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Research paper

Hydrogen sulfide metabolism regulates endothelial solute barrier function

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ABSTRACT

Hydrogen sulfide (H_2S) is an important gaseous signaling molecule in the cardiovascular system. In addition to free H_2S, H_2S can be oxidized to polysulfide which can be biologically active. Since the impact of H_2S on endothelial solute barrier function is not known, we sought to determine whether H_2S and its various metabolites affect endothelial permeability. In vitro permeability was evaluated using albumin flux and transendothelial electrical resistance. Different H_2S donors were used to examine the effects of exogenous H_2S. To evaluate the role of endogenous H_2S, mouse aortic endothelial cells (MAECs) were isolated from wild type mice and mice lacking cystathionine γ-lyase (CSE), a predominant source of H_2S in endothelial cells. In vivo permeability was evaluated using the Miles assay. We observed that polysulfide donors induced rapid albumin flux across endothelium. Comparatively, free sulfide donors increased permeability only with higher concentrations and at later time points. Increased solute permeability was associated with disruption of endothelial junction proteins claudin 5 and VE-cadherin, along with enhanced actin stress fiber formation. Importantly, sulfide donors that increase permeability elicited a preferential increase in polysulfide levels within endothelium. Similarly, CSE deficient MAECs showed enhanced solute barrier function along with reduced endogenous bound sulfane sulfur. CSE siRNA knockdown also enhanced endothelial junction structures with increased claudin 5 protein expression. In vivo, CSE genetic deficiency significantly blunted VEGF induced hyperpermeability revealing an important role of the enzyme for barrier function. In summary, endothelial solute permeability is critically regulated via exogenous and endogenous sulfide bioavailability with a prominent role of polysulfides.

1. Introduction

Endothelial cells form the innermost layer of blood vessels and serve as a barrier confining blood within the vessel. This barrier function exhibits heterogeneity across different vascular beds to meet local tissue microenvironments and functions [3]. Endothelial barriers in large to medium vessels throughout the body are considered to be largely impermeable to various molecules in the blood under physiological conditions. In contrast, as the major exchange vessels, capillaries witness continuous exchange of soluble contents between blood and the interstitial tissue. On the other hand, permeability can be induced in other vascular beds (predominantly in the post-capillary vanules) where basal permeability is low. Increased permeability is an essential part of acute and chronic inflammation and is critical for compartmentalization and the recruitment of inflammatory cells. Increased permeability may also favor angiogenesis and tissue repair by changing extracellular matrix composition and obliterate contact inhibition between cells [2,15]. Therefore, endothelial permeability plays critical roles in vascular functions. The integrity and complexity of intercellular junctions, including tight junctions and adherens junctions, regulate endothelial permeability. Permeability enhancers, such as vascular endothelial growth factor (VEGF), cause cell contraction and junction disruption resulting in intercellular gaps and increased permeability[21].

Hydrogen sulfide (H_2S), as a gaseous signaling molecule, has been demonstrated to be beneficial for a range of cardiovascular diseases, including peripheral and cardiac ischemia [6,24,25], atherosclerosis[30]. Although we know that H_2S is important for

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cardiovascular health, its effects on endothelial barrier function remain unclear. A few studies investigated the role of H_2S on hyperpermeability induced by particulate matter in the lung vasculature and ischemia/reperfusion injury in brain microvessels [14,19,41]. Yet the complexity of H_2S metabolism has not been taken into consideration.

In endothelial cells, cystathionine γ-lyase (CSE) is considered to be a predominant source of H_2S [40]. As a by-product of the transsulfuration pathway, the level of free sulfide is low (i.e. H_2S, HS^- and S^{2-}). However, in the presence of free radicals, metal ions and myeloperoxidase, thiyl radicals can be formed leading to persulfide and polysulfide compound. Others and we have also reported that H_2S can be mobilized from biochemical reservoirs such as acid labile sulfur (iron sulfur clusters), and sulfane sulfur (e.g. thiosulfide, protein per/polysulfide) [23,36,37]. Moreover, increasing evidence suggests that sulfane sulfur serves as an active form of H_2S that is highly potent and may be responsible for some of H_2S biological effects [18,29]. To better understand the effect of H_2S on endothelial permeability, we examined how exogenous and CSE derived endogenous H_2S could regulate endothelial permeability.

2. Materials and methods

2.1. Animals

All mice used in this study were housed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. 12–20 week old male C57BL/6 J wild-type and CSE knockout mice were used for experiments. All animal studies were approved by the LSU Health-Shreveport institutional animal care and use committee (Protocol Number: P-14-040).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) (FC-0044, Lifeline Cell Technology) were maintained in endothelial growth medium (LL-0003, Lifeline Cell Technology) with 10% fetal bovine serum (FBS) at 37 °C. Mouse aortic endothelial cells (MAECs) were isolated from wild-type and CSE knockout mice. Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (8 mg/kg) and perfused with 5 ml phosphate buffered saline (PBS). Thoracic aortae were dissected and cut into 0.5 mm thick rings. Aortic rings pooled from 3 mice were seeded on Matrigel (354,234, Corning) in a 6-well plate and cultured in endothelial growth medium for 5–7 days for endothelial cells to sprout. After removal of aortic ring, Matrigel was digested by dispase (354,235, Corning). Cells were sorted for endoglin (CD105) (13–1051-85, eBioscience) using Dynabeads M-280 Streptavidin (11205D, Invitrogen) and immortalized using temperature sensitive SV40 large T antigen by retrovirus. Isolated MAECs were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) (15–013-CV, Corning) with 10% FBS supplemented with Recombinant mouse interferon gamma (IFN-γ) (50-990-843, Fisher Scientific) at 33 °C. For experiments, MAECs are kept at 37 °C without INF-γ for at least 48 h for inactivation of large T-antigens. Both HUVECs and MAECs were starved overnight with low serum medium before experiments. Transfection was performed using siRNA oligos and lipofectamine 2000 (11,668-019, Thero Fisher Scientific).

2.3. In vitro permeability assay

Solute permeability was tested using 6.5 mm transwell inserts with 0.4 μm pore size (07–200-154, Fisher Scientific) as previously described [42]. Transwell inserts were coated with fibronectin. Endothelial cells were trypsinized and reconstituted in medium at the concentration of 40,000 cells/ml. 250 μl cells (10,000 cells) were seeded in fibronectin coated transwell inserts and 1370 μl medium was added to the bottom chamber. Cells were cultured for 36 h without changing medium. At the time of study, 25 μl albumin–fluorescein isothiocyanate (FITC) conjugate (10 mg/ml) (A9771–100MG, Sigma-Aldrich) with or without sulfide donors was added to inserts. At certain time points, 50 μl medium was collected from bottom chambers with 50 μl fresh medium added back immediately. Sampled medium was diluted and fluorescence was measured at 495 nm/519 nm. For MAECs, phenol red free DMEM (17–205-CV, Corning) was used for transwell permeability assays. For transendothelial electrical resistance (TEER), transwell inserts with endothelial monolayers were transferred to a cell culture plate embedded with chopstick electrodes [11]. Cells were allowed to rest for 20 min before the resistance was measured using EVOM (World Precision Instruments).

2.4. In vivo permeability assay

In vivo permeability was examined by the modified Miles assay as previously reported [8]. Evans blue (EB) (100 μl, 1% w/v in normal saline) was delivered by tail vein immediately followed by saline, vascular endothelial growth factor 165 (VEGF 165) (293-VE-010) and sulfide donor injections in the ear pinna. After 30 min, mice were euthanized and perfused with 5 ml PBS. Ears and internal organs were harvested. Samples were kept in microcentrifuge tubes and dried at 95 °C overnight on a heat block. EB were extracted from dried tissue in 500 μl formamide at 55 °C for 2 days. EB concentration was determined by absorbance at 630 nm.

2.5. Immunocytochemistry and immunoblotting

For immunocytochemistry (ICC), cells were cultured on a glass coverslip and fixed with 4% formaldehyde and permeabilized using 0.1% Triton X-100. Coverslips were blocked with 10% serum and incubated with primary antibodies for 16 h. After thorough rinsing in Tris buffered saline with 0.1% Tween-20, coverslips were incubated with secondary antibodies for one hour. Images were taken using Nikon NIS Elements. For western blotting (WB), cells were lysed in 2X Laemmli sample buffer and boiled for 5 min. Protein sample was then loaded on 10% SDS denaturing gels and transferred to a PVDF membrane. Nonspecific proteins are blocked with 5% non-fat milk. Proteins of interest are blotted for and appropriate secondary antibodies are used for detection. Antibodies and reagents used can be found in Supplementary Table 1.

2.6. Cellular hydrogen sulfide measurement in situ

Free sulfide was measured in cells using a specific fluorescent probe, sulfide fluor-7 acetoxymethylester (SF7-AM) (748,110-1MG, Sigma-Aldrich) [28]. Endothelial cells were incubated with 2.5 μM SF7-AM in phenol red free for 30 min and rinsed with medium. Cells were then treated with sulfide donors. Fluorescence was measured at 495 nm/519 nm. The fluorescent intensity at a certain time point (F0) was divided by fluorescent intensity at zero-time point (Fi), denoted as F0/Fi. Result was presented as the fold change of F0/Fi over control group.

2.7. Measurement of hydrogen sulfide metabolites

Sulfide metabolites were measured using HPLC as previously reported (36,37). Briefly, cells were lysed in the reaction buffer (0.1 mM DTPA in 100 mM Tris, pH 9.5) with 0.1% Triton-X-100. For