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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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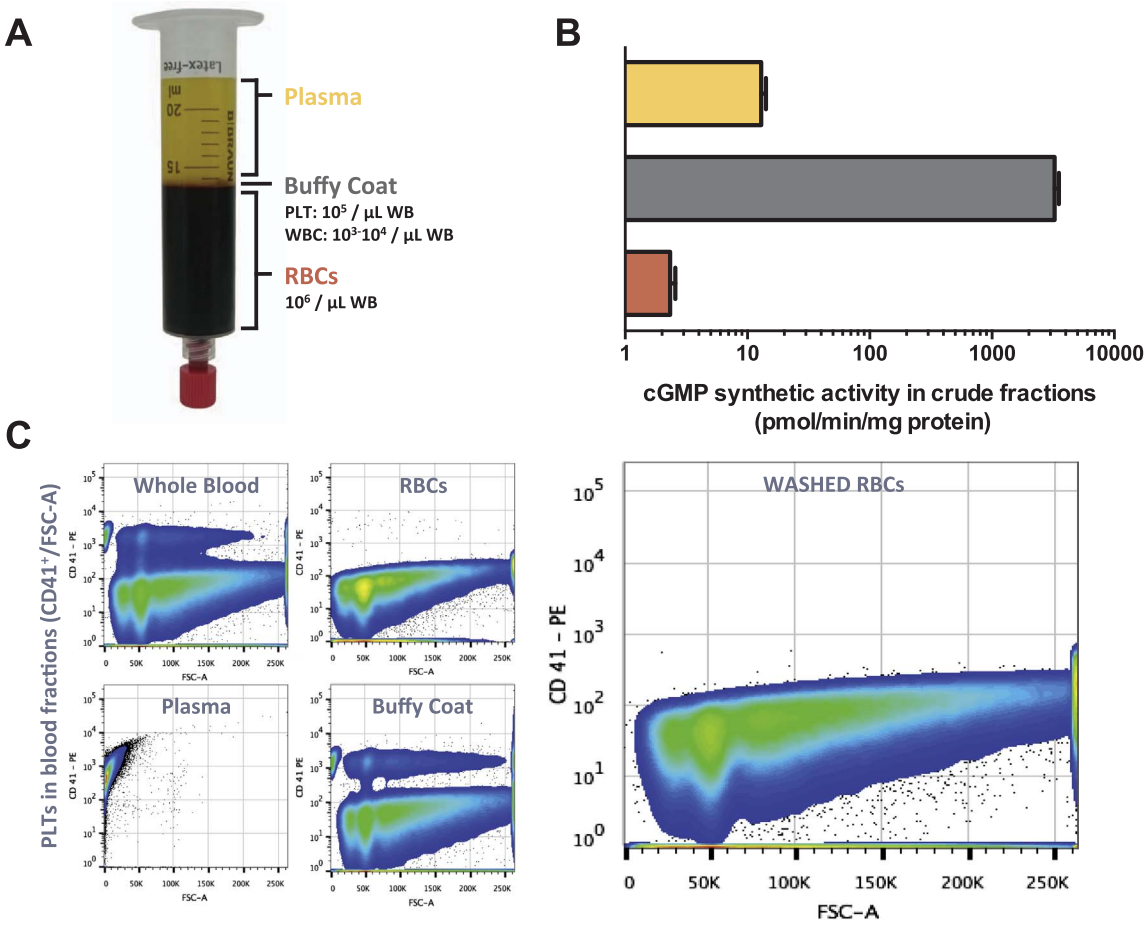

Fig. 1. NO-dependent cGMP-forming activities differ markedly between blood compartments. (A) Representative picture of whole blood after fractionation by centrifugation. **(B)** Rate of NO-induced catalytic conversion of GTP into cGMP in crude lysate from blood fractions; sGC activity was induced by 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

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