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The effect of hydrogen sulfide on the contractility of cerebral arterioles. A pilot study

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ABSTRACT

Background and Aims: Endogenous gaseous substances, such as NO and CO have been found to be effective vasodilators earlier. H_2S has been identified as an additional one, however, for that substance both vasodilatory and vasoconstrictor responses have been described in different vascular territories. Our aim was to examine the effect of hydrogen sulfide on the tone of cerebral arterioles and some aspects of its mechanism. *Methods:* The work was performed on excised rat anterior cerebral artery segments in vitro (diameter range 150–250 μm), using a pressure myograph system. We used NaHS as exogenous H_2S donor, propargylglycine (PAG) to abolish the endogenous synthesis of hydrogen sulfide and 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) to examine the potential role of Cl^-/HCO_3^- exchanger in the effects of H_2S. The time course of the events after application of exogenous H_2S was also evaluated. *Results:* Our findings revealed that in these pathologically important vessels *(1)* endogenously produced H_2S is not a vasodilator, but a moderate vasoconstrictor; *(2)* H_2S has a biphasic effect: low concentrations are moderate vasoconstrictors, while at higher concentrations the initial contraction is followed by dilatation; *(3)* that vasodilation is prevented by DIDS (4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium, an inhibitor of the Cl^-/HCO_3^- exchanger). *Conclusion:* These studies confirm that H_2S should be taken into consideration as a modulator of cerebral arteriolar tone in mammals.

KEYWORDS

hydrogen sulfide, H_2S, propargylglycine, DIDS, cerebral circulation

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INTRODUCTION

Research on the regulation of cerebrovascular tone has an outstanding significance from clinical point of view. Stroke and other cerebrovascular diseases are a serious burden for healthcare all over the world, their epidemiologic importance as a cause of chronic disability and death continues increasing despite improving preventive therapeutic means [1, 2].

As it is known, cerebral vascular resistance is controlled by myogenic mechanisms, local metabolites and blood-borne substances. Its control is not homogenous throughout the brain, substantial territorial differences exist. In general, the acetylcholine-induced vasodilation is far more pronounced in the anterior circulation, while vasoconstrictors are more effective in the vertebrobasilar region [3]. There are other important findings, which show further territorial differences in the activity of cerebral the vessels. It was revealed that vasoconstrictor responses (to noradrenaline, dopamine, 5-HT, prostaglandin F2) were more effective in the anterior cerebral artery, while these vessels were less sensitive to bradykinine-induced vasodilation [3].

After centuries in the past, when hydrogen sulfide (H_2S) was considered merely a hazardous gas, today it is widely accepted that this can be considered a third gasotransmitter in mammalians. It has widespread effects both in physiological and pathophysiological conditions [4]. H_2S is a weak acid, therefore it is mostly dissociated (approx. in 80%) to hydrosulfide anions (HS^-) and hydrogen ions (H^+) at physiological pH values [5]. The mechanism of action of hydrogen sulfide is diverse, *(1)* persulfidation/polysulfide generation, *(2)* interaction with reactive oxygen and nitrogen species, *(3)* reaction with metalloproteins [6]. Hydrogen sulfide is produced in the mammalian body by four enzymatic pathways, cystathionine-gamma-lyase (CSE) being the most important one [4, 7–9]. Although there are numerous attempts with different techniques for the correct and precise measurement of endogenous hydrogen sulfide production, the exact level of H_2S is different tissue and, what is also very important, in different cell compartments, is still under debate. The serum and tissue concentrations are suspected to be in the nanomolar range [10]. It is also probable that although the tissue concentration of H_2S is very low in general, in distinct intracellular microenvironments it can reach higher values and exert important biological effects [10]. Tissue levels can be different in the wall of larger and smaller arteries: H_2S level was higher in the tail artery compared to the aorta of the rat [11, 12].

It is known that this molecule has important effect not only on of vascular tone, but also on angiogenesis, vascular permeability and atherogenesis [13]. Considering vascular tone, mostly vasodilatation was observed, but two-phase effects and vasoconstriction also has been observed [6, 14]. Vasorelaxation is more pronounced in the peripheral resistance arteries, compared with the large, conductive vessels [15]. In genetically modified mice lacking one of the H_2S producing enzymes, CSE, cholinergic vasorelaxation and hyperpolarization was found to be reduced proving the physiological significance of that pathway [16]. H_2S has even been suggested to be one of the endothelial hyperpolarization factor (EDHF) molecules by some authors [15, 16].

The Cl^-/HCO_3^- channels were found to be part of the vascular response by one key publication [13]. The Cl-transport inhibitor DIDS ameliorated the H_2S-induced vasorelaxation in murine models which further proves the role of this pathway [17, 18]. Nevertheless, vasoconstrictor responses after H_2S treatment were also observed on different specimens [19].

The aim of the present study was to test using pressure myography whether endogenous H_2S produced in the wall of cerebral resistance vessels, and the same substance, given externally has a vasoactive effect and whether the anion exchanger plays an important role in its action.

MATERIALS AND METHODS

Animals

All the applied procedures conformed to the Guide for the Care and Use of Laboratory Animals (8th edition, ELAR/NRC 2011), the legal and institutional guidelines for animal care and were approved by the Animal Care Committee of the Semmelweis University and Hungarian authorities (PE/EA/1430-7/2018).

Experiments were performed on freshly isolated anterior cerebral artery segments from the A2 segment, prepared from 3 to 4 months old/295–385 g weighted male Wistar rats (n= 4–9). All animals had rat chow (S8106-S011 SM, Ssniff Spezialdiaten, Soest, Germany) and water ad libitum.

Materials

U46619 was purchased from Tocris Bioscience (Bristol, UK). Sodium hydrosulfide (NaHS), DL-propargylglycine (PAG) and 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium (DIDS) was obtained from Sigma-Aldrich/Merck KGaA (Darmstadt, Germany). Drug solutions were freshly prepared.

The composition (in mmol l^{-1}) of the Krebs-Ringer solution used was the following: Na^+ 144; K^+ 4.7; SO_4^- 1,2; $H_2PO_4^-$ 1.2; Mg^{2+} 1.2; HCO_3^- 24; Ca^{2+} 2.5; glucose 5.5; and EDTA 0.02, and for the Ca-free solution: Na^+ 144; K^+ 4.7; SO_4^- 1,2; $H_2PO_4^-$ 1.2; Mg^{2+} 1.2; HCO_3^- 24; glucose 5.5; and EDTA 0.025, EGTA 2.0.

Preparation of anterior cerebral artery segments

Male Wistar rats were anaesthetised with pentobarbital (Nembutal, Ceva Santé Animale, Libourne, France, 45 mg kg^{-1} body weight, administered i.p.). Then the right atrium was cut open, and all the systemic circulation was perfused from the left ventricle with heparinized cold Krebs-Ringer solution. The head was removed, the brain exposed and removed together and put in ice-cold Krebs solution. The course of the right anterior cerebral artery (ACA) was identified under the preparation microscope (Leica) and the A2 section carefully cleared from surrounding tissue and cut out. All preparation processes were performed under cold, oxygenated Krebs-Ringer solution. An approx. 2 mm long segment from the A2 section was used in the further experiments. Smaller side-branches, if any, were ligated, vessels were cannulated at both ends with plastic microcannulas and extended to their in vivo length. Vessels were pressurized with Krebs-Ringer solution as described below.

Pressure arteriography of arteriolar segments

The glass-bottomed organ chamber containing the already cannulated vessel segment was placed on the stage of an inverted microscope (Leica, Wetzlar, Germany). Servo-controlled pumps

(Living Systems, Burlington, VT, USA) were used to set the intraluminar pressure. Calibration of the experimental system was done with a mercury manometer. The vessels mounted in Krebs-Ringer solution were bubbled with a gas mixture containing 5% CO_2, 20% O_2 and 75% N_2, and were pressurized to 50 mmHg. Then the segments were left in the system for equilibration for 30 min. During the examinations, temperature of the bath solutions was kept at 37 °C. Pictures of the transparent, mounted segment were taken by a digital camera (Leica DFC 320) and were photographed using the Leica Qwin V3 software. The inner and outer diameters of the vessels were measured from these pictures. Calibration was made using a micrometer etalon (Wild, Heelbrugg, Switzerland). At the end of the drug tests, vessel segments were washed out, they were incubated in Ca^{2+}-free Krebs solution for 30 min to determine passive, fully relaxed diameters. After 30 min equilibration at 50 mmHg, pressure-diameter in normal Krebs-Ringer solution, pressure-diameter curves were recorded (0–10–20–30–40–50–60–70–80–90–100 mmHg). Inner and outer diameters of the vessels were measured offline using the ImageJ software.

Pharmacological studies

Pressure diameter curves were recorded in myogenic contraction as above and the procedure was repeated with the PAG in the bath. In other specimens, at 50 mmHg intraluminal pressure the thromboxane-A2-receptor ($T\times A_2r$) agonist U46619 was added (3×10^{-6} M) and the contraction recorded. Than the H_2S donor NaHS was added in increasing concentrations, 10–30–100–300–1,000 µM, and the diameter responses were recorded. In another group the specimens were pretreated with the anion exchanger inhibitor DIDS (300 µM), U46619 contraction was induced, and the vasodilatory action of NaHS (1,000 µM) was tested at this concentration.

Statistical analysis

Results are shown as mean ± SEM. One-way and two-way ANOVA was used with Tukey post-hoc tests. Paired *t* test was applied when diameter changes from reference value were evaluated. The *P* value <0.05 was considered as statistically significant. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

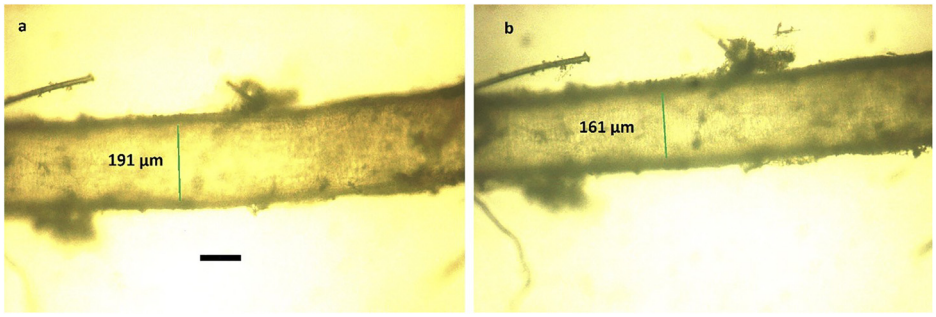

Fig. 1. Rat anterior cerebral artery segment mounted for pressure arteriography, 50 mmHg intraluminal pressure. **A**. Before and **B**. with 10 µM NaHS in the bath. Note moderate contraction at micromolar concentrations of hydrogen sulfide. Bar, 100 µm

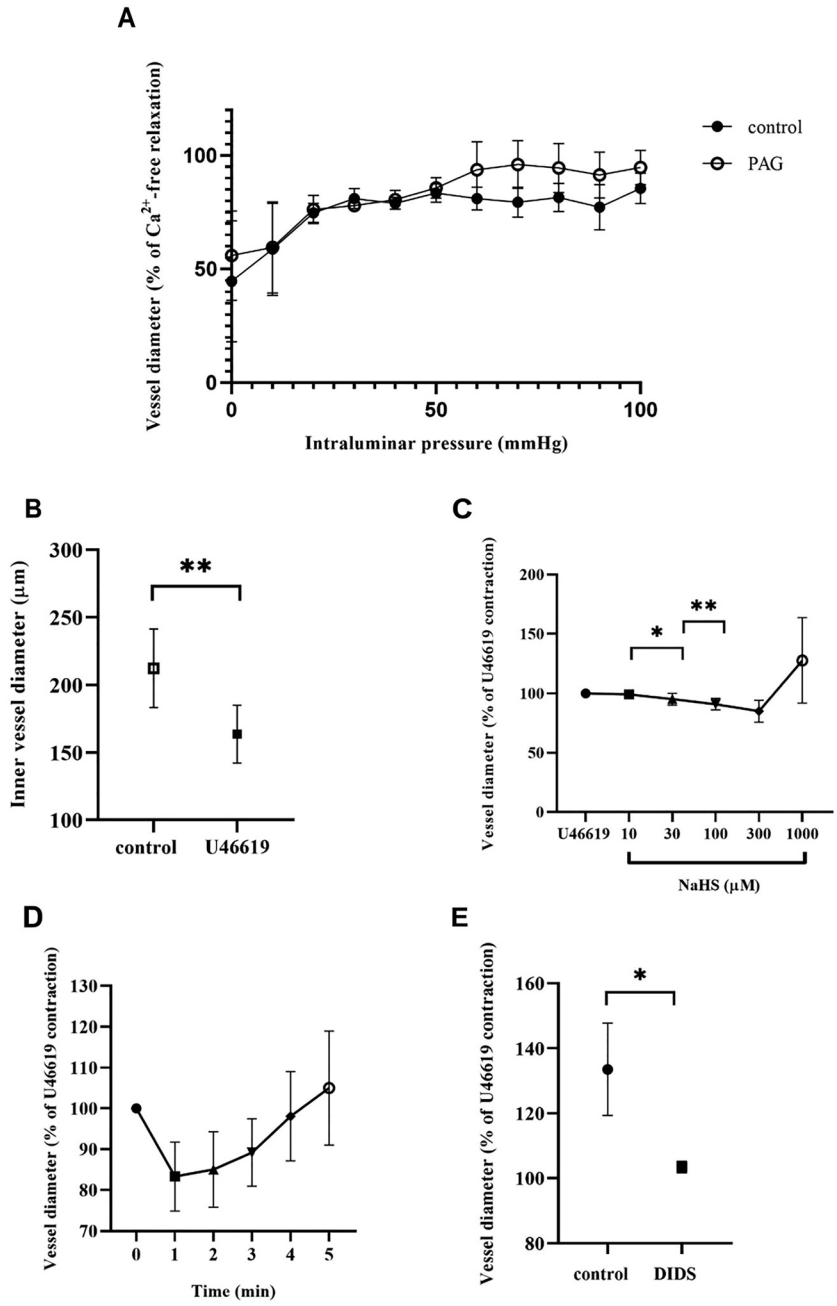

Fig. 2. **A**. Pressure-diameter characteristics of rat anterior cerebral arterioles (150–250 μm) in spontaneous contraction. Effect of the PAG (propargylglycine, an inhibitor of endogenous H_2S production) treatment on the vascular tone at different intraluminal pressures ($n = 4$, $P < 0.05$ with the paired t test).

Note moderate vasodilator effect at higher pressures (n.s.). **B**. Precontraction of rat cerebral arterioles with the TxA_2 agonist U46619 (3 μM, $n = 9$, ** $P < 0.01$ with ANOVA), **C.** Effect of increasing concentrations of the H_2S donor NaHS on the diameter of precontracted segments (2 min after addition of the drug to the bath). Note moderate contraction at 30–300 μM, but relaxation at 1,000 μM ($n = 5$, $P < 0.05$ with the paired t test). **D**. Time course of the effect of 300 μM NaHS treatment on the diameter of precontracted segments, 1–5 min after addition of the drug. Note initial wave of contraction followed by relaxation (statistically significant with the paired t test). U46619-contracted diameters are considered as units. **E.** Effect of DIDS pretreatment (4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium, an anion exchanger blocker) on the vasodilation exerted by 1,000 μM NaHS ($n = 5$, paired t test *$P < 0.05$). U46619 contracted diameters are considered as unit. Note inhibition of H_2S relaxation.

RESULTS

Segments had a typical for these small arterioles myogenic tone (approx. 20% at higher pressures, Fig. 2A). Addition of PAG induced a slight relaxation at higher intraluminal pressures, which did not reach the level of statistical significance (Fig. 2A). All segments produced significant in vitro contraction with U46619 (3 μM, Fig. 2B **, $P < 0.01$ with the paired t test). The H_2S donor NaHS had a biphasic effect: a vasoconstriction at lower concentrations (Fig. 1A and B; Fig. 2C), while if applied in higher concentrations (300–1,000 μM, an initial vasoconstriction was followed by vasorelaxation (Fig. 2C and D) significant with the paired t test $P < 0.05$).

Pretreatment of the vessel segments with DIDS inhibited the vasorelaxation exerted by the administration of 1,000 μM NaHS; this effect was statistically significant (Fig. 2E).

DISCUSSION

In our experiments, the inhibition of endogenous H_2S production by the CSE-inhibitor molecule PAG resulted only moderate, non-significant relaxation of rat cerebral arterioles in the intraluminal pressure range of 50–100 mmHg in vitro. Our result strengthens the observation that moderate vasoconstrictor response might be the consequence of this substance which might be important when considering sulfide releasing molecules as drugs [20]. Vascular consequences of the presence of H_2S in the brain tissue have been reviewed by us lately [21]. Compared to the previous publications in this field, our recent work has particular characteristics: while majority of the former studies were performed on rat middle cerebral artery segments, using mostly wire myography in the ex vivo experiments, we studied the effect of H2S on rat anterior cerebral artery segments, using pressure myography, which allows the direct examination of vasoconstriction or dilatation in resistance vessels. These differences in the type of the used specimens and methods underline the importance of our findings in this pilot study.

When NaHS was administered in increasing concentrations to cerebral arteriolar segments precontracted with 3 μM U46619 (a T×A2 agonist), a biphasic effect was detected, which consisted of a vasoconstriction at lower doses of H_2S, followed by vasorelaxation after the H_2S concentration increased above the level of 300 μM. Vasoconstrictor effect of H_2S is a well-known phenomenon from the related literature: it was formerly observed in other cerebral vessel studies as well, while the biphasic effect was proven by measurements on vascular segments from the

systemic circulation [6, 21] A possible underlying mechanism behind this phenomenon is the regulatory effect of H_2S on NO production and bioavailability, resulting in decreased NO-mediated vasorelaxation [14, 22].

After pretreatment with DIDS, NaHS-induced relaxation diminished. While the effect of DIDS on the vasorelaxant effect of NaHS has been described in other vessels earlier by other studies using wire myography on mouse mesenteric arteries, rat middle cerebral arteries or on rat thoracic aortic rings, the underlying mechanisms are still not clearly understood [18, 23] and there are only few publications on this so far, especially related to the cerebrovascular circulation [21]. According to these previous studies it is possible that H_2S acts directly on the Cl^-/HCO_3^- channels - supposedly through PKA or PKC activation [24, 25]. However, based on results of other studies it is also possible that H_2S treatment leads to the activation of the Cl^-/HCO_3^- channels not directly, but through intracellular acidosis, which is a result of the blocked ATP generation after inhibition of cytochrome *c* [23].

CONCLUSION

Taken together, the two most important aspects of our current work are *(1)* the description of the vasoconstrictive nature of endogenous H_2S in the modulation of the tone of the rat cerebral circulation and *(2)* the biphasic vascular effect after exogenous H_2S treatment, which was only reported in the systemic circulation previously. Furthermore, the H_2S induced relaxation is significantly inhibited by an anionic exchanger blocker in these vessels as well which reveals one, albeit not deciphered, detail regarding the vasodilatory mechanism of H_2S in the cerebral circulation.

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