Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 559 (2007) 65–68
www.elsevier.com/locate/ejphar

Short communication

Hydrogen sulfide inhibits human platelet aggregation

Giovanni Zagli [a,*], Riccardo Patacchini [b], Marcello Trevisani [c], Rosanna Abbate [a], Sandro Cinotti [d], Gian Franco Gensini [a], Giulio Masotti [a], Pierangelo Geppetti [a]

[a] Department of Critical Care Medicine and Surgery, University of Florence, Florence, Italy
[b] Department of Pharmacology, Chiesi Pharmaceuticals, Parma, Italy
[c] Department of Experimental Medicine and Clinical Medicine, Pharmacology Unit, University of Ferrara, Ferrara, Italy
[d] Hemophilia Center, Azienda Ospedaliero—Universitaria Careggi, Florence, Italy

Received 22 September 2006; received in revised form 16 November 2006; accepted 11 December 2006
Available online 17 January 2007

Abstract

Gaseous mediators such as nitric oxide (NO) play a major regulatory role in the cardiovascular system homeostasis, including platelet aggregation. Here, we investigated whether hydrogen sulfide (H_2S), a newly recognized endogenous mediator, can affects aggregation of human platelets, using sodium hydrogen sulfide (NaHS) as H_2S-donor. NaHS inhibited platelet aggregation induced by ADP, collagen, epinephrine, arachidonic acid, thromboxane mimetic, U46619, and thrombin. H_2S effect was not dependent by cAMP/cGMP generation, NO production or potassium-channels opening. NaHS concentrations (up to 10 mM) did not exert toxic effects on platelet viability. The possible protective role of endogenous H_2S in cardiovascular system is discussed.

Keywords: Hydrogen sulfide; H_2S; Platelet inhibition; Thromboembolic disease; Cardiovascular risk factor

1. Introduction

Hydrogen sulfide (H_2S) is a malodorous gas that, following acute/massive inhalation, may produce toxic effects (Beauchamp et al., 1984). However, H_2S is also endogenously generated from the metabolism of both cysteine and homocysteine (Moore et al., 2003). Micromolar levels of H_2S have been detected in brain, blood and other peripheral organs, mostly produced through two enzymatic pathways: cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) (Hosoki et al., 1997; Eto and Kimura, 2002). It has been shown that exogenously administered H_2S relaxes isolated blood vessels and induces transient hypotension *via* activation of ATP-sensitive K^+-channels (K_{ATP}) (Zhao et al., 2001). It has been speculated that endogenous H_2S, similarly to nitric oxide (NO), might contribute to the maintenance of the cardiovascular homeostasis. In fact, decreased of plasma level H_2S were found in systemic or pulmonary hypertensive rats (Chunyu et al., 2003; Yan et al., 2004), while normal blood pressure was restored by administration of NaHS, an H_2S generating salt (Yan et al., 2004). In contrast, massive hyperproduction of endogenous H_2S seems to be responsible for hypotension caused by experimental septic/endotoxic shock (Collin et al., 2005). The discovery that H_2S is endogenously generated within the heart, where it might play a cardioprotective function against ischemic injury (Pan et al., 2005; Bian et al., 2006), reinforces the hypothesis that H_2S might act as a protective agent in the cardiovascular system.

In the present study we have addressed the question whether H_2S can affect platelet aggregation. Our results indicate that H_2S inhibits aggregation of human platelets *in vitro* in a concentration-dependent manner, and suggest the possibility that changes of circulating H_2S might have a role in thromboembolic diseases.

2. Materials and methods

2.1. Human subjects

Approval of this study was obtained previously from the local Ethics Committee. Informed consent was provided

* Corresponding author. Postal address: Department of Pharmacology, Viale Pieraccini 6, 50134 Florence, Italy. Tel.: +39 055 4271417; fax: +39 055 4271280.
E-mail address: giovanni.zagli@unifi.it (G. Zagli).

0014-2999/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2006.12.011

according to the Declaration of Helsinki. All subjects were healthy volunteers of both sex, age 28–42 years.

2.2. Platelet aggregation assay

Human platelets were obtained from whole fresh blood drawn from donors who had not consumed any medication two weeks prior the test. Blood was collected in vacuum cuvettes with citrate-buffer (sodium citrate, 3.8%). Platelet-rich plasma (PRP) was obtained from blood after centrifugation at 180 ×*g* for 10 min at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation of the remaining blood at 1200 ×*g* for 10 min. Washed platelets (WP) were prepared as previously reported (Mustard et al., 1972). Briefly, PRP was diluted with ACD-buffer solution (sodium citrate 75 mM, citrate acid 42 mM, glucose 136 mM, pH 4.5), and centrifuged at 1000 ×*g* for 10 min. The pellet was gently re-suspended (2×10^8 cells/ml) in Hepes-buffer containing NaCl 145 mM, KCl 5 mM, Mg_2SO_4 1 mM, D-glucose10 mM, HEPES 10 mM, $CaCl_2$ 2 mM, bovine serum albumin 0.1%, pH 7.4.

All the experiments were performed using PRP, except for thrombin experiments in which WP was used. Platelet aggregation was monitored at 37 °C by measuring the variation of light transmission with Platelet Aggregation Profiler 4 (Biodata, Horsham, U.S.A.) as previously reported (Born, 1962). As source of H_2S in sodium-chloride solution (0.9%, vehicle), we used sodium hydrogen sulfide (NaHS), which, reacting with water, produces H_2S (Hosoki et al., 1997). All solution of NaHS were buffered to a pH of 7.4. In all experiments, NaHS was administered 20 s after each stimulus. In preliminary experiments NaHS, added 20 s before the aggregating stimulus, produced inhibitory effects that were indistinguishable from those obtained with NaHS added after the stimulus (data not shown). NaHS, ADP, epinephrine, collagen, SQ 22,536 (9-(Tetrahydro-2-furanyl)-9H-purin-6-amine), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), tetraethyl ammonium (TEA), arachidonic acid, thrombin, thromboxane-mimetic U46619, and L-NAME (*N*(G)-L-nitro-arginine methyl ester), were obtained from Sigma-Aldrich Srl (Milano, Italy). Forskolin and SNAP (*S*-nitroso-*N*-acetylpenicillamine) were obtained from Tocris Cookson Ltd (Bristol, UK).

2.3. Statistical analysis

All values are mean ± standard error of mean (S.E.M.) of 6–10 observations in duplicate. Each observation refers to a platelet sample taken from an individual volunteer. Comparisons were performed using the ANOVA test and the Bonferroni *post hoc* test when appropriate.

3. Results

NaHS prevented in a concentration-dependent manner platelet aggregation induced by different stimuli: ADP, U46619, collagen, epinephrine, thrombin and arachidonic acid (Figs. 1 panel A–D and 2 panel A). The highest concentration (10 mM) of NaHS was able to completely inhibit platelet aggregation whatever the agonist employed. IC_{50}s (concentration of NaHS producing 50% inhibition of full aggregation) of NaHS antiaggregating activity ranged from 698 μM (against ADP, 2 μM) to 5.62 mM (against U46619, 0.5 μM) (Fig. 1 panel D and C). Further, we tested the possibility that inhibition of aggregation by NaHS was due to a toxic effect. In the

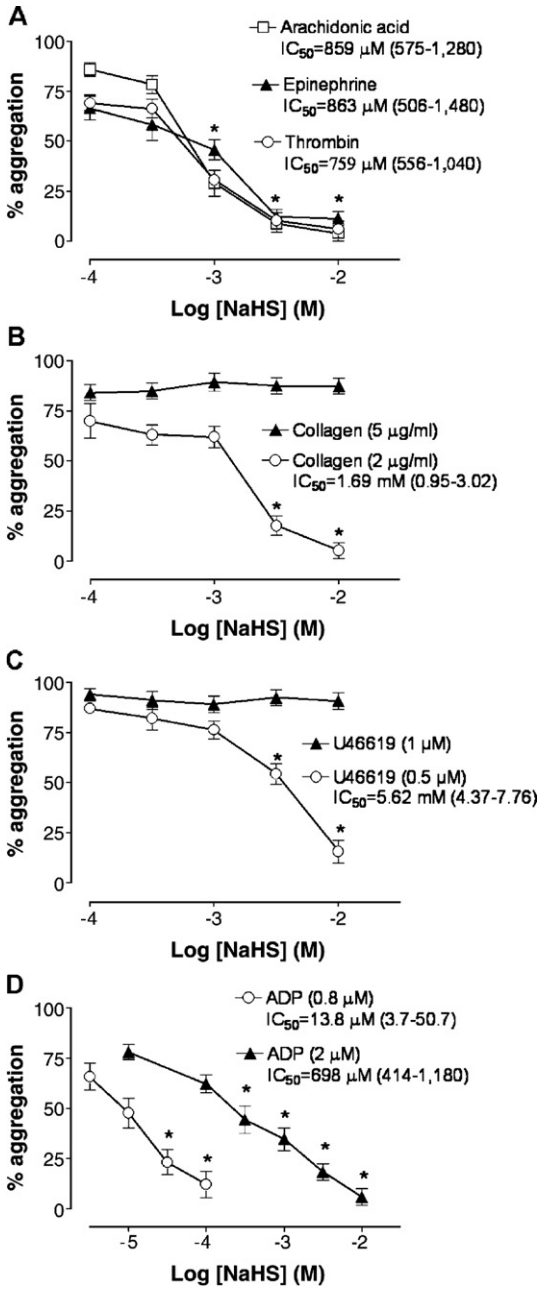

Fig. 1. Concentration-dependent inhibition by the H_2S donor, NaHS, of aggregation of human platelets induced by various stimuli. Concentration-response curves and IC_{50}s of NaHS on platelet aggregation induced by: arachidonic acid (□, 145 μg/ml), epinephrine (▲, 7 μM) and thrombin (○, 0.5 U/ml) (panel A); collagen (○, 2 μg/ml) and collagen (▲, 5 μg/ml) (panel B); U46619 (○, 0.5 μM) and U46619 (▲, 1 μM) (panel C); ADP (○, 0.8 μM) and ADP (▲, 2 μM) (panel D). Data are expressed as percent inhibition of aggregation and represent the mean ± S.E.M. of at least 6 experiments. *, $P<0.001$ *vs.* vehicle (NaCl 0.9%). IC_{50}s (concentration of NaHS producing 50% inhibition of full aggregation) are expressed as mean (95% confidence limits, in brackets).

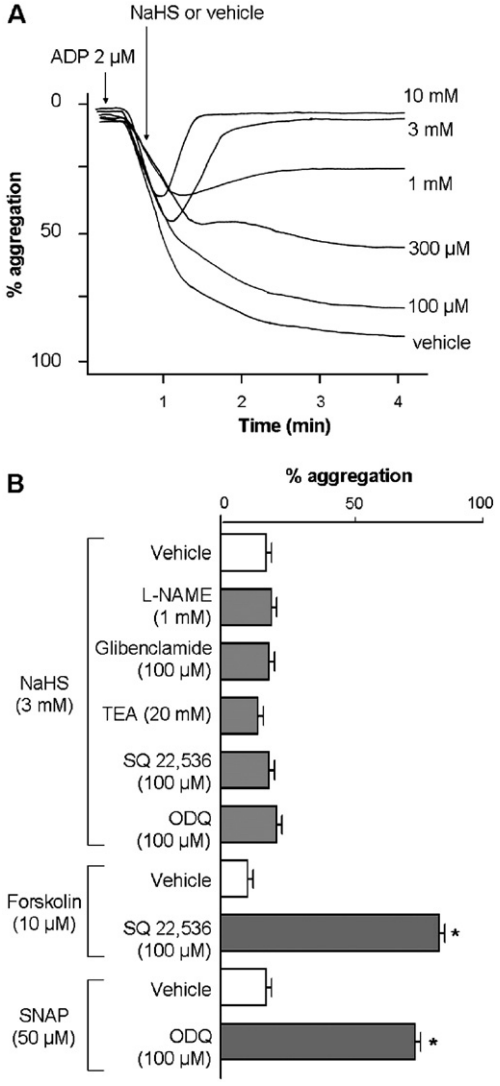

Fig. 2. Glibenclamide, TEA, SQ 22,536, ODQ and L-NAME do not reduce NaHS-induced platelet disaggregation. Inhibitory effect of NaHS on human platelet aggregation induced by ADP (2 μM) (panel A). Effect of glibenclamide, TEA, SQ 22,536, ODQ and L-NAME (added 30 min before) on the inhibitory effect produced by NaHS, forskolin or SNAP on aggregation of human platelets induced by ADP (2 μM) (panel B). Data are expressed as percent inhibition of aggregation and represent the mean ± S.E.M. of at least 6 experiments. *, $P<0.001$ *vs.* vehicle (NaCl 0.9%).

presence of 10 mM of NaHS, aggregation induced by collagen, 2 μg/ml (Fig. 1 panel B) and U46619, 0.5 μM (Fig. 1 panel C) was abated, but it was completely restored if the stimulus concentration was slightly increased (collagen, 5 μg/ml; U46619, 1 μM) (Fig. 1 panel B and C). Moreover, treatment of platelets with 10 mM of NaHS for 10 min did not show toxic effects determined by a trypan blue dye exclusion test (data not shown). Overall these data suggest that it is unlikely that NaHS inhibits platelet aggregation by affecting platelet viability.

To understand the mechanism(s) underlying H_2S-induced inhibition of platelet aggregation, we tested different inhibitor compounds (30 min incubation each) against NaHS (3 mM). The NO-synthase inhibitor, L-NAME (100 μM), was not able to affect the inhibitory action of NaHS on the aggregation induced by ADP (2 μM) (Fig. 2 panel B), thus, excluding the possibility that NaHS acts *via* endogenous NO generation. Inhibition of adenylyl cyclase by SQ 22,536 (100 μM) and of guanylyl cyclase by ODQ (100 μM) did not affect NaHS-induced inhibition of ADP-induced aggregation (2 μM). As expected, these interventions completely prevented the antiaggregating effects produced by forskolin (10 μM) and SNAP (50 μM), respectively (Fig. 2 panel B). These results rule out cGMP or cAMP generation as mediators of the antiaggregating effect of NaHS. A reported mechanism by which H_2S produces vascular smooth muscle relaxation, and that might contribute to the antiplatelet activity of NaHS, is the opening of K_{ATP}-channels (Zhao et al., 2001). However, neither glibenclamide (100 μM) nor the unselective K^+-channel blocker, TEA (20 mM), was able to affect the inhibition induced by NaHS (Fig. 2 panel B). These results exclude the role of K_{ATP} and other K^+-channel in H_2S-induced antiaggregation.

The IC_{50} values of NaHS against the different aggregating stimuli employed, exceed the physiological concentrations of H_2S detected in human plasma (10–100 μM) (Richardson et al., 2000). Therefore, we wondered whether concentrations close to those measured in human plasma could be sufficient to determine a measurable inhibition of platelet aggregation. Significant inhibition of platelet aggregation induced by a moderate ADP concentration (0.8 μM), was obtained with NaHS concentrations as low as 30 μM (Fig. 1 panel D).

4. Discussion

The mechanism by which NaHS/H_2S inhibits platelet aggregation remains undetermined. Disulfide bond rearrangement, a dynamic process in cell and protein function, has been recently emphasized for platelet function (Essex, 2004). The importance of disulfide-metabolism in platelets has been highlighted by the demonstration that redox potentials modulate platelet activation by the modification of sulfhydryl exposure in integrins (Walsh et al., 2004). As H_2S is known to reduce disulfide bridge in proteins (Beauchamp et al., 1984; Beck et al., 1983), it could be hypothesized that H_2S might exert antiaggregating effect by interfering with disulfide metabolism of critical proteins for platelet activation.

These observations could have important implications. Firstly, the hypothesis may be advanced that, as for H_2S, other molecules that have the capability to release H_2S may exert antiaggregating properties. Thus, the present study may burst pharmacological research on novel H_2S-donors as a new class of antiaggregating agents. A second main consequence of the present results considers the possibility that endogenous H_2S regulates platelet aggregability, and by this mechanism contributes to cardiovascular homeostasis. H_2S concentrations that completely inhibit platelet aggregation are clearly unphysiological. However, the potency of H_2S to inhibit platelet aggregation depends on the strength of the stimulus, and H_2S concentrations close to those found in human plasma exhibited antiaggregating effects (Fig. 1 panel D). Thus, it could be theorized that physiological H_2S concentrations in plasma may be sufficient to prevent platelet aggregation induced by moderate stimulation.

A consequence of this hypothesis is that high levels of H_2S may favour platelet disaggregation and *vice versa*. In this respect, it is intriguing to note that patients with Down's Syndrome, who have (≈50%) increased H_2S production and low homocysteine level because of CBS hyperactivity (Belardinelli et al., 2001; Pogribna et al., 2001), show a low incidence of thromboembolic fatal events such as myocardial infarction (Ho and Nguyen, 2003). Consistently with this observation, a 30 year-long retrospective study, performed on 25,292 petroleum workers exposed to H_2S-inhalation, has shown that the mortality for myocardial infarction was reduced by ≈50% compared to that expected in normal population (Lewis et al., 2003).

Further studies are needed to confirm the role of endogenous H_2S in platelets homeostasis and to assess whether low H_2S plasma level may represent a risk factor for cardiovascular/thromboembolic diseases.

Acknowledgements

The study was partially supported by Grants from *Fondazione Cassa di Risparmio di Firenze* and from *Fondazione DEI-Onlus*.

References

Beauchamp Jr., R.O., Bus, J.S., Popp, J.A., Boreiko, C.J., Andjelkovich, D.A., 1984. A critical review of the literature on hydrogen sulfide toxicity. Crit. Rev. Toxicol. 13, 25–97.

Beck, J.F., Donini, J.C., Maneckjee, A., 1983. The influence of sulfide and cyanide on axonal function. Toxicology 26, 37–45.

Belardinelli, M.C., Chabli, A., Chadefaux-Vekemans, B., Kamoun, P., 2001. Urinary sulfur compounds in Down syndrome. Clin. Chem. 47, 1500–1501.

Bian, J.S., Yong, Q.C., Pan, T.T., Feng, Z.N., Ali, M.Y., Zhou, S., Moore, P.K., 2006. Role of hydrogen sulfide in the cardioprotection caused by ischemic preconditioning in the rat heart and cardiac myocytes. J. Pharmacol. Exp. Ther. 316, 670–678.

Born, G.V., 1962. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 194, 927–929.

Chunyu, Z., Junbao, D., Dingfang, B., Hui, Y., Xiuying, T., Chaoshu, T., 2003. The regulatory effect of hydrogen sulfide on hypoxic pulmonary hypertension in rats. Biochem. Biophys. Res. Commun. 302, 810–816.

Collin, M., Anuar, F.B., Murch, O., Bhatia, M., Moore, P.K., Thiemermann, C., 2005. Inhibition of endogenous hydrogen sulfide formation reduces the organ injury caused by endotoxemia. Br. J. Pharmacol. 146, 498–505.

Essex, D.W., 2004. The role of thiols and disulfides in platelet function. Antioxid. Redox Signal. 6, 736–746.

Eto, K., Kimura, H., 2002. A novel enhancing mechanism for hydrogen sulfide-producing activity of cystathionine beta-synthase. J. Biol. Chem. 277, 42680–42685.

Ho, P.C., Nguyen, M., 2003. Acute two-vessel coronary closure in a patient with Down's syndrome. Int. J. Cardiovasc. Interv. 5, 95–97.

Hosoki, R., Matsuki, N., Kimura, H., 1997. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. Biochem. Biophys. Res. Commun. 237, 527–531.

Lewis, R.J., Schnatter, A.R., Drummond, I., Murray, N., Thompson, F.S., Katz, A.M., Jorgensen, G., Nicolich, M.J., Dahlman, D., Theriault, G., 2003. Mortality and cancer morbidity in a cohort of Canadian petroleum workers. Occup. Environ. Med. 60, 918–928.

Moore, P.K., Bhatia, M., Moochhala, S., 2003. Hydrogen sulfide: from the smell of the past to the mediator of the future? Trends Pharmacol. Sci. 24, 609–611.

Mustard, J.F., Perry, D.W., Ardlie, N.G., Packham, M.A., 1972. Preparation of suspensions of washed platelets from humans. Br. J. Haematol. 22, 193–204.

Pan, T.T., Feng, Z.N., Lee, S.W., Moore, P.K., Bian, J.S., 2005. Endogenous hydrogen sulfide contributes to the cardioprotection by metabolic inhibition preconditioning in the rat ventricular myocytes. J. Mol. Cell. Cardiol. 30, 30.

Pogribna, M., Melnyk, S., Pogribny, I., Chango, A., Yi, P., James, S.J., 2001. Homocysteine metabolism in children with Down syndrome: in vitro modulation. Am. J. Hum. Genet. 69, 88–95.

Richardson, C.J., Magee, E.A., Cummings, J.H., 2000. A new method for the determination of sulphide in gastrointestinal contents and whole blood by microdistillation and ion chromatography. Clin. Chim. Acta 293, 115–125.

Walsh, G.M., Sheehan, D., Kinsella, A., Moran, N., O'Neill, S., 2004. Redox modulation of integrin [correction of integin] alpha IIb beta 3 involves a novel allosteric regulation of its thiol isomerase activity. Biochemistry 43, 473–480.

Yan, H., Du, J., Tang, C., 2004. The possible role of hydrogen sulfide on the pathogenesis of spontaneous hypertension in rats. Biochem. Biophys. Res. Commun. 313, 22–27.

Zhao, W., Zhang, J., Lu, Y., Wang, R., 2001. The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO J. 20, 6008–6016.