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The possible role of hydrogen sulfide as a modulator of hemostatic parameters of plasma

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ABSTRACT

Hydrogen sulfide (H_2S) is a well known toxic gas at high levels. However, at physiological levels, H_2S may play a role in the pathogenesis of various cardiovascular diseases. The objective was to study the effects of exogenous H_2S on the hemostatic parameters (coagulation and fibrinolytic activity) of human plasma. Human plasma was incubated (5, 15 and 30 min) with NaHS as a H_2S donor at the final concentration of 0.01–100 μM. Hemostatic factors, such as maximum velocity of clot formation, fibrin lysis half-time, the activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) were estimated. Moreover, the aim of our study was to establish the influence of NaHS (10 μM; 5, 15 and 30 min) on the clot formation using the purified fibrinogen. We demonstrated that coagulation/fibrinolytic properties of human plasma incubated with NaHS were changed. APPT, PT and TT of plasma treated with NaHS at tested concentrations – 0.01–100 μM were prolonged. We observed that NaHS (0.01–100 μM) reduced fibrin polymerization in whole plasma and 10 μM NaHS also reduced polymerization of purified fibrinogen. In the presence of NaHS (at the low tested concentration – 1 μM) the decrease was about 18% (in plasma, $p < 0.05$). Our experiments also showed that NaHS (0.01–100 μM) stimulated the fibrin lysis in whole plasma. However, the time-dependent (5, 15 and 30 min) reduction of fibrin/fibrinogen polymerization and stimulation of fibrin lysis by NaHS (10 μM) was not observed. In conclusion, the present study demonstrates the anticoagulant properties of exogenous H_2S *in vitro*.

1. Introduction

Hydrogen sulfide (H_2S), as endogenous gas, has been investigated widely in recent years. H_2S, like carbon monoxide (CO) and nitric oxide ($NO^{\cdot}$) is a molecule that has been implicated in different physiological and pathological processes in mammals, including circulation system and cardiovascular diseases [1–6]. Results of Wang et al. [7] showed correlation between atherosclerosis and endogenous H_2S. H_2S plays a role in vascular smooth muscle cell proliferation and apoptosis [8]. It is involved in hyperhomocysteinemia and inhibition of atherogenic modification of LDL [8]. Other experiments demonstrated the inhibitory action of H_2S on various steps of blood platelet activation (platelet adhesion, secretion and aggregation) [9–13]. Moreover, H_2S modifies the adhesive properties of collagen and fibrinogen [14]. H_2S may be also a therapeutic agent in cardiovascular illnesses – ischemia reperfusion [3,5,15]. The major source of H_2S in plasma is likely produced by vascular smooth muscle cells and erythrocytes [16,17]. The physiological concentration of H_2S in human plasma and in tissues is about 50 μM (range from 34 μM to 65 μM) [18], but in brain is about 150 μM [19], depending on the method used for measurement and the donor's age. Experiments of Olson [20] have reported plasma or blood H_2S concentrations in this range, typically between 30 and 300 μM.

There are only a few studies addressing the anti-platelet and anti-coagulatory effects of H_2S [10,12,21,22]. The results of Grambow et al. [21] have reported that H_2S acts anti-thrombotic by reduction of platelet adhesion molecule expression and platelet granule exocytosis. Because, mechanism(s) involved the relationship between the action of hydrogen sulfide and different elements of hemostasis (blood vessels, blood platelets, the coagulation and fibrinolytic system) are still unknown, the aim of our study was to explain the effects of sodium hydrosulfide (NaHS, as a fast-releasing H_2S donor; at tested concentrations – 0.01–100 μM) on coagulation and fibrinolytic activities of human plasma by determinating such parameters as maximum velocity of polymerization and fibrin lysis half-time. We also measured the amidolytic activity of plasmin in human plasma. Moreover, the aim of our experiments was to examine the effect of NaHS on other hemostatic parameters – the activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) of human plasma

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in vitro. The aim of our experiment was also to establish the influence of NaHS (10 μM; 5, 15 and 30 min) on the hemostatic activity of the purified fibrinogen (the precursor of a fibrin clot; Fg). Because, results of Olson et al. [23] have demonstrated that 250 μM NaHS produces about 88 μM H_2S, used concentrations of NaHS (0.01–100 μM) may release about 0.003–35 μM H_2S. 100 μM NaHS tested in our experiments can be assumed to cause H_2S concentration about 35 μM (corresponds to physiological levels of H_2S in human plasma).

2. Materials and methods

Sodium hydrosulfide, which has been well established as a reliable H_2S donor [24,25], and thrombin were purchased from Sigma Chemical Co. (Germany). Fibrinogen isolated from pooled citrated human plasma by the cold ethanol precipitation technique followed by ammonium sulfate fractionation at 26% saturation at 4 °C, according to Doolittle [26]. Its concentration was determined spectrophotometrically at 280 nm using an extinction coefficient 1.55 for 1 mg/ml solution. The concentration of purified Fg in the reaction system was about 2 mg/ml. Other coagulation reagent were obtained from Diagon Ltd. (Hungary) and Boehringer Ingelheim. All other chemicals were reagent grade products purchased from POCh (Gliwice, Poland).

2.1. Isolation of human plasma

Blood samples were taken from 10 healthy volunteers without cardiovascular disorders, allergy and lipid or carbohydrate metabolism disorders, untreated with drugs. Healthy subjects did not use addictive substances and antioxidant supplementation, their diet was balanced (meat and vegetables), lived in similar socio-economic conditions. Subjects with significant medical illness were excluded. They were no smokers.

Human blood was collected into sodium citrate (0.32% final concentration) and immediately centrifuged (2000×*g*, 15 min) to get plasma.

The protocol was accepted by the Committee for Research on Human Subjects of the University of Lodz number KBBN-UŁ/II/18/2011.

2.2. Exposure of human plasma and human fibrinogen to NaHS

Samples of human plasma were exposed to NaHS at a final concentration between 0.01 and 100 μM. Samples were incubated for 5, 15 and 30 min at 37 °C (in air-tight tubes).

Samples of human purified Fg (2 mg/ml) in 50 mM Tris/HCl, 140 mM NaCl, pH 7.4 were exposed to 10 μM NaHS. Samples were incubated for 5, 15 and 30 min at 37 °C (in air-tight tubes).

2.3. The measurement of prothrombin time

Human plasma (50 μl) was incubated with 50 μl of Dia-PT liquid (commercial thromboplastin) for 1 min at 37 °C on block heater. After incubation, the measuring cuvette was transferred to the measuring holes and 50 μl of 25 mM $CaCl_2$ was added. The PT was determined coagulometrically (Optic Coagulation Analyser model K-3002; Kselmed, Grudziadz, Poland) [27].

2.4. The measurement of thrombin time

Human plasma (50 μl) was added to measuring cuvette and incubated for 1 min at 37 °C on block heater. The measuring cuvette was transferred to the measuring holes and 100 μl of thrombin was added (final concentration – 1 U/ml). The TT was determined coagulometrically (Optic Coagulation Analyser model K-3002; Kselmed, Grudziadz, Poland) [27].

2.5. The measurement of APTT

Human plasma (50 μl) was added to measuring cuvette and incubated with Dia-PTT liquid (commercial preparation) for 3 min at 37 °C on block heater. The measuring cuvette was transferred to the measuring holes and 50 μl of 25 mM $CaCl_2$ was added. The APTT was determined coagulometrically (Optic Coagulation Analyser model K-3002; Kselmed, Grudziadz, Poland) [27].

2.6. The measurement of fibrin polymerization and lysis in plasma

Human plasma was trebly diluted and clotted with thrombin (at the final concentration of 0.5 U/ml), at the presence of 62 ng/ml of t-PA. Thrombin-catalyzed polymerization and the subsequent tissue plasminogen activator (t-PA)-catalyzed lysis were monitored for 50 min, as the change of turbidity at 405 nm (BioRad 550). The maximal velocity (V_{max}, mOD/min) and maximal absorbance (A_{max}) were recorded for each absorbance curve. The half-lysis time was defined as the time for the elastic modulus to decline to 50% its peak value (½ A_{max}) [28].

2.7. The measurement of fibrinogen polymerization

Control fibrinogen, NaHS-treated fibrinogen (2 mg/ml) in the presence of 0.025 M $CaCl_2$ were added to the wells of a microtiter plate. To initiate fibrin formation, we added thrombin (final concentration – 0.25 U/ml) to some of the wells. Fibrinogen polymerization was monitored for 15 min at 405 nm using microplate spectrophotometer (BioRad 550). The maximally velocity and maximally absorbance were recorded for each absorbance curve [28].

2.8. Plasmin activity in plasma

Plasmin activity in human plasma was estimated by the hydrolysis of chromogenic substrate by streptokinase (SK). After 5 min incubation (at 37 °C) the absorbance measurements were performed in a microplate reader (Bio-Rad Microplate Reader, model 550) at 415 nm. No activity of generated plasmin was detected in the absence of SK [29].

2.9. Gel electrophoresis

Samples were prepared for electrophoresis in Laemmli sample buffer [30] in the absence or presence of β-mercaptoethanol and were separated on SDS–PAGE using a Mini-Protean Electrophoresis Cell (BioRad, Hercules, CA).

2.10. Data analysis

All the values in this study were expressed as means ± SD. The statistical analysis was performed with ANOVA test and POST Hoc test (Bonferroni). The statistically significant differences were also assessed by applying the paired Student's *t* test and the significance level was $p < 0.05$. In order to eliminate uncertain data, the Q-Dixon test was performed.

3. Results

Incubation (5–30 min) with NaHS resulted in changes of coagulation activity of human plasma (Fig. 1, Table 1). We showed that incubation of plasma (5 min) with NaHS (at tested concentrations: 0.01–100 μM) significantly prolonged the activated partial