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The anti-thrombotic effect of hydrogen sulfide is partly mediated by an upregulation of nitric oxide synthases ☆

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ABSTRACT

Introduction: Hydrogen sulfide (H_2S) known as a gasotransmitter is increasingly recognized for its anti-adhesive, anti-inflammatory and vasoactive properties. Due to these properties, we analysed anti-thrombotic effects of H_2S and the participation of the nitric oxide synthase (NOS)-pathway.
Materials and Methods: In individual venules of the ear of hairless SKH1-hr mice, thrombus formation was induced using a phototoxic light/dye-injury model and intravital fluorescence microscopy. Animals were treated intravenously with the H_2S donor Na_2S or NaCl as control. In a second setting, the NOS inhibitor L-NAME was applied intraperitoneally as a bolus 12 h prior to Na_2S treatment and thrombus induction. Blood and ear tissue were sampled after microscopy for assessment of plasma concentrations of soluble (s)P-selectin, sE-selectin, sVCAM-1 and sICAM-1 and expression of endothelial (e)NOS and inducible (i)NOS, respectively.
Results: When mice were treated with Na_2S, venular thrombus formation was significantly delayed versus that in animals of the NaCl-treated control group. While plasma levels of pro-thrombotic adhesion molecules were not affected by Na_2S, immunohistochemistry of the vessel walls showed a significant up-regulation of eNOS and iNOS expression within the Na_2S-treated group. The delay of thrombus formation in the Na_2S-group was partly but significantly reverted by application of L-NAME.
Conclusions: The anti-thrombotic efficacy of H_2S involves the NOS-pathway and may be of preventive and therapeutic value for clinical disorders with increased risk of thrombotic events.

Introduction

Thrombus formation and thrombosis related complications such as pulmonary embolism are still a major cause of morbidity and mortality and result from the coincidence of multiple disorders of haemostasis and endothelial lesions with inflammation. Further, multiple risk factors, like immobilization after trauma, apoplexy and daily strenuous posture also known as the “economy class syndrome” as well as lifestyle risk factors with smoking, contraceptive drug intake, metabolic syndrome and drug abuse are able to trigger thrombus formation. Despite prophylactic treatment, thrombotic events display a major cause of in-hospital lethality, especially after major surgery, such as prosthetic knee and hip joint implantation, in elderly patients. Due to limitations of the most common anti-coagulant substances [1] there is a need for new anti-coagulants with more benefit and less side effects for patients.

Hydrogen sulfide (H_2S) is a gas, which is noted for its smell of rotten eggs and has traditionally been known as an air pollutant and poisonous gas [2,3]. In the past, however, the physiological relevance of H_2S gained more recognition as it was detected in various mammalian tissues, mediating cytoprotective and pleiotropic effects [2–4]. H_2S is enzymatically synthesized by the two pyridoxal-5-phosphate-dependent enzymes, i.e. cysthathionine-γ-lyase (CSE) and cystathionine-ß-synthase (CBS) [5]. 3-mercaptopyruvate sulfurtransferase (3-MST) along with cysteine aminotransferase is a further H_2S-producing enzyme, as demonstrated by Kimura [6]. Most recently, 3-MST together with D-amino acid oxidase emerged as the fourth way for endogenous H_2S production from D-cysteine, predominantly in the cerebellum and the kidney [7].

There are at least two mechanisms for the release of H_2S. First, H_2S is immediately released after its production by enzymes. Another

Abbreviations: ADP, adenosine diphosphate; CO, carbon monoxide; H_2S, hydrogen sulfide; NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; GP_{Ib}, glycoprotein Ib; $GP_{IIb/IIIa}$, glycoprotein IIb/IIIa; CSE, cystathionine-γ-lyase; CBS, cystathionine-β-synthase; 3-MST, 3-mercaptopyruvate sulfurtransferase; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; L-NAME, NG-nitro-L-arginin-methylester; Na_2S, sodium sulfide; NaHS, sodium hydrogen sulfide; $FeCl_3$, ferric (III) chloride; NaCl, sodium chloride; s, soluble.

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mechanism is that the produced H_2S is stored intracellular in a sulfane sulfur bond and is released in response to a physiological signal [8]. Endogenous H_2S concentrations in human plasma are described with a range from 34 μM to 65 μM [9]. H_2S easily permeates through biological membranes due to its lipophilic solubility and dissociates to hydrosulfide and sulfide ions. In aqueous solution, about one-third of H_2S remains undissociated at physiological pH values [4]. Next to nitric oxide (NO) and carbon monoxide (CO), H_2S has been recommended to be the third gasotransmitter [3,4]. As the research in gas biology commenced with NO some 30 years ago, it has become clear that cells are caught in the crossfire of different gaseous transmitters. NO and CO share similar biological effects, including vasodilatation as well as inhibition of platelet aggregation and leukocyte adhesion. The study on H_2S is at an earlier stage, but seems to resemble the specific effects of both other gases [10–12]. In line with this, H_2S is suggested to play an important role in vascular homeostasis [13,14] as well as in anti-inflammation and anti-adhesion [2,15]. While most studies so far focus on inflammatory processes, organ cell injury and postischemic reperfusion disorders [2], there are only a few studies addressing the anti-aggregatory and anti-coagulatory effects of H_2S [16,17]. Most recently our group could demonstrate that a slow releasing H_2S donor acts anti-thrombotic by reduction of platelet adhesion molecule expression and platelet granule exocytosis [17]. However, the effects of H_2S on the complex coagulation system could be manifold due to its pleiotropic character. To further extend the knowledge about the mechanisms of H_2S on microvascular thrombus formation, the current study addresses the interactions of H_2S with the vascular endothelium, focusing on its anti-thrombotic property mediated by the synthesis and release of NO.

Materials and Methods

Animals

23 female SKH1-hr hairless mice (10 to 12 weeks old with a body weight (bw) of 22 to 26 g) were purchased from Charles River Laboratories (Sulzfeld, Germany). The animals were housed in a specific pathogen free facility with a 12 h light-dark cycle and had access to standard laboratory chow and water ad libitum. The experiments were conducted in accordance with the German legislation on protection of animals and the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council).

Chemicals

The H_2S donor sodium sulfide (Na_2S; Sigma Chemical, Deisenhofen, Germany) was dissolved in physiological saline to a stock solution of 5 mmol x l^{-1} and was infused intravenously at a concentration of 125 μmol x kg^{-1} bw x h^{-1}. The unspecific nitric oxide synthase (NOS) inhibitor L-NAME (NG-nitro-L-arginin-methylester) was dissolved in physiological saline to a concentration of 100 mg x kg^{-1} bw and was administered intraperitoneally 12 h prior to the experiment (Sigma Chemical). All solutions were freshly prepared at the day of the experiment. Dose and application mode of drugs were chosen in accordance to previous work of our group [18,19].

In Vivo Thrombosis Model

Mice were anesthetized by an intraperitoneal injection of ketamine (90 mg x kg^{-1} bw) and xylazine (25 mg x kg^{-1} bw) and placed on a plexiglas pad with an integrated heating plate for maintaining the body temperature at 37 °C. The ear was spread cautiously into position with the help of a moisturized translucent plastic wrap for visualization of the microcirculation by intravital fluorescence microscopy (Axiotech vario; Zeiss, Jena, Germany) equipped with a 100 W mercury lamp and a blue filter set (465-495 nm/>505 nm excitation/emission wavelength). Thrombus induction and intravital microscopy of the ear of the mouse were conducted upon injection of 0.1 ml 5 % fluorescein isothiocyanate-labeled dextran (FITC-dextran) (MW 150 kDa; Sigma) via a venous jugular catheter. The present thrombus model has the advantage that there is no need for any surgical preparation to investigate the microcirculation within the ear. Therefore, thrombus induction is not affected by inflammation due to a surgical trauma and can be studied under almost physiological conditions. Photochemical thrombus formation was induced by continuous blue light exposure to individual venules using an x63 water immersion objective lens (Zeiss). Up to five venules (diameter range 45 to 75 μm) per ear were studied. Light exposure started 10 min after start of the continuous Na_2S or NaCl infusion and was discontinued after blood flow in the vessel ceased for at least 30 s due to complete vessel occlusion. Microscopic images were recorded by a video system (S-VHS Panasonic AG 7350-E, Matsushita, Tokyo, Japan) for off-line evaluation using a charge-coupled device video camera (FK 6990A-IQ, Pieper, Berlin, Germany) and were monitored on a television screen.

Experimental Design and Experimental Groups

The following three groups of animals were studied: a saline-treated control group (n = 8), a Na_2S-treated group (n = 10) and a Na_2S + L-NAME-treated group (n = 5). Ten minutes before induction of thrombus formation intravenous infusion via the venous jugular catheter of either Na_2S (125 μmol x kg^{-1} bw x h^{-1}; Na_2S) or equivalent volumes of physiological saline (control) was started and continued until the end of the experiment. The third group received a continuous infusion of Na_2S, as described, but was pre-treated with a single intraperitoneal bolus of L-NAME (100 mg x kg^{-1} bw) 12 h prior to the experiments (Na_2S + L-NAME).

Microcirculatory Analysis

Microcirculatory parameters, such as initial vessel diameter (D), red blood flow velocity (v_{RBC}) and wall shear rate (γ) were assessed prior to the thrombus induction. The calculation of the wall shear rate is based on the Newtonian definition $\gamma = 8 \times v_{RBC} \times D^{-1}$, with v_{RBC} representing the red blood cell centerline velocity divided by 1.6, according to the Baker-Wayland factor [20] and D representing the individual inner vessel diameter. Analysis of kinetics of thrombus formation included the time periods until initial occlusion of perfusion and sustained cessation of blood flow due to complete vessel occlusion. Time periods were assessed off-line by analyzing the videotaped images using the image analyzing system CapImage (Dr. Zeintl software, Heidelberg, Germany). If a vessel did not occlude within 25 min of continuous light exposure, observation was stopped and the vessel was considered as patent.

Histology and Immunohistochemistry

At the end of the experiments both ears of all animals were sampled and fixed in 4% phosphate buffered formaldehyde for 2 to 3 days. After paraffin embedding tissue blocks were cut in 4 μm slices and stained with hematoxylin-eosin for routine histological analysis. For immunohistochemical demonstration of eNOS and iNOS, tissue sections collected on poly-L-lysine-coated glass slides were treated by microwave for antigen unmasking. A mouse anti-mouse eNOS and a rabbit anti-mouse iNOS antibody (both Calbiochem, Darmstadt, Germany) were used as primary antibodies at dilutions of 1:50 (eNOS) and 1:500 (iNOS) and incubated overnight at 4 °C, followed by incubation with the appropriate secondary horseradish-peroxidase labeled antibodies in accordance to the instructions of the LSAB + System HRP kit (DAKO, Hamburg, Germany) and development using ACE as chromogen. The sections were counterstained with hematoxylin and examined