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Role of endogenous hydrogen sulfide in nerve-evoked relaxation of pig terminal bronchioles☆

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ABSTRACT

Hydrogen sulfide (H_2S) is a gasotransmitter employed for intra- and inter-cellular communication in almost all organ systems. This study investigates the role of endogenous H_2S in nerve-evoked relaxation of pig terminal bronchioles with 260 μm medium internal lumen diameter. High expression of the H_2S synthesis enzyme cystathionine γ-lyase (CSE) in the bronchiolar muscle layer and strong CSE-immunoreactivity within nerve fibers distributed along smooth muscle bundles were observed. Further, endogenous H_2S generated in bronchiolar membranes was reduced by CSE inhibition. In contrast, cystathionine β-synthase expression, another H_2S synthesis enzyme, however was not consistently detected in the bronchiolar smooth muscle layer. Electrical field stimulation (EFS) and the H_2S donor *P*-(4-methoxyphenyl)-*P*-4-morpholinylphosphinodithioic acid (GYY4137) evoked smooth muscle relaxation. Inhibition of CSE, nitric oxide (NO) synthase, soluble guanylyl cyclase (sGC) and of ATP-dependent K^+, transient receptor potential A1 ($TRPA_1$) and transient receptor potential vanilloid 1 ($TRPV_1$) channels reduced the EFS relaxation but failed to modify the GYY4137 response. Raising extracellular K^+ concentration inhibited the GYY4137 relaxation. Large conductance Ca^{2+}-activated K^+ channel blockade reduced both EFS and GYY4137 responses. GYY4137 inhibited the contractions induced by histamine and reduced to a lesser extent the histamine-induced increases in intracellular $[Ca^{2+}]$. These results suggest that relaxation induced by EFS in the pig terminal bronchioles partly involves the H_2S/CSE pathway. H_2S response is produced via NO/sGC-independent mechanisms involving K^+ channels and intracellular Ca^{2+} desensitization-dependent pathways. Thus, based on our current results H_2S donors might be useful as bronchodilator agents for the treatment of lung diseases with persistent airflow limitation, such as asthma and chronic obstructive lung disease.

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Abbreviations: $[Ca^{2+}]_i$, intracellular calcium (Ca^{2+}) concentration; AMG9810, (2*E*)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide; BK, large conductance Ca^{2+}-activated K^+ channels; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; EFS, electrical field stimulation; GYY4137, *P*-(4-methoxyphenyl)-*P*-4-morpholinyl phosphinedithioic acid; HC030031, 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7*H*-purin-7-yl)-*N*-(4-isopropylphenyl)acetamide; H_2S, hydrogen sulfide; IbTX, iberiotoxin; L-NOARG, N^G-nitro-L-arginine; NaHS, sodium hydrosulfide; NO, nitric oxide; ODQ, 1*H*-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one; PPG, DL-propargylglycine; sGC, soluble guanylyl cyclase; $TRPA_1$, transient receptor potential A1; $TRPV_1$, transient receptor potential vanilloid 1.

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1. Introduction

Hydrogen sulfide (H_2S) has been proposed to be the third endogenous gaseous transmitter besides nitric oxide (NO) and carbon monoxide (CO) [1,2]. In mammalian tissues, endogenous H_2S is synthesized from L-cysteine by the action of two pyridoxal-5′-phosphate-dependent enzymes, cystathionine γ-lyase (CSE) or cystathionine β-synthase (CBS) [1–4]. The CBS-linked route is a predominant source of H_2S in the central nervous system, whereas CSE is the H_2S synthesis major enzyme in the cardiovascular system [5]. H_2S exerts a wide range of peripheral biological actions, such as neurotransmission, insulin secretion regulation and vasorelaxation [6]. H_2S has also anti-inflammatory and anti-proliferative effects, with inhibitory effects in models of lung inflammation and fibrosis [7,8].

The effect of gaseous compounds on airways reactivity has widely been studied. For instance, endogenously-released [9,10] or exogenously-added [11] nitric oxide (NO), has been shown to relax bronchial strips via soluble guanylyl cyclase (sGC)-dependent mechanisms involving the activation of large conductance Ca^{2+}-activated K^+ (BK) channels. However, NO-releasing compounds, such as nitrates and nitrosothiols, have been found to be relatively ineffective as bronchodilators in lung diseases such as asthma [12] due to either a short half-life and the development of nitrate tolerance. Thus, compounds not inducing nitrate tolerance and with a longer duration than classic organic nitrate esters have been developed [13]. In this sense, NO donors such as GEA3175 induce a slow-developing relaxation of bovine bronchioles, via the NO/sGC/cGMP pathway, and involving K^+ channels [14].

Recently, Rashid et al. [15] demonstrated H_2S production, CSE and CBS expression and sodium hydrosulfide (NaHS)-induced smooth muscle relaxation in 5 mm diameter porcine airways. Therefore understanding the mechanism involved in the regulation of the bronchiolar smooth muscle tension is essential in order to provide bronchodilator agents effective for the treatment of lung diseases with persistent airflow limitation, such as asthma and chronic obstructive lung disease. Thus, the current study investigates the role of H_2S in the neurogenic relaxation elicited by electrical field stimulation (EFS) in the porcine terminal bronchioles.

2. Material and methods

2.1. Tissue

Pigs of either sex with no lesions in their respiratory tract were selected, under veterinary supervision, from the local slaughterhouse. Apices of the lungs were removed immediately after the animals were killed and transported to the laboratory in cold (4 °C) physiological saline solution. Bronchiolar rings were carefully dissected removing the adhering pulmonary parenchymal tissue and bronchiolar artery.

2.2. Western blot

Bronchiole smooth muscle was homogenized in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 1% SDS, 1 mM sodium vanadate and 0.01% protease inhibitor cocktail (all from Sigma-Aldrich, St Louis, MO, USA). 50 μg proteins were separated in a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Madrid, Spain). All membranes were blocked by 5% non-fat dry milk for 1 h at room temperature. For immunodetection, membranes were incubated overnight at 4 °C with rabbit anti-CSE (CTH antibody, H-167: sc-135203) or anti-CBS (H-300: sc-67154) (1:500 dilution, Santa Cruz Biotechnology Inc., Heidelberg, Germany) and mouse anti-β-actin (1:20,000 dilution, from Santa Cruz Biotechnology Heidelberg, Germany) antibodies. Membranes were then washed in 0.05% Tween-20, incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and then washed and visualized by chemiluminescence (ECL advance-kit, GE Healthcare, Madrid, Spain). Anti-CSE and CBS bands were normalized to those of β-actin. CSE and CBS expression in pig urinary bladder neck membranes were included as positive controls [16].

2.3. Immunohistochemistry

Bronchiolar segments were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2–4 h at 4 °C, and subsequently placed in 30% sucrose in phosphate buffer for cryoprotection. The tissue was embedded and frozen in optimum cutting temperature compound (Sakura Finetek, Europe BV, Alphen aan den Rijn, The Netherlands), and stored at −80 °C. Transversal sections 5 μm thick were obtained by means of a cryostat and preincubated in 10% normal goat serum in PB containing 0.3% Triton-X-100, for 2–3 h. Then, sections were incubated with rabbit anti-CSE or anti-CBS antibodies at 4–8 μg/ml final concentration, plus a mouse anti-protein gene product 9.5 (anti-PGP 9.5, Abcam, Cambridge, UK), as neuronal marker, diluted 1:50, during 48 h at 4 °C, washed and reacted with the secondary antibodies Alexa Fluor 594 goat-antirabbit, 1:200 dilution, to detect CSE and CBS, and Alexa Fluor 488 goat-antimouse, 1:200 dilution, to detect PGP 9.5, (both antibodies from Invitrogen, Life Technologies, Madrid, Spain), for 2 h at room temperature. The slides were covered with a specific mounting medium with 4′6-diamidine-2-phenylindole (DAPI) (Invitrogen, Life Technologies, Madrid, Spain), which stains all cell nuclei. Observations were made with a fluorescence microscope (Olympus IX51, Olympus Europe SE & Co., Hamburg, Germany). No immunoreactivity could be detected in sections incubated in the absence of the primary antiserum [16,17].

2.4. Endogenous H_2S measurement

Endogenous bronchiolar H_2S production and release was measured essentially as previously described [18]. Briefly, tissues were homogenized (1:10 w/v) in ice-cold PB. The assay mixture for the tissue homogenate consisted of 10 mM L-cysteine, 2 mM pyridoxal 5′-phosphate, and saline. To examine the role of an irreversible CSE inhibitor, a NO synthase inhibitor, and a NO donor, 1 mM DL-propargylglycine (PPG), 100 μM N^G-nitro-L-arginine (L-NOARG), or 100 μM (*S*)-nitroso-*N*-acetylpenicillamine (SNAP), respectively, were added separately to the tissue homogenate assay mixture. The reaction was carried out in air-tight cryopreservation tubes. It was initiated by transfer of the tubes from ice to a shaking water bath at 37 °C. After incubation for 30 min, 1% zinc acetate was injected to trap-generated H_2S, and this was followed by 10% trichloroacetic acid to denature the protein and thus stop the reaction. Subsequently, 20 μM *N*,*N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 M HCl was added, and this was immediately followed by 30 μM $FeCl_3$ in 1.2 M HCl. This reaction mixture was then centrifuged at 4.500*g* for 10 min. After 20 min, 200 μl aliquots of the supernatants were transferred to a 96-well microplate and the absorbance was measured at 670 nm (ELx800 microplate reader, Izasa S.A., Barcelona, Spain). The H_2S concentration of each sample was calculated against a calibration curve of NaHS (0.12–250 μM). Results are expressed as nanomoles of H_2S formed per mg of soluble protein per 20 min. Protein was determined with the Lowry assay (DC Protein Assay Kit, Bio-Rad, Madrid, Spain).

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