Redox Biology 18 (2018) 295–304

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

A selective and sensitive method for quantification of endogenous polysulfide production in biological samples

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ARTICLE INFO

Keywords:
Polysulfide quantification
LC-MS/MS
SSP4
Biological samples

ABSTRACT

Hydrogen sulfide (H_2S) is a gasotransmitter that regulates cellular homeostasis and impacts on multiple physiological and pathophysiological processes. However, it exerts many of its biological actions indirectly via the formation of H_2S-derived sulfane sulfur species/polysulfides. Because of the high reactivity of sulfur species, the detection of H_2S-derived polysulfides in biological systems is challenging and currently used methods are neither sensitive nor quantitative. Herein, we describe a LC-MS/MS-based method that makes use of Sulfane Sulfur Probe 4 to detect endogenously generated polysulfides in biological samples in a selective, sensitive and quantitative manner. The results indicate a large variability in the activity of the H_2S-generating enzymes in different murine organs, but the method described was able to detect intracellular levels of polysulfides in the nanomolar range and identify cystathionine γ-lyase as the major intracellular source of sulfane sulfur species/polysulfides in murine endothelial cells and hearts. The protocol described can be applied to a variety of biological samples for the quantification of the H_2S-derived polysulfides and has the potential to increase understanding on the control and consequences of this gaseous transmitter.

1. Introduction

Hydrogen sulfide (H_2S) is a ubiquitously generated gaseous signaling mediator, or "gasotransmitter" that plays an important role in the regulation of numerous cellular functions, as well as physiological and pathophysiological processes [1]. In mammalian cells H_2S is predominately produced during the metabolism of L-cysteine by two pyridoxal-5′-phosphate-dependent enzymes i.e. cystathionine-β-synthase (CBS), and cystathionine-γ-lyase (CSE), or during the catabolism of 3-mercaptopyruvate by 3-mercaptopyruvate sulfurtransferase (3MST) [2,3]. H_2S generating enzymes show distinct tissue-specific distribution patterns and intracellular compartmentalization [1].

Abnormal H_2S production has been associated with different pathological states in the gastrointestinal, cardiovascular and central nervous systems [4]. However, it is currently difficult to convincingly link H_2S with specific pathologies because of the lack of a widely accepted, easy to use, sensitive and selective assay for its quantification. The reliable detection of H_2S in biological samples is hampered by the fact that the gas is volatile, redox reactive and steady-state concentrations are low [3]. These difficulties, combined with the use of methods that are not specific for measurement of free sulfides at physiological pH, have resulted in reports of H_2S concentrations spanning five orders of magnitude i.e. ranging from the low nanomolar to hundreds of micromolar range [5,6]. This ambiguity in the physiologically relevant concentration of H_2S has, in turn, resulted in the use of widely varying concentrations of sulfide donors to elicit physiological effects.

H_2S-related sulfane sulfur compounds include persulfides (R-S-SH), polysulfides (R-S_n-SH or R-S-S_n-S-R), inorganic hydrogen polysulfides (H_2S_n, n ≥ 2) and protein-bound elemental sulfur (S8). Recent studies have suggested that polysulfides are generated endogenously following oxidation of the endogenously released H_2S and can serve as a sink of H_2S. Indeed, many of the biological effects currently attributed to H_2S

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https://doi.org/10.1016/j.redox.2018.07.016
Received 10 June 2018; Received in revised form 8 July 2018; Accepted 19 July 2018
Available online 21 July 2018

may in fact be due to polysulfides [7]. Current methods used for the detection of H_2S and polysulfides include the colorimetric assay of methylene blue formation, a sulfide ion-selective or a polarographic electrode, gas chromatography with flame photometric or sulfur chemiluminescence detection, ion chromatography, HPLC analysis of the monobromobimane derivative of sulfide, and the use of sulfide-sensitive fluorescent dyes (for review see [8]). Few of these different methods can be applied to living cells because of their destructive nature. More recently, luminescence probes have been used to detect H_2S, however, the biological applications of this technique require advanced imaging equipment and the selectivity of the method has been questioned [7]. The aim of this study, therefore, was to develop a sensitive LC-MS/MS based method for the detection of endogenously generated H_2S-derived polysulfides and hydropersulfides in living cells and tissues as well as in frozen samples. The assay is based on the reaction of polysulfides with Sulfane Sulfur Probe 4 (SPP4), which has been used by several research groups [9–11].

2. Materials and methods

2.1. Materials

Sulfane Sulfur Probe 4 (SSP4) and sodium trisulfide (Na_2S_3) were from Sulfobiotics (Dojindo EU GmbH, Munich, Germany), cell culture media were from Gibco (Invitrogen; Darmstadt, Germany) and the phenol red-free endothelial cell growth medium (EGM) was from PELObiotech (Planegg/Martinsried, Germany). Sodium polysulthionate (SG1002) was from Sulfagenix Inc. (Melbourne, Australia) and 2-[(4-hydroxy-6-methylpyrimidin-2-yl)sulfanyl]-1-(naphthalen-1-yl)ethanone (Inhibitor 3) was from Molport (AppliChem, Darmstadt, Germany). The anti-CSE antibody was from Proteintech (Manchester, UK), anti-CBS was from Abnova (Germany), anti-3MST was from Atlas (Bromma, Sweden), the antibody against telomerase was from Abcam (Cambridge, UK). Secondary anti-rabbit and anti-mouse antibodies were obtained from Calbiochem (Darmstadt, Germany). The antibody against β-actin was from Sigma-Aldrich (Darmstadt, Germany) and all other chemicals and reagents were purchased either from Sigma-Aldrich (Darmstadt, Germany) or AppliChem (Darmstadt, Germany).

2.2. Animals

$CSE^{-/-}$ mice and floxed CSE (C57/Bl6J $CSE^{fl/fl}$) mice [12] were kindly provided by Josef Pfeilschifter (Frankfurt, Germany). $CSE^{fl/fl}$ mice were crossed with tamoxifen-inducible Cdh5-CreERT2 mice [13], to generate animals specifically lacking CSE in endothelial cells ($CSE^{i\Delta EC}$ mice) as described [14]. Mice were housed in conditions that conform to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication no. 85-23). Animals received standard chow and 8–10 week old mice of both genders were studied.

2.3. Cell isolation and culture

Human umbilical vein endothelial cells were isolated and cultured as described [15], and confluent cells up to passage 1 were used. The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki and the isolation of endothelial cells was approved in written form by the ethics committee of the Goethe University. Murine lung endothelial cells were isolated from either wild-type or $CSE^{i\Delta EC}$ mice and cultured up to passage 8, as described [16].

For H_2S_n determination, cells were seeded to 48 well plates pre-coated with fibronectin. To induce CSE deletion, cells from untreated $CSE^{i\Delta EC}$ mice (passage 4) were treated *in vitro* with 4-OH-tamoxifen (Sigma, Darmstadt, Germany) for 7 days. Thereafter, 4-OH-tamoxifen was removed and the cells were passaged for additional 3 times before use, as a control, endothelial cells from wild-type mice were also treated with 4-OH-tamoxifen.

2.4. Adenoviral generation and infection protocols

Adenoviruses for the overexpression of green fluorescent protein (GFP), CBS, CSE and 3MST were generated as described [17–19]. Human endothelial cells (passage 1, 80% confluent) and murine endothelial cells (passage 5–8, 80% confluent) were starved of serum for 4 h in endothelial growth medium (EGM) containing 0.1% BSA. Adenoviruses (10 MOI) were incubated for 30 min with AdenoBoost (Sirion Biotech GmbH, Martinsried, Germany) and then added to the endothelial cells for 4 h (37 °C). The culture medium was then replaced and cells were maintained in culture for 24 h before H_2S_n generation was measured.

2.5. Platelet isolation

Platelets were obtained by centrifugation (900*g*, 7 min) of platelet-rich plasma, as described [20]. The resulting pellet was washed in Ca^{2+}-free HEPES buffer (mM: NaCl, 136; KCl, 2.6; $MgCl_2$, 0.93; NaH_2PO_4, 3.26; glucose, 5.5; HEPES, 3.7; pH 7.4 at 37 °C) and samples were re-suspended in HEPES buffer.

2.6. Immunoblotting

Samples (cells or tissues) were lysed in ice-cold RIPA buffer (mM: 50 Tris HCl pH 7.5, 150 NaCl, 25 NaF, 10 $Na_4P_2O_7$, 1% Triton X-100 and 0.5% sodium deoxycholate) and total proteins were separated by SDS-PAGE, subjected to Western blotting and visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, Germany), as described [16].

2.7. Methylene Blue measurement

Methylene Blue detection was performed as described [21] and a standard curve of NaHS in ddH_2O (up to 100 μM) was used for quantification.

2.8. 7-Azido-4-Methylcoumarine (AzMc) assay

The reaction of AzMC to sulfur products was evaluated as described [22] and a standard curve of NaHS in ddH_2O (up to 100 μM) was used for quantification.

2.9. Sample preparation

2.9.1. Assay of H_2S_n in cultured cells

Culture medium was removed from confluent cultures of cells and washed once with warm Hanks buffer. EGM supplemented with 0.1% BSA (200 μL) was added and after 2 h was substituted with phenol-free EGM containing SSP4 (10 μM; always prepared freshly and kept in the dark). Depending on the aims of particular experiments the medium was supplemented with either substrates; L-cysteine (L-Cys, 100 μM), L-homocysteine (L-Hcy, 100 μM), 3-mercaptopyruvate (3MP, 100 μM) and pyridoxal-5′-phosphate (PLP, 10 μM) or inhibitors; aminoxy-acetate (AOAA, 1 mM), DL-proparglycine (PAG, 1 mM) and Inhibitor 3 (10 μM). Solvent-treated groups contained a final concentration of 0.1% DMSO. After 60 min (37 °C), the cell supernatant was collected in Eppendorf tubes and any detached cells were removed by centrifugation at 16,000*g* (4 °C, 10 min). Then 80% of the supernatant was collected for LC-MS/MS or fluorescence measurements. Thereafter, the cell pellet was washed twice with phosphate buffered saline (PBS) and lysed in ice-cold RIPA buffer for immunoblotting.

To determine whether or not SSP4 was cell permeable, experiments were repeated as above, with the exception that the cell pellet was washed twice with PBS and snap frozen in liquid N_2. For the