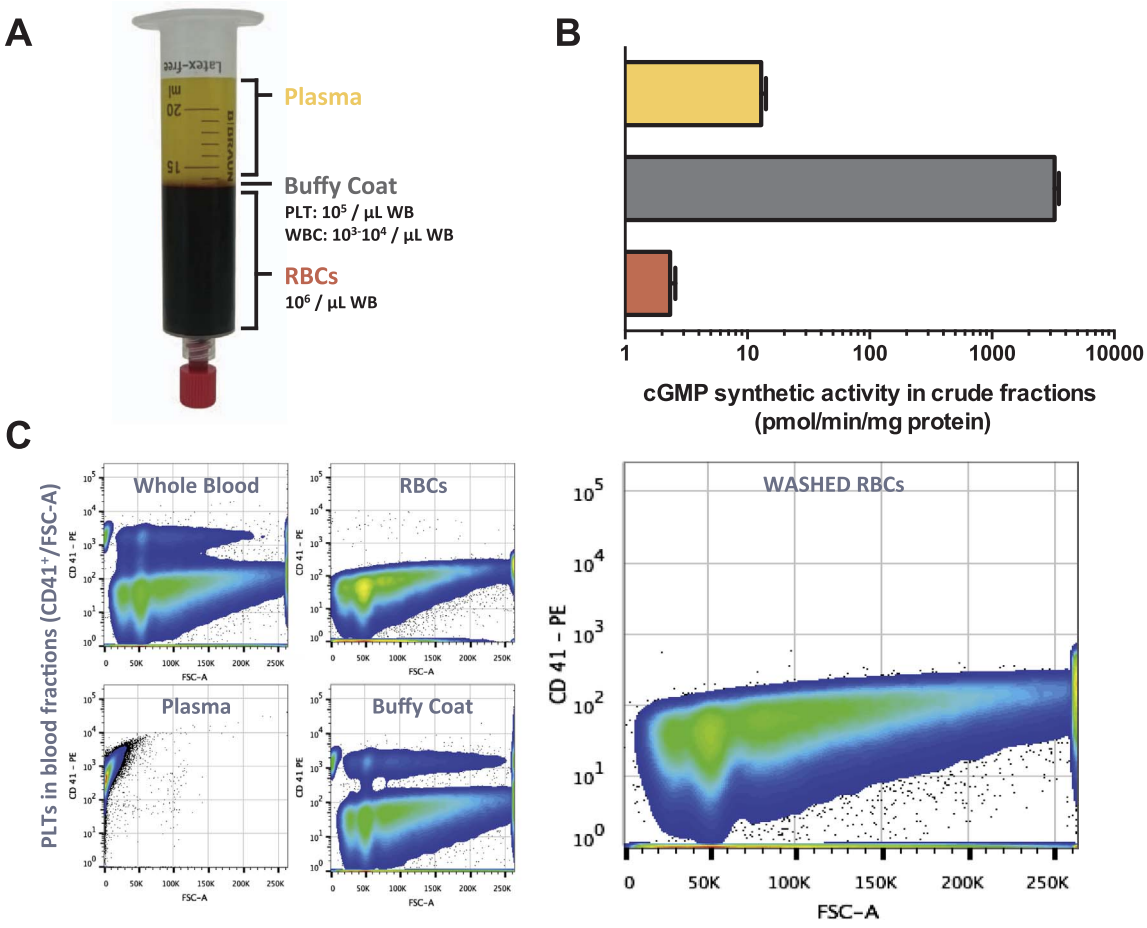

Fig. 1. NO-dependent cGMP-forming activities differ markedly between blood compartments. (A) Representative picture of whole blood after fractionation by centrifugation. **(B)** Rate of NO-induced catalytic conversion of GTP into cGMP in crude lysate from blood fractions; sGC activity was induced by stimulation with DEA/NO (200 µM) and YC-1 (100 µM) for 30 min at 37 °C in the presence of the PDE inhibitor IBMX (1 mM), and cGMP was assessed by radioimmunoassay in triplicate. Data are representative of 5 independent preparations. **(C)** Purification of RBC fraction from platelet (PLT) contamination by centrifugation. The presence of platelets in whole blood, in the crude fractions (RBCs, buffy coat and plasma) and the washed RBC fraction was assessed by analyzing percentage and size of $CD41^+$ events by flow cytometry in a fluorescence vs. forward scatter (FSC) dot plot. Left panels: presence of platelets ($CD41^+$ events) in whole blood and in the buffy coat. In plasma, some smaller cellular $CD41^+$ components (smaller platelets and microparticles) are also present. Right panel: after three wash steps at lower speed (300 × g), the RBC fraction is free from platelet contamination. Threshold count within the RBC population: 100,000 events.

The term “soluble” GC was introduced to differentiate the NO-sensitive sGC from the particulate/membrane-bound GCs, which are transmembrane receptors activated by natriuretic peptides. Structurally, the sGC enzyme exists in two independent isoforms with indistinguishable enzymatic activity, containing either $\alpha_1\beta_1$- or $\alpha_2\beta_1$-subunits, also known as GC-1 and GC-2, respectively [10,11]. While the $\alpha_1\beta_1$-type sGC is almost ubiquitous and localized mainly in the cytoplasm, $\alpha_2\beta_1$-sGC is expressed in specific tissues including the brain and the vasculature [10].

Together with the endothelial isoform of NO synthase (eNOS), sGC plays a key role in the regulation of cardiovascular homeostasis by participating in the control of vascular tone [10,12] and cardiac function [13], rendering it a promising pharmacological target in cardiovascular disease [14,15]. In the blood, sGC plays a central role in regulating platelet aggregation and hemostasis [16–18], while in the bone marrow NO-mediated sGC activation regulates the committed differentiation of erythroid cells [19,20], mainly via cGMP-dependent activation of the transcription factor GATA2, which regulates the expression of fetal hemoglobin [19,20]. The presence of sGC in mature RBCs was proposed two decades ago by Petrov and Lijnen [21], who observed that treating RBC suspensions with NO donors increase intracellular cGMP-levels and affect H^+/Na^+ transport [21]. Increase in cGMP-levels were observed in patients with sickle cell anemia [22,23]; cGMP was also proposed to affect membrane fluidity [24] and symmetry [25], as well as RBC deformability [26].

The above findings are not without controversy, and to date attempts aimed to assess a sGC catalytic activity in RBC lysates remain unsuccessful [17]. Indeed, changes in cGMP-levels in RBCs may also be dependent on the cAMP/cGMP cross-talk regulated by PDEs [27], regulation of cGMP export [28,29] or the activity of particulate GC rather than sGC signaling [21]. However, a functional role for a cGMP-dependent pathway in RBCs is corroborated by the fact that mice lacking cGMP-dependent protein kinase (cGK1 or protein kinase G, PKG1) are anemic [30,31], though the presence of PKG in these RBCs was questioned recently [31]. To the best of our knowledge, no proteomic studies ever confirmed that sGC exists in mature RBCs.

Based on these observations, we hypothesized that RBCs may carry sGC, initiating the canonical cGMP-signaling cascade. Using two independent chromatographic procedures to enrich sGC and other soluble cytoplasmic proteins and to remove hemoglobin from crude RBC preparations, we were able to provide conclusive evidence that RBCs carry a catalytically active sGC, regulate intracellular cGMP-levels and activate PKG-dependent phosphorylation in a PDE5-dependent fashion. NO responsiveness is blunted in $\alpha_1\beta_1$-sGC KO mice, but is preserved in $\alpha_2\beta_1$-sGC KO, indicating that RBCs carry the isoform 1 of sGC. Moreover, sGC activity is preserved under conditions of decreased NO bioavailability like in eNOS KO mice and in patients with CAD and endothelial dysfunction. Therefore, the proteins belonging to the sGC/PDE/PKG pathway in RBCs may be considered as drugable targets in diseases with reduced NO availability. This may hold promise also in

sickle cell anemia [19,32] or pulmonary hypertension [33], which are well-characterized diseases with NO-dependent microcirculatory dysfunction.

2. Methods

Please refer to Supplementary information (SI) for more details.

2.1. Reagents and materials

Unless otherwise specified, all chemicals were of the highest purity available purchased from Sigma-Aldrich Co. LLC. (Deisenhofen, Germany). Water was Millipore quality (EMD Millipore Corporation, San Diego, CA, USA). Materials for Western blotting were purchased from Life Technologies (Invitrogen, Darmstadt, Germany), cell culture material from GE Healthcare GmbH (Solingen, Germany). BAY 41-2272 and BAY 60-2270 were generously provided by Bayer Pharma AG (Wuppertal, Germany).

2.2. Study subjects

Venous blood was obtained from healthy subjects (25–35 years old), patients with CAD (n = 20, age 59 ± 10 years), and age-matched control subjects (n = 20, age 54 ± 10 years) (**SI** Table S3). Specific red cell sGC activity was assessed in a randomized subset (n = 6 / group). Flow-mediated dilation (FMD) and glyceryl trinitrate-induced dilation were measured by high-resolution ultrasound [34]. Procedures were conducted in accordance with the Declaration of Helsinki and approved by the ethic committee of the Heinrich Heine University (Study-ID 4460; Trial registration #NCT02265016, www.clinicaltrials.gov). All authors have access to primary clinical trial data on request.

2.3. Assessment of NO-induced cGMP-forming activity and sGC expression in blood fractions

Blood fractionation procedure is described in Figs. 1A and 2A (see also SI); sGC catalytic activity was assessed in triplicate in each fraction as NO-induced cGMP formation by radioimmunoassay, as well as by direct conversion of ^{32}P-GTP into ^{32}P-cGMP [35] (Fig. 2B inset). The entire procedure was repeated 3-times using blood from three donors. Proteins were separated on a SDS-PAGE and stained with Coomassie blue, or analyzed by Western blot analysis as described [2,3]. Identity of sGC was confirmed by reverse-phase liquid chromatography – tandem mass spectrometry (LC−MS/MS) analysis on Ultimate 3000 RSLCnano system coupled to an Orbitrap Elite mass spectrometer, using Proteome Discoverer Software for ver. 1.4.1.14 peptide identification (Thermo Fisher Scientific, Bremen, Germany). Mass tolerance was set to 5 ppm for precursor ions and 0.4 Da for fragment ions, and false discovery rate to < 1% (q-value < 0.01).

2.4. Measurement of sGC activation and phosphorylation of VASP in intact RBCs

RBCs were pre-incubated for 10 min at 37 °C in Ca^{2+}-containing HBSS commercial buffer (HBSS+; Thermo Fisher Scientific) under gentle shaking in the absence or presence of the PDE inhibitor sildenafil (100 μM) and/or of the sGC-inhibitor ODQ (5 μM). Afterwards, RBCs were treated with 200 μM DEA/NO, 10 μM BAY 41-2272, 10 μM BAY 60-2770 or vehicle control (DMSO) and incubated for 10 min at 37 °C (or as indicated). RBCs were pelleted by centrifugation and snap frozen in liquid nitrogen. The cGMP and cAMP concentrations were assessed by radioimmunoassay [36]. Phosphorylation of VASP at Ser239 was analyzed in Hb-depleted and concentrated extracts by Western blot analysis as described [37].

2.5. Enzymatic assays of red cell sGC, PDE and PKG activity

Activity of sGC, PDE, and PKG were assessed in Hb-free extracts prepared by using HemoVoid™ (Biotech Support Group LLC, Monmouth, NJ, USA) respectively by analysis of formation of cGMP by radioimmunoassay [36], by analyzing the conversion of ^{32}P-cGMP to guanosine and ^{32}P-phosphate in the presence of alkaline phosphatase [10] and by using a cGK Assay Kit (Cyclex Co., Ltd., Nagano, Japan), and activities were normalized by protein content.

2.6. Statistical analysis

If not differently specified, the results are given as mean ± standard error of the mean (SEM). Statistical significance was tested by using GraphPad Prism 6.0 (Graphpad Software Inc., La Jolla, CA, US) or with SPSS 23 (IBM, Armonk, NY, US). Multiple comparisons were tested with ANOVA followed by an appropriate *post hoc* test as indicated in the figure legends. Where indicated, paired or unpaired Student's *t*-test was used to determine if two groups of data were significantly different. Only groups with similar variance were statistically compared. Outliers were identified graphically by analyzing box & whiskers plots according to Tukey. Normal distribution was tested by D'Agostino-Pearson test. $p < 0.05$ was considered as statistically significant. The sample size was chosen based on our previous in vitro and in vivo studies.

3. Results

3.1. NO-dependent cGMP-forming activities differ markedly among blood compartments

Aiming to verify whether RBCs carry an active sGC we began by screening crude blood fractions, obtained by differential centrifugation of whole blood, for their ability to produce cGMP in response to NO (Fig. 1A,B). For this purpose crude homogenates of each fraction were treated with the NO donor DEA/NO (200 μM) and the sGC stimulator YC-1 (100 μM) in the presence of the PDE inhibitor IBMX (1 mM). As expected, the highest NO-stimulated cGMP-forming activity (3000 pmol/min/mg protein) was found in the buffy coat, the fraction containing platelets and leukocytes (Fig. 1C-panel "buffy coat"); platelets are known to have abundant sGC expression and activity [17]. Much lower NO-stimulated cGMP-forming activity was detected in plasma (Fig. 1B), which contains some platelets and platelet-derived microparticles [38] (Fig. 1C–panel "plasma"). In the crude RBC fraction NO-stimulated cGMP-synthesis was very low or not detectable (Fig. 1B). However, the relationship between NO-dependent cGMP-synthesis and dilution of RBC lysate (and thus protein content) was non-linear, suggesting the presence of an inhibitory contaminant. We suspected hemoglobin, which is present in high concentrations in RBC lysates (90–100 mg/ml; $30.99 \pm 0.12 \times 10^{-12}$ g/cell; > 97% of dry weight [39], as also evidenced by the intense rubin red color), to be the contaminant preventing sGC stimulation by NO during the assay.

3.2. Human RBCs carry a soluble guanylate cyclase

To investigate whether human RBCs contain a functional sGC, it was therefore necessary to assure that the RBC fraction was free from platelet contamination, and to remove hemoglobin from the cell lysate. Platelets were fully removed from intact RBC suspensions by repeated, low-speed centrifugation steps, as verified by counting CD41-positive ($CD41^+$) events on a total of 100,000 events by flow cytometry (Fig. 1C–panel "washed RBCs"). RBC lysates devoid of hemoglobin were obtained as depicted in Fig. 2A by ion exchange chromatography [35], followed by a concentration step. In the sGC-enriched fraction we measured a sGC specific activity of 50–200 pmol cGMP/min/mg protein, as assessed by radioimmunoassay in four independent preparations (Fig. 2A) or by measuring direct conversion of ^{32}P-GTP into ^{32}P-

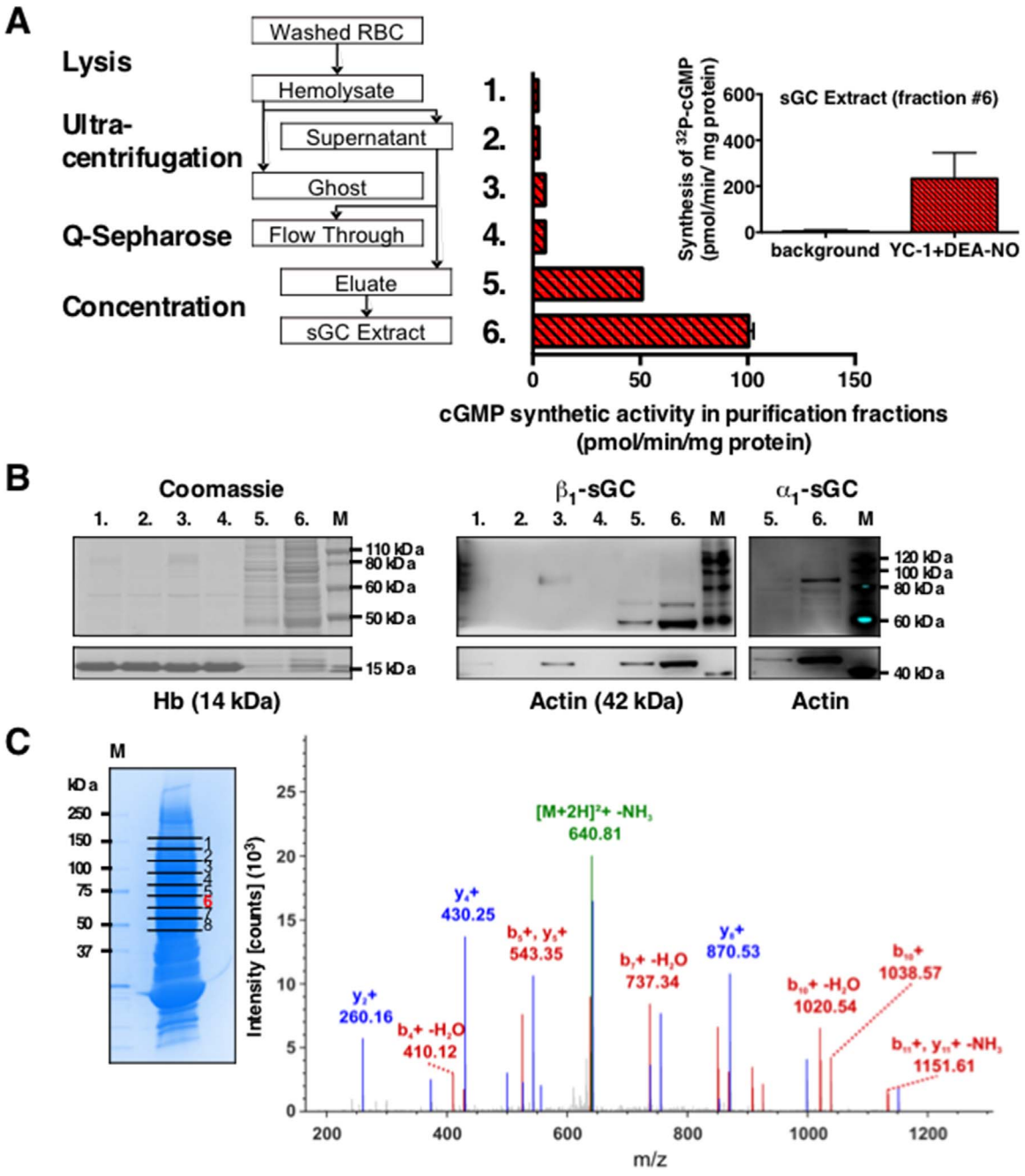

Fig. 2. Identification of a sGC in human RBCs. (A) Procedure for extraction and partial purification of a red cell sGC from human RBC lysates by ion exchange chromatography (left panel). Catalytic sGC activity in each fraction (numbered 1–6) as assessed as NO-induced formation of cGMP by radioimmunoassay (representative of 5 independent preparations; right panel). **Inset:** catalytic sGC activity in RBC extracts (fraction 6) as assessed in duplicate by direct conversion of ^{32}P-GTP into ^{32}P-cGMP (n = 5). **(B)** Analysis of protein composition of the purification fractions (#1–6) shows loss of hemoglobin (Hb, 14 kDa) and enrichment of soluble cytoplasmic proteins, including the α_1- and β_1-subunits of sGC as demonstrated by SDS-PAGE (stained with Coomassie blue) and Western blot analysis. Data representative of 5 independent preparations. **(C)** Mass spectrometric identification of the sGC β_1-subunit (UniProt #Q02153) by LC-MS/MS analysis. Left panel: SDS-PAGE (100 μg), the black lines represent the cut-out regions. Two distinct peptides belonging to the sGC β_1-subunit could be identified in gel band #6 representing a mass area of 70–75 kDa. The precursor ions are depicted in green, *b* ions in red and *y* ions in blue. Please refer to SI Tab. S1, S2 for further information.

cGMP (Fig. 2A-inset). Since the assessment of activity in crude hemolysates in the presence of hemoglobin was not quantitative, purification yields of sGC from hemolysate can only be estimated to be on the order of 1,000-fold (from 0.07 to 100 pmol/min/mg protein). Enrichment of soluble proteins in the eluate, and loss of hemoglobin in the flow-through of the chromatographic procedure can be observed on a SDS-PAGE stained with Coomassie blue (Fig. 2B, left panel; Fig. S1). The presence of both α_1- and β_1-subunits of sGC in the eluate was demonstrated by Western blot analysis (Fig. 2B, right panel); the presence and identity of the β_1-chain of sGC was confirmed by ESI-MS/MS analysis (Fig. 2C, see Table S1, S2 for details on peptide matches). Taken together, these results demonstrate that human RBCs carry a catalytically active sGC isoform 1, composed of α_1- and β_1-subunits.

3.3. NO activates sGC and elicits cGMP-dependent signaling in intact human RBCs

Next, we aimed to assess NO-induced increases in cGMP-levels in intact RBCs. For that purpose, human RBC suspensions were treated with NO donors with or without PDE inhibitors, and changes in

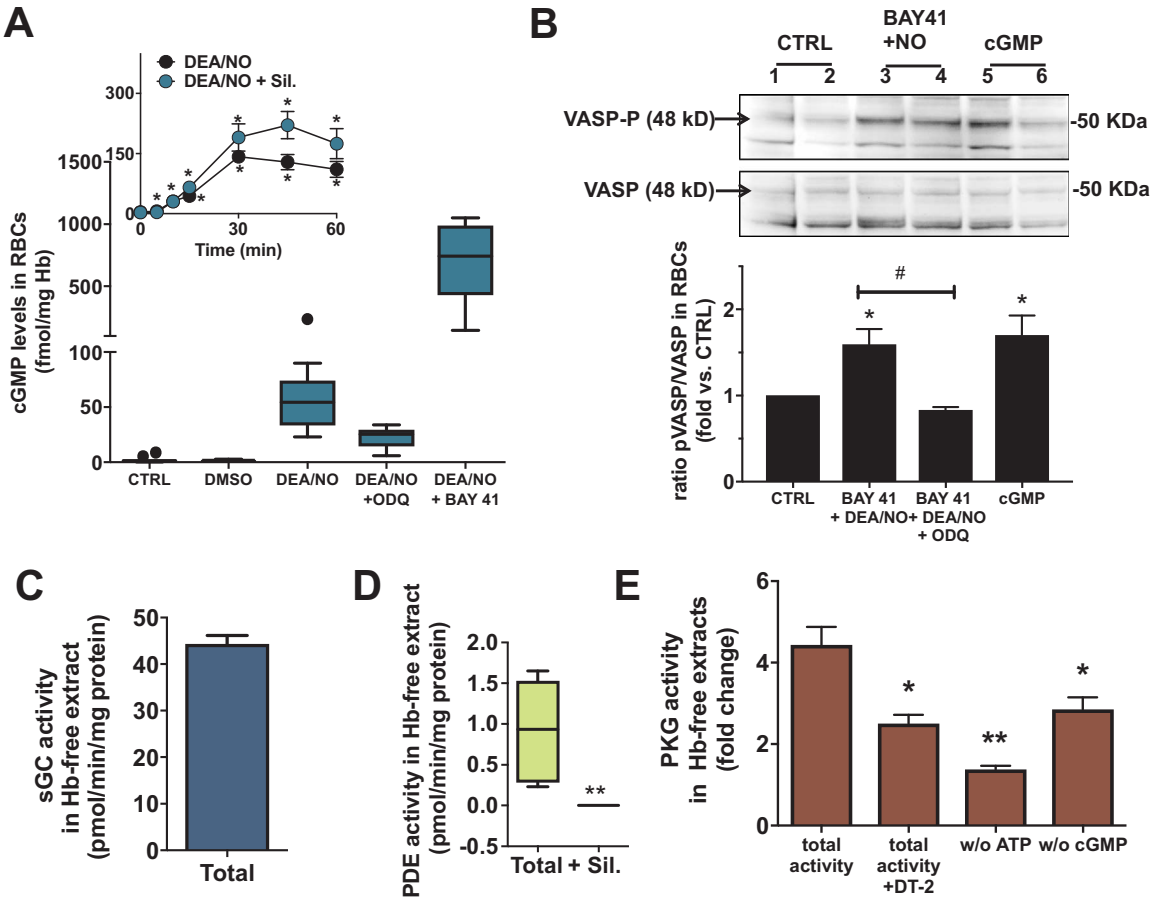

Fig. 3. Functional activation of sGC in human RBCs. (A) Treatment with DEA/NO (200 μM) activates red cell sGC and increases cGMP-levels in intact RBCs as assessed by radioimmunoassay, and it is blocked by the sGC inhibitor ODQ (5 μM). Treatment with the sGC stimulator BAY 41-2272 (10 μM) + DEA/NO (200 μM) increased the cGMP concentrations in RBCs of 500 - 1000 folds (n = 10; RM 1-way ANOVA p = 0.0012; Tukey's * p < 0.05, ** p < 0.01, *** p < 0.001; # paired *t*-test p = 0.0338). **Inset:** Time-dependent increases in intracellular cGMP-levels in intact human RBCs treated with 200 μM DEA/NO in the absence or presence of the PDE5 inhibitor sildenafil (Sil., 100 μM; n = 6; RM 1-way ANOVA p < 0.0001; Dunnett's vs. untreated t = 0, * p < 0.05). **(B)** Treatment of intact RBCs with DEA/NO (200 μM) + BAY 41-2272 (10 μM) increases VASP phosphorylation at Ser239 as compared to untreated control (CTRL), and is blocked by addition of ODQ (5 μM). Treatment with 100 μM 8-pCPT-cGMP increased VASP phosphorylation. Densitometry is the results of 2 independent experiments carried out with the blood collected from three independent donors and treated as indicated. In the Western blot samples obtained by two independent donors were treated and loaded in parallel (Donor 1, lanes 1, 3, 5, 7; Donor 2, lanes 2, 4, 6, 8). In each lane were loaded 40 μg Hb-free RBC lysates **(C)** sGC catalytic activity (n = 3) in 50 μg hemoglobin (Hb)-depleted cytoplasmatic preparations of human RBCs as assessed by radioimmunoassay **(D)** PDE5 catalytic activity of PDE5 in 50 μg Hb-free extracts of human RBCs as assessed by radioactive conversion (n = 6). **(E)** PKG catalytic activity in 50 μg Hb-free extracts of human RBCs (n = 12; RM 1-way ANOVA p < 0.0001; Dunnett's vs. total activity, *p < 0.001, **p < 0.0001).

intracellular cGMP-levels were assessed by radioimunoassay (Fig. 3). Indeed, the NO donor DEA/NO (200 μM) induced time-dependent changes of intracellular cGMP (maximum 200-fold over control), as shown in the inset of Fig. 3A. Similar effects were observed with other NO donors (Sper/NO 1–500 μM) and nitrosothiols (SNAP 1–500 μM). The relatively high concentrations of NO donors required to demonstrate a clear cGMP increase are explained by the reaction of NO with oxyhemoglobin (10 mM heme within RBCs) to form methemoglobin, which can be easily followed by the characteristic color change from red to brown. Moreover, intracellular cGMP-levels in RBCs were dependent on the activity of the cGMP-specific PDE5, as shown by treatment with the PDE5 inhibitor sildenafil (Fig. 3A-inset). Addition of the sGC inhibitor ODQ reduced the NO-dependent rises in intra-erythrocytic cGMP-levels (Fig. 3A–DEA/NO + ODQ), confirming that cGMP increases were due to sGC activation. Moreover, we found that the sGC-specific modulator BAY 41-2272, which is known to sensitize sGC towards NO via stabilization of the nitrosyl-heme complex, induced up to 1000-fold increases in cGMP-levels and strongly potentiated the effects of DEA/NO (Fig. 3a–DEA/NO + BAY 41). Functionally, the cGMP increases in RBCs induced by DEA/NO + BAY 41-2272 led to activation of PKG, as monitored by the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser239 (Fig. 3B). Although the occurrence of cGMP-inhibited PDE3 in RBCs and cGMP-mediated increase in cAMP in RBCs has been reported [27], in our hands cGMP increases did not affect cAMP levels (Fig. S2). Using a commercial resin (HemoVoid™), which removes hemoglobin (and partially carbonic anhydrase)[40] and allows enrichment of soluble cytoplasmic proteins, we established a procedure that allows fast and reliable preparation of hemoglobin-free cell lysates from as little as 1–2 ml blood. In those samples, expression and activity of the cGMP-generating sGC, cGMP-hydrolyzing PDE5 and cGMP-transducing PKG was assessed by enzymatic assays and Western blot analysis (Fig. 3C, D, E). Of note, sGC specific activity in these preparations was lower as compared to sGC preparations obtained by ion exchange chromatography (Fig. 1B), optimized for extraction and enrichment of sGC (44 pmol/min/mg in HemoVoid™ eluates vs. 50–200 pmol/min/mg in eluates form ion exchange chromatography).

It was proposed that PKG activation may participate in control of RBC membrane properties and RBC clearance; in fact, RBCs from PKG KO mice are less deformable, and mice show splenomegaly [30,31]. We here find that treating RBC suspension with low NO concentrations in the presence or in the absence of BAY 41-2272 lead to inhibition of membrane scrambling and exposure of phosphatidylserine induced by Ca^{2+} overload and NEM-mediated alkylation of critical thiols (SI Fig. S3). However, treatment with stable cGMP did not block the Ca^{2+}/

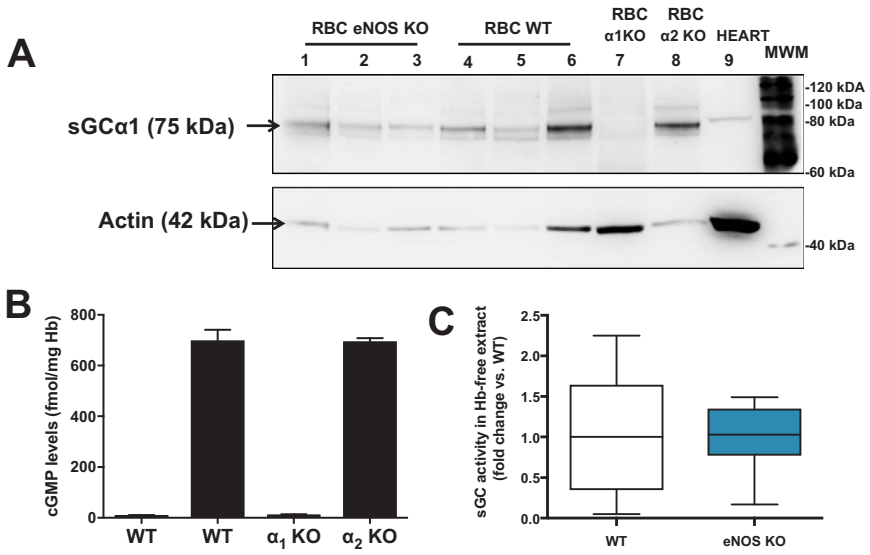

Fig. 4. Expression and activity of red cell sGC is blunted in RBCs from α_1-sGC KO mice but preserved in α_2-sGC KO and eNOS KO mice. (A) Western blot analysis of whole RBC lysates (775 μg, containing Hb) show the presence of the α_1-subunit in RBCs from WT mice, and its absence in RBCs from α_1-sGC KO mice; sGC expression is preserved in RBCs from eNOS KO mice. Heart lysate (H, 10 μg) from WT mice; molecular weight marker (M). Representative of n = 3 independent experiments. **(B)** Treatment of RBC suspensions with 200 μM DEA/NO and 10 μM BAY 41-2272 increases intracellular cGMP-levels in RBCs from WT mice; the NO-mediated cGMP response is blunted in RBCs from α_1-sGC KO (α_1 KO) mice, and conserved in RBCs from α_2-sGC KO mice (α_2 KO; n = 3). **(C)** Specific sGC catalytic activity measured as cGMP formation in response to DEA/NO and BAY 41-2272 in Hb-depleted RBC lysates is also conserved in eNOS KO mice (n = 9, WT: n = 9), shown as normalized data in fold change vs. WT.

NEM-induced membrane scrambling (SI Fig S3), or affect RBC deformability (SI Fig S4) in our experimental conditions, indicating that these ex vivo experiments do not fully reproduce the in vivo situation.

Collectively, these results demonstrate that stimulation of sGC by NO leads to cGMP increases and activation of the canonical sGC-downstream signaling pathway in intact human RBCs.

3.4. NO responsiveness is blunted in RBCs from α1-sGC KO mice but preserved in RBCs from eNOS KO mice

Next we analyzed expression, catalytical activity and NO-dependent increases in cGMP-levels in RBCs from wild type mice, and mice lacking the α_1-subunit of sGC (and thus $\alpha_1\beta_1$-sGC, isoform 1) or α_2-sGC (and thus the $\alpha_2\beta_1$-sGC, isoform 2). We found that RBCs from α_1-sGC KO mice lack both α_1 and β_1 subunits in RBCs as determined by Western blots of Hb-free cytoplasmic extracts (Fig. 4A-upper panel–lane α_1-sGC KO), while RBCs from α_2-sGC KO carried both α_1 and β_1 subunits (Fig. 4A-upper panel-lane α_2-sGC KO). Interestingly, treatment with DEA/NO + BAY 41-2272 failed to increase cGMP-levels in RBCs from α_1-sGC KO mice, but produced similar cGMP-levels in RBCs from α_2-sGC KO (Fig. 4A-bottom). In addition, expression and catalytical sGC activity is fully preserved in eNOS KO mice (Fig. 4B), when compared to WT-RBCs. Collectively, these experiments demonstrate that human and murine RBCs both carry a NO-sensitive $\alpha_1\beta_1$-sGC (isoform 1) and not $\alpha_2\beta_1$-sGC (isoform 2), whose expression and activity remains fully preserved following complete loss of eNOS as intracellular enzymatic NO source in RBCs [3].

3.5. Activity and NO responsiveness of sGC is preserved in RBCs from patients with endothelial dysfunction and CAD

Next, we explored the activity of sGC in RBCs from patients with CAD (see Table S3 for clinical characteristics of the study population). CAD patients typically present a significantly decreased FMD, but unaltered glyceryl trinitrate-induced vasodilation (Fig. 5A), indicating decreased endothelium-dependent vasodilation with conserved NO-mediated vascular smooth muscle responses. In RBCs obtained from these individuals we found that the expression of red cell eNOS is decreased (Fig. S6), confirming our and other groups' previous observations [3,41]. Of note, the specific sGC catalytic activity assessed in hemoglobin-free cytoplasmic extracts from RBCs (Fig. 5C) is fully preserved in CAD patients and did not differ when compared to RBCs from the age-matched control group. Yet, DEA/NO + BAY 41-2272 increased cGMP-levels to a greater extent in intact RBCs from CAD patients as compared to aged-matched controls (Fig. 5B). Hypersensitivity for NO-mediated vasorelaxation [42,43] under conditions of abrogated NO production by eNOS was described for endothelium-denuded vessels [42] and vascular tissue of eNOS KO mice [43]. Taken together, not only NO donor-dependent vascular function, but also the functionality of red cell sGC is fully preserved in this patient cohort with CAD.

3.6. RBCs from CAD patients and aged-matched controls carry low levels of NO insensitive oxidized/heme-free sGC

In CAD patients the pool of oxidized/heme-free sGC in platelets is significantly increased as a consequence of oxidative stress [18]. To explain why RBCs from CAD patients have a comparable response to NO as aged-matched healthy controls, we analyzed RBC redox status and responsiveness of red cell sGC to the sGC activator BAY 60-2770, a compound that specifically targets the inactive, oxidized/heme-free form of sGC [44]. The intracellular redox status of RBCs did not differ between groups, as assessed by measuring GSH by enzymatic assay (Fig. 5D), or free reactive thiols by ThiolTracker (Fig. S7). Interestingly, BAY 60-2770 increased cGMP in RBCs from CAD patients and age-matched healthy individuals to about the same extent (Fig. 5E). By comparison, in RBCs from young healthy volunteers BAY 60-2770 did not significantly increase cGMP-levels (Fig. 5F) unless cells were pre-incubated with ODQ, which promotes oxidation of the heme iron of sGC with subsequent loss of the heme-group. Taken together, these results demonstrate that very low levels of oxidized/heme-free sGC are present in RBCs of both CAD and age-matched controls.

4. Discussion

The role of RBCs in scavenging, transporting and metabolizing NO is now well established [1]. In this work we aimed to analyze whether RBCs also sense and respond to NO via activation of sGC-dependent pathways and effectors, and whether those pathways are affected by clinical conditions associated with compromised endothelial function due to impaired NO availability. Although it has been shown that treatment with NO donors increases in cGMP in mature RBCs [19,21], and a sGC is known to be present in erythroid precursor cells [19], to the best of our knowledge evidence of sGC protein expression and/or catalytical activity in mature RBCs have not being presented so far [17]; instead, doubts about a functional significance of cGMP-dependent pathways in RBCs have been put forward recently [30,31].

We here demonstrate that i) human and murine RBCs carry catalytically active $\alpha_1\beta_1$-sGC (isoform 1), PKG and PDE5; ii) NO-stimulation

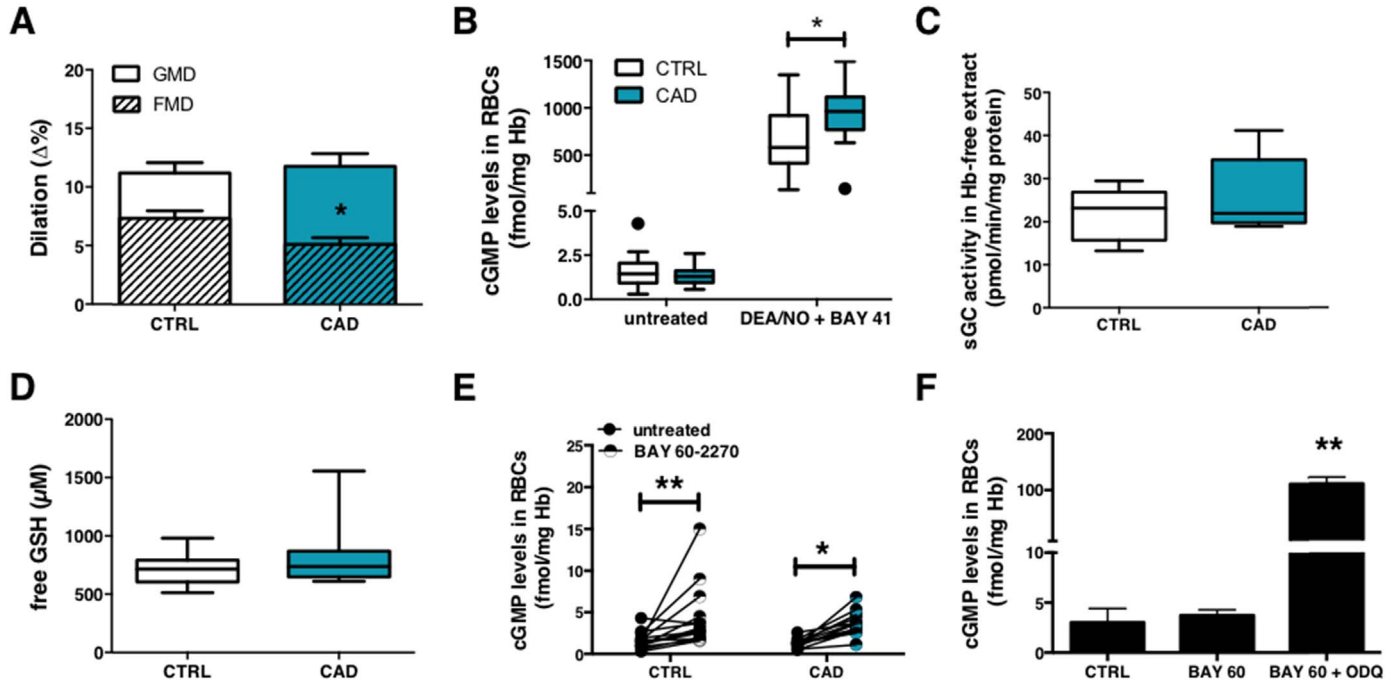

Fig. 5. Activity, expression and redox state of sGC is fully preserved in RBCs of CAD patients. (A) Patients with coronary artery disease (CAD) and endothelial dysfunction show decreased flow-mediated dilation (FMD; CAD: n = 27, CTRL n = 25) and conserved glyceryl trinitrate-mediated dilation (GMD; CAD: n = 22; CTRL n = 19) as assessed by high-resolution ultrasound (FMD CAD vs. CTRL, Sidak * p = 0.0122; see **SI Tab. S3** for patient characteristics and SI for exclusion criteria). **(B)** 10 μM BAY 41-2272 + 200 μM DEA/NO in the presence of 300 μM IBMX increase cGMP-levels in intact RBCs of CAD patients to a greater extent as compared to aged-matched controls, as assessed by radioimmunoassay and normalized for Hb content (CAD: n = 12, CTRL: n = 14; 2-way ANOVA CAD vs. CTRL $p < 0.001$; Sidak's * $p < 0.05$). **(C)** Specific sGC catalytic activity measured as cGMP formation in response to DEA/NO and BAY 41-2272 in Hb-depleted RBC lysates is conserved in patients with CAD (n = 6, CTRL: n = 6). **(D)** The levels of free GSH in RBCs in patients with CAD are not different from aged-matched controls (CAD: n = 9, CTRL: n = 14). **(E)** The oxidized/heme-free sGC activator BAY 60-2770 increases intracellular cGMP-levels in RBCs from patients with CAD and aged-matched healthy controls to a similar extent, indicating that RBCs from both groups carry a small amount of oxidized/heme-free sGC (CAD: n = 11, CTRL: n = 14; 2-way ANOVA treatment p = 0.0001; Sidak's BAY 60 vs. CTRL * $p < 0.05$; ** $p < 0.01$). **(F)** The sGC activator BAY 60-2770 increases intracellular cGMP up to 200-fold only following preincubation of RBCs from healthy young volunteers with ODQ, which oxidizes Fe^{2+} to Fe^{3+} and leads to formation of heme-free sGC (n = 6, Friedman test p = 0.0055; Dunn's ** p 0.01).

increases cGMP-levels in intact RBCs in a sGC and PDE-dependent manner, inducing canonical PKG/VASP-dependent signaling; iii) NO responsiveness of sGC is blunted in α_1-sGC KO mice, but not in α_2-sGC KO and eNOS KO mice; and that iv) red cell sGC activity is fully preserved in patients with stable CAD. Future studies specially focusing on changes in perfusion within the microcirculation should determine the effects of pharmacological therapies with sGC stimulators and activators, or PDE5 inhibitors on RBC signaling, which may allow to overcome decreased NO availability and sGC activation in patients with established NO-dependent dysfunction of the microcirculation such as sickle cell anemia, heart failure, pulmonary hypertension [33].

4.1. Identification of a NO-sensitive sGC in RBCs

It is well known that platelets are the blood compartment containing the bulk of sGC activity, as also shown here by our fractionation experiment. Therefore, to demonstrate that RBCs carry an active sGC, it was necessary to exclude platelet contamination from RBC preparations; this was achieved here by serial low-speed centrifugations and by analyzing the presence of $CD41^+$ events by counting at least 100,000 cells by flow cytometric analysis. In addition, RBC lysates contain millimolar concentration of hemoglobin, which is known to limit the sensitivity of methods for protein detection, like Western blot or LC-MS/MS, and several other assays [45]. In the present study, we succeeded in demonstrating that RBCs carry an active sGC by applying two independent methods to prepare hemoglobin-free, sGC enriched extracts. We first applied an optimized protocol for classical sGC protein enrichment based on anion exchange chromatography, and obtained an extract with a sGC-specific activity of 50–200 pmol/min/mg; thereafter, we applied a method designed to remove hemoglobin and enrich soluble cytoplasmic proteins based on exclusion affinity chromatography and found a sGC specific activity of 44 pmol/min/mg. Although the specific activity of sGC in the extracts obtained by the latter method is somewhat lower than in preparations obtained by ion exchange chromatography, this method enabled us to readily assess (in a relative low volume of RBC lysate) both expression and activity of PDE5 (reported using another method earlier [10]) and importantly PKG, whose presence in RBCs was recently questioned [30,31].

The existence of both the α_1- and β_1-subunits of sGC in human and murine RBCs and their absence in RBCs from α_1-sGC KO mice was demonstrated by Western blot analysis, and by confirming the presence of the β_1-subunit by LC-MS/MS in human samples. Interestingly, RBCs of α_2-sGC KO mice show a fully preserved NO-induced cGMP response, and carry both α_1- and β_1-subunits of sGC. Moreover, we could not find any evidence for the presence of the α_2 subunit in RBC extracts by any of the techniques applied.

These results collectively show that human and murine RBCs carry a catalytically active NO-sensitive sGC, which comprises the $\alpha_1\beta_1$-heterodimer (sGC isoform 1).

4.2. Red cell sGC signaling in RBCs

The functional role of red cell sGC in controlling cGMP-levels in RBCs was tested by treating human and mouse red cell suspensions with NO donors, the specific sGC stimulator BAY 41-2272, and the sGC inhibitor ODQ, in the presence or absence of the specific PDE5 inhibitor sildenafil; these results were confirmed using RBCs harvested from α_1-sGC KO and α_2-sGC KO mice. These experiments revealed that NO-mediated increases in cGMP in RBCs are dependent on both intracellular sGC and PDE activity. Treating human and murine RBCs with NO donors increased intracellular cGMP-levels in a time and concentration-dependent fashion, which was abrogated by treatment

with the sGC inhibitor ODQ. Maximal stimulation of intracellular sGC activity was induced by treatment with a NO donor and the sGC stimulator BAY 41-2272 in the presence of the PDE5 inhibitor sildenafil. This treatment resulted in up to 1000-fold increases in cGMP in both human and murine RBCs. Compared to the stimulator YC-1, which was used in the fractionation experiments, BAY 41-2272 is considerably more potent and specific for sGC (high concentrations of YC-1 were shown to have additional inhibitory effects on PDEs) [44]. We further demonstrate that human RBCs also carry an active PKG (confirming data by Föller et al. [30], in murine RBCs) and the stimulation of their sGC induces PKG-dependent phosphorylation of VASP at Ser239. PKG activation may participate in control of RBC membrane properties and RBC clearance; in fact, RBCs from PKG KO mice are less deformable, and mice show splenomegaly [30,31]. We here find that treating RBCs suspension with low NO concentrations in the presence or in the absence of BAY 41-2272 lead to inhibition of membrane scrambling and exposure of phosphatidylserine induced by Ca^{2+} overload and NEM-mediated alkylation of critical thiols (SI Fig. S3). However, treatment with stable cGMP did not block the Ca^{2+}/NEM-induced membrane scrambling (SI Fig S3), or affect RBC deformability (SI Fig S4) in our experimental conditions, indicating that these ex vivo experiments do not fully reproduce the in vivo situation. Considering that both eNOS KO [25] and PKG KO [30] mice show increased RBC uptake by the spleen, it is tempting to speculate that the eNOS/sGC/PKG pathway contributes to regulate the half-life of RBCs in circulation. It is intriguing to speculate that RBC size distribution width, which reflects functionally distinct and heterogeneous circulating RBC populations and was recognized to be of prognostic value for cardiovascular disease [46], might be associated with differences in NO/sGC signaling.

4.3. Levels, activity, and NO responsiveness of sGC in RBCs from patients with CAD

We here present compelling evidence that differently from eNOS expression and activity [3,41,45], red cell sGC expression, sGC responsiveness to NO and sGC specific activity were found to be fully preserved in RBCs from patients affected by CAD and endothelial dysfunction with conserved glyceryl trinitrate-mediated vascular response. Of note, RBCs from this cohort of CAD patients showed an increased responsiveness to sGC stimulation when compared to aged-matched controls. This finding is reminiscent of the well-known hypersensitivity for NO-mediated vasorelaxation [42,43] in endothelium-denuded vessels [42] or vascular tissue of eNOS KO mice [43], which is not due to changes in expression. Similarly we found that protein levels and specific activity of red cell sGC were unaffected by the lack of basal NO tone in RBCs from eNOS KO mice as compared to WT animals. Thus, red cell sGC expression remains pharmacologically targetable by sGC stimulators and sGC activators [15,44] even if NO bioavailability is decreased.

NO can activate sGC only if the iron-heme is in the reduced (ferrous, Fe^{2+}) state. The sGC activators Cinaciguat (BAY 58-2667) and its close chemical analogue BAY 60-2770 (used in the present study) belong to a class of molecules able to bind and activate the NO-insensitive oxidized (ferric, Fe^{3+})/heme-free sGC [47]. By analyzing the effects of sGC activators on cGMP-levels, Stasch and colleagues discovered that an NO-insensitive/heme-free form of sGC is found in vascular tissues, platelets and many other cell types [14], especially under conditions of increased oxidative stress [12] like in CAD, making them promising drugs for the treatment of cardiovascular disease [48,49]. Here we find that treating RBC suspensions with BAY 60-2770 increased intracellular cGMP-levels in RBCs from CAD and aged-matched controls to the same extent, but not in RBCs from young healthy individuals. However, BAY 60-2770 increased cGMP-levels in all RBCs following oxidation of red cell sGC by ODQ, confirming the specificity of the target interaction with regard to redox status. BAY 60-2770 increased cGMP-levels considerably less than NO (100 fold) or NO + BAY 41-2272 (1000 fold) as compared to control. The response to the sGC activator BAY 60-2770 in RBCs is smaller as compared to vascular tissue or vascular cells [12], indicating that NO-insensitive oxidized/heme-free sGC is present in RBCs, albeit at lower levels when compared to diseased vessels. Accordingly, in RBCs from CAD patients we did not find any changes in redox state or levels of free thiols, indicating that the rich antioxidant systems in RBCs (which are optimized to keeping hemoglobin in its reduced, O_2-binding form) [1] are fully functional in CAD and may also contribute to keeping sGC (and probably other redox sensitive Fe^{2+}-heme proteins in RBCs) in their reduced NO-sensitive form in both health and disease. Taken together, these results indicate that levels, catalytic activity and NO responsiveness of sGC and redox state are fully preserved in RBCs from patients with stable CAD. It is tempting to speculate that modification of the capacity of RBCs to cope with oxidative modifications (as in anemia induced by hemoglobinopathies or modification in redox enzymes [1]) may affect RBC function also by sGC oxidation and inhibition of cGMP-mediated pathways.

4.4. Summary and outlook: what is the functional role of sGC in RBCs?

In summary, our results demonstrate that RBCs are not only scavengers, transporters and producers of NO, but they are also able to sense NO via the NO receptor sGC and its fully functional cGMP-dependent signaling pathway, which remains fully responsive to NO and sGC stimulators/activators in patients with CAD.

One argument which could be raised to invalidate these conclusions might be that we had no way to test purity of our RBC preparations form platelet contamination, other than checking the presence of platelets in our washed RBC preparation by flow cytometric analysis (although we counted 100 000 events). There are several arguments speaking in favor of the conclusion that RBCs carry their own sGC and against the fact we are only measuring sGC activity from platelet contamination: i) the presence of active sGC was observed in erythroid cells [19]; therefore, similar to numerous other cytoplasmic enzymes [1,45], it is very likely that mature RBCs carry sGC too; ii) the lack of responsiveness of red cell sGC to BAY60-2770 (especially in CAD) is something that seems to be typical of RBCs and to the best of the authors knowledge is not found in platelets or anywhere else [12,14,18]; this indicates that sGC is kept in its NO-responsive Fe^{2+} from in RBCs and is likely to be related to the high antioxidant capacity and redox reserve of RBCs [1], which is mainly needed to keep hemoglobin in its Fe^{2+} oxygen-binding form. Of interest, a recent paper by the group of A. Straub [50] showed that cytochrome b5 reductase 3 (also known as methemoglobin reductase, which reduce methemoglobin (Fe^{3+}) into deoxyhemoglobin (Fe^{2+}), and very abundant in RBCs) keeps also sGC in the Fe^{2+}-reduced state in the smooth muscle cell; therefore, it is tempting to speculate that it may well do the same with red cell sGC in RBCs. Third, in patients with CAD we observed that both NO-responsiveness and sGC activity in RBCs are fully preserved, whether the group of Stasch in a similar collective observed a decreased in sGC activity in platelets patients with CAD [18]; future follow up study should be carried out to compare sGC activity in platelets and RBCs from the same patient cohort.

However, the question remain about what are the sources of NO leading to activation of red cell sGC in vivo, or in more general terms, what is the functional role of this pathway in RBCs. Our working hypothesis (Fig. 6) is that the sGC pathway may be activated in RBCs by endothelial-derived NO (similar to what occur in platelets), as well as erythrocytic NO produced in normoxia by eNOS [3,6] or hypoxia by nitrite-derived NO production [51,52], as depicted in Fig. 6; however at present we are not able to present any valid experimental evidence that an intracellular source of NO in RBCs is regulating this pathway. Interestingly, there is a rapidly accumulating body of evidence that RBC-mediated inhibition of platelet aggregation by nitrite-derived NO production and activation of the sGC/PKG signaling pathway in platelets ex vivo and in vivo [4,52–57]. As discussed previously [1,45],

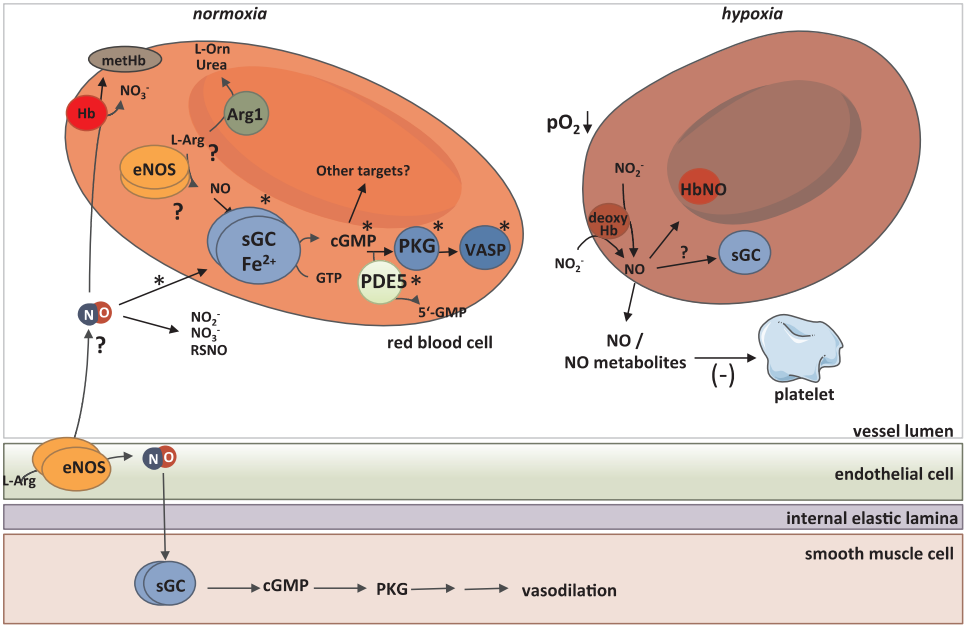

Fig. 6. Summary of findings and working hypothesis of possible role of red cell sGC. RBCs were considered for a long time to scavenge endothelial-derived NO, in a reaction where hemoglobin is oxidized to methemoglobin, while NO is converted into nitrate. RBCs were shown to carry enzymes regulating NO synthesis including eNOS and arginase 1 (Arg1). In this manuscript, we show that RBCs carry a isoform 1 of soluble guanylate cyclase (sGC or GC1), catalyzing NO-depended cGMP formation in a phopshodiesterase 5 dependent fashion, leading to activation of protein kinase G (PKG) and probably to other targets. Potential NO sources leading to activation of red cell sGC may be endothelial NO, or erythrocytic NO produced under normoxic conditions (e.g. by eNOS) or under hypoxic conditions from nitrite by deoxyhemoglobin catalyzed nitrite reduction. This reaction was shown to inhibit platelet aggregation in vivo. The functional significance of the NO – cGMP pathway in RBCs should be further investigated in vivo.

* Shown in the present manuscript
? Hypothesized pathway

interactions between RBCs and platelets may strongly contribute to maintain hemostatsis and participate in cardioprotective pathways. Evidence of an in vivo relevance of this pathway (and many other signaling pathways in RBCs and platelets), as well as RBC-compartment specificity of biochemical assays may only be obtained by analyzing erythroid-specific or megakaryocyte specific gene targeted mice.

Nevertheless, since as demonstrated here sGC/PDE5/PKG pathway is preserved in CAD, its components may represent potential drugable targets not only in patients with CAD but also in other diseases associated with functionally relevant NO deficiency such as heart failure, anemia, and pulmonary hypertension [19,22,23], deserving further study in these patient cohorts.

Authorship contribution

M.M.C.-K., E.M., C.M.K., W.L., G.W., C.P., T.P., B.S., J.-P.S., M.F., D.K., M.K. designed the experiments and analyzed the data; data from study subjects were analyzed by M.M.C.-K., E.M., C.M.K., W.L., G.W., C.P., M.K, .E.M., D.K. contributed with essential reagents; M.M.C.-K., E.M., C.M.K., W.L., G.W., C.P., T.P., B.S. performed research; M.M.C.-K., E.M., C.M.K., W.L., J.Y., G.W., C.P., J.P., J.-P.S., M.F., D.K., M.K. contributed to paper draft and concept; M.M.C.-K., E.M., J.-P.S., M.F., D.K., M.K. wrote the paper.

Disclosure of conflict of interest

The authors declare no competing financial interests. J.-P.S. is an employee at Bayer Pharma AG.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.08.020.

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