Clinical Biochemistry 45 (2012) 1678–1682

Contents lists available at SciVerse ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Antioxidative properties of hydrogen sulfide may involve in its antiadhesive action on blood platelets

Agnieszka Morel, Joanna Malinowska, Beata Olas *

Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska 141/3, 90–236 Lodz, Poland

ARTICLE INFO

Article history:
Received 5 July 2012
Received in revised form 29 August 2012
Accepted 30 August 2012
Available online 8 September 2012

Keywords:
Hydrogen sulfide
Blood platelets
Adhesion
Adhesive proteins

ABSTRACT

Background: Hydrogen sulfide (H_2S) is a signaling molecule in different systems, including the cardiovascular system. However, mechanisms involved in the relationship between the action of H_2S and hemostasis process are still unclear.

Objective and methods: The present work was designed to study the effects of hydrogen sulfide on adhesion of blood platelets *in vitro*. Platelet suspensions were preincubated (5–30 min) with NaHS as a hydrogen sulfide donor at the final concentrations of 0.00001–10 mM. Then, for platelet activation thrombin (0.1 U/mL) or TRAP, peptide with the sequence Ser-Phe-Leu-Leu-Arg-Asn (SFLLRN; 20 μM) was used. We also measured the effects of H_2S on superoxide anion radicals ($O_2^{-\bullet}$) production in blood platelets.

Results: We observed that adhesion to collagen and to fibrinogen of resting platelets preincubated with NaHS was changed, and this process was statistically significant (for 0.00001–5 mM NaHS, $p<0.05$; 10 mM, $p<0.01$). The inhibitory effect of NaHS on adhesion of thrombin – or TRAP – stimulated platelets to collagen was found (for 0.00001 and 0.0001 mM NaHS, $p<0.05$; 0.001–1 mM NaHS, $p<0.01$; 5 and 10 mM NaHS, $p<0.001$). Hydrogen sulfide reduced also the thrombin- or TRAP-induced platelet adhesion to fibrinogen (for 0.00001 and 0.0001 mM NaHS, $p<0.05$; 0.001–1 mM NaHS, $p<0.01$; 5 and 10 mM NaHS, $p<0.001$). Moreover, H_2S caused a dose-dependent reduction of $O_2^{-\bullet}$ produced in platelets ($p<0.05$).

Conclusion: The results obtained that the antioxidative activity of H_2S may involve in its antiadhesive properties on blood platelets.

Introduction

Hydrogen sulfide (H_2S) is a gaseous messenger molecule, which in normal conditions has a characteristic disagreeable odour of rotten eggs. It is a highly reactive molecule as it dissociates to form HS^- and S^{2-}. H_2S is estimated 81.5% exists as HS^- and 18.5% as H_2S under physiological conditions. Undissociated H_2S, similar to the other gaseous transmitters such as nitric oxide ($NO^{\cdot}$) and carbon monoxide (CO), is a lipophilic compound, easily passes through the cell membrane [1]. Hydrogen sulfide is synthesized from the amino acids: cysteine (Cys) and homocysteine (Hcy) by two enzymes: cystathionine-β-synthase and cystathionine-γ-lyase. The physiological concentration of H_2S in plasma and in majority tissue is about 50 μM. Its physiological level in the brain is up to three-fold higher than in plasma. Endogenous concentration of H_2S is regulated by many metabolic pathways, such as mitochondrial oxidation and cytosine methylation. Hydrogen sulfide can be scavenged by methemoglobin or metallo- or disulfide-containing molecules (such as: oxidized glutathione – GSSG) [1–4].

The physiological effects of endogenous H_2S may be multifaceted. Hydrogen sulfide, like nitric oxide and carbon monoxide, is a signaling molecule in different systems, including the inflammatory and nervous system, and in particular the cardiovascular system [1,2,4,5]. It regulates vascular tone and cardiac work and exerts cardioprotection [2–4,6,7]. Moreover, hydrogen sulfide has other various biological properties [8,9]. However, the role of H_2S in hemostasis is still unexplained. In the literature, there are only few papers describing studies on the effects of hydrogen sulfide on the hemostasis, including blood platelet aggregation [6,10,11]. Some studies have shown the role of H_2S as therapeutic agent in cardiovascular diseases. An injectable Na_2S (IK-1001), which is an H_2S donor, has developed for clinical use. It is currently undergoing phase I and II trials for therapy in ischemia reperfusion injury and renal injury [12]. Garlic derived compounds (at dietary doses) S-allylcysteine exhibit a range of cardioprotective effects which include the inhibition of platelet aggregation, and these effects may be mediated through H_2S [12–14].

In the present study we investigated the effects of hydrogen sulfide on blood platelet adhesion to evaluate the anti-platelet properties of H_2S. In order to clarify the mode of anti-platelet action of hydrogen sulfide, we used (1) TRAP - peptide which is “tethered” ligand domain of thrombin receptor, and begins with the sequence Ser-Phe-Leu-Leu-Arg-Asn (SFLLRN) and (2) thrombin. Moreover, we

* Corresponding author. Fax: +48 42 635 44 84.
E-mail address: olasb@biol.uni.lodz.pl (B. Olas).

0009-9120/$ – see front matter
http://dx.doi.org/10.1016/j.clinbiochem.2012.08.025

measured the effects of H_2S on superoxide anion radicals ($O_2^{-\bullet}$) production in resting blood platelets and platelets stimulated by TRAP or thrombin.

Materials

Reagents

Thrombin, collagen type I, bovine serum albumin (BSA), cytochrome C and bicinchoninic acid solution (BCA) were purchased from Sigma (St Louis, MO). Sodium hydrosulfide (NaHS), which has been well established as a reliable H_2S donor [15,16], was from Sigma (St Louis, MO). Thrombin receptor activating peptide (TRAP) SFLLRN was also obtained from Sigma (St Louis, MO) and it was stored at −20 °C. TRAP was diluted with 0.15 M NaCl to desired concentrations just before the experiments. Fibrinogen was prepared from human blood according to Doolittle et al. [17]. All other reagents were of analytical grade and were provided by commercial suppliers.

Blood collection

Human blood was taken from healthy volunteers aged 23–31 years (average: 24; SD = 7.2 years) not taking any medications or addictive substances (including tobacco, alcohol and aspirin or any other anti-platelet drugs) and keeping a balanced diet (meat and vegetables), with similar socio-economic background, using no antioxidant supplementation.

Isolation of human blood platelets

Human blood was collected into ACD solution (citric acid/citrate/dextrose; 5:1 v/v) and platelets were isolated by differential centrifugation of blood as described by Wachowicz and Kustroń [18]. The platelets were counted by the photometric method according to Walkowiak et al. [19]. Washed human platelet suspensions in the modified Tyrode's Ca^{+2}/Mg^{+2} free buffer (127 mM NaCl, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM HEPES, 5.6 mM glucose, pH 7.4) were exposed to NaHS at a final concentration between 0.00001 and 10 mM.

Some samples of washed platelets were activated by thrombin or TRAP.

Platelet adhesion

Adhesion of blood platelets to fibrinogen and collagen type I was determined according to Tuszyński and Murphy [20]. Platelets (3×10^8 platelets/mL) after preincubation (5, 15 and 30 min, 37 °C) with NaHS at the final concentrations of 0.00001–10 mM or without this compound (control) were activated by thrombin (0.1 U/mL) or by TRAP (20 μM). Wells of a 96-well microtiter dish (CLINIPLATE EB FB 50 PCS/CRS, Labsystems) were incubated for 2–3 h with 50 μL of fibrinogen (final concentration of 2 mg/mL), dissolved in phosphate-buffered saline, pH 7.5 (PBS) or with 40 μg/mL collagen dissolved in 0.05% CH_3-COOH. The wells were aspirated, treated with 200 μL PBS containing 1% BSA for 1 h and then washed three more times with 200 μL of PBS. Immediately after washing, the wells were supplemented with 50 μL of the test agonist: thrombin (final concentration, 0.1 U/mL) in PBS or by TRAP (final concentration, 20 μM) in 0.15 M NaCl. Then platelet suspension 100 μL was added to each well and the plate was incubated at 37 °C for 1 h. Nonadherent cells were removed by aspiration and the wells were washed three times with 200 μL of PBS. The total cell-associated protein was determined by dissolving the attached blood platelets directly in the microtiter wells with 200 μL of the Sigma BCA working solution and incubated at 37 °C for 60 min. Plates were allowed to cool to room temperature, cover sheets were removed, and the absorbance of each well was determined at 540 nm with a microtiter plate reader (BioRad, Model 550). Before adding the Sigma BCA working solution, microscopic examination of adherent blood platelet to microplate wells coated with collagen type I or fibrinogen was done.

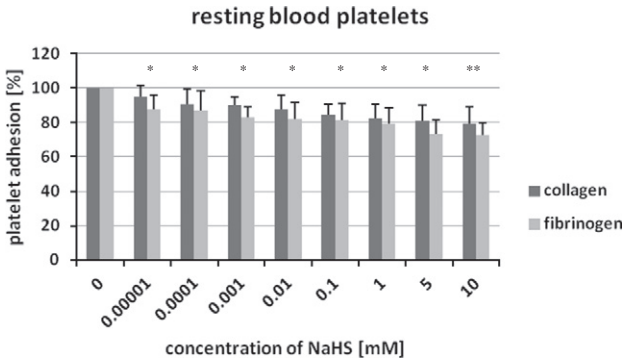

Fig. 1. Effect of NaHS on adhesion of resting platelets to collagen or to fibrinogen. Data represent means ± SD of eight experiments done in quadruplicate. NaHS was preincubated for 5 min at 37 °C with platelets at final concentrations of 0.00001–10 mM. *$p<0.05$ and **$p<0.01$ statistically significant different from controls (incubated in the absence of NaHS) by ANOVA and Bonferroni test.

$O_2^{-\bullet}$ generation in blood platelets

Generation of superoxide anion radicals ($O_2^{-\bullet}$) in resting platelets and platelets activated by agonist (thrombin (0.1 U/mL) or TRAP (20 μM)) was measured by cytochrome c reduction, as described earlier [21,22]. Briefly, an equal volume of modified Tyrode's buffer, containing cytochrome c (160 μM), was added to a 1 mL suspension of platelets. After incubation, the platelets were sedimented by centrifugation at 2000 ×*g* for 5 min and the supernatants were transferred to cuvettes. Reduction of cytochrome c was measured spectrophotometrically at 550 nm. To calculate the molar concentration of $O_2^{-\bullet}$ an extinction coefficient for cytochrome c of 18700 M^{-1} cm^{-1} was used.

Cell viability

The activity of lactic dehydrogenase (marker of platelet lysis) in the extracellular medium after treatment of blood platelets with NaHS was measured spectrophotometrically according to Wroblewski and La Due [23].

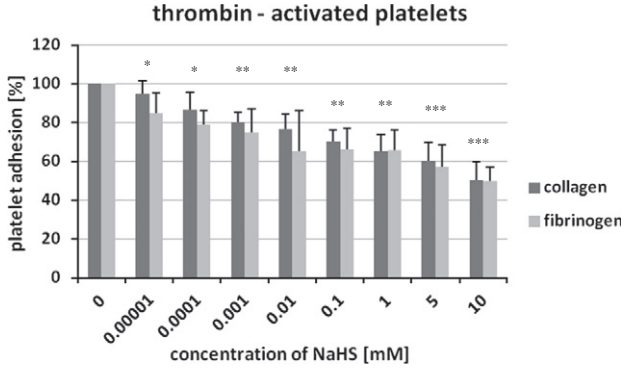

Fig. 2. Effect of NaHS on adhesion of thrombin (0.1 U/mL)-activated platelets to collagen or to fibrinogen. Data represent means ± SD of eight experiments done in quadruplicate. NaHS was preincubated for 5 min at 37 °C with platelets at final concentrations of 0.00001–10 mM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ statistically significantly different from controls (incubated in the absence of NaHS) by ANOVA and Bonferroni test.