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Laminar shear flow increases hydrogen sulfide and activates a nitric oxide producing signaling cascade in endothelial cells

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ABSTRACT

Laminar shear flow triggers a signaling cascade that maintains the integrity of endothelial cells (ECs). Hydrogen sulfide (H_2S), a new gasotransmitter is regarded as an upstream regulator of nitric oxide (NO). Whether the H_2S-generating enzymes are correlated to the enzymes involved in NO production under shear flow conditions remains unclear as yet. In the present study, the cultured ECs were subjected to a constant shear flow (12 dyn/cm^2) in a parallel flow chamber system. We investigated the expression of three key enzymes for H_2S biosynthesis, cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercapto-sulfurtransferase (3-MST). Shear flow markedly increased the level of 3-MST. Shear flow enhanced the production of H_2S was determined by NBD-SCN reagent that can bind to cysteine/homocystein. Exogenous treatment of NaHS that can release gaseous H_2S, ECs showed an increase of phosphorylation in Akt^{S473}, ERK$^{T202/Y204}$ and eNOS^{S1177}. This indicated that H_2S can trigger the NO-production signaling cascade. Silencing of *CSE*, *CBS* and *3-MST* genes by siRNA separately attenuated the phosphorylation levels of Akt^{S473} and eNOS^{S1177} under shear flow conditions. The particular mode of shear flow increased H_2S production. The interplay between H_2S and NO-generating enzymes were discussed in the present study.

1. Introduction

Shear flow is a mechanical stressor that can be sensed by mechano-sensors located on membranes of endothelial cells (ECs). It triggers a network of signaling pathways in the effects of anti-atherosclerogenesis and vascular homeostasis [1]. Among the various shear-flow induced signaling molecules, reactive oxygen species (ROS)/reactive nitrogen species (RNS) are most discussed [2]. Under irregular shear flow, an elevated level of ROS increases the risk of atherogenesis [3]. However, during laminar shear flow, reversible ROS such as nitric oxide (NO) is abundantly generated through protein kinase B (Akt)-mediated phosphorylation of endothelial nitric oxide synthase (eNOS) ser-1177 residues [4,5]. NO can temporarily bind to the catalytic sites of enzymes, also known as S-nitrosylation, and then protects enzymes from irreversible inactivation caused by strong oxidative modifications [6]. Through the suppression of NADPH oxidase (Nox), an enzyme that promotes ROS synthesis, laminar shear flow can reduce the formation of superoxide and oxidative stress in arteries [7]. Under disturbed flow, the level of thioredoxin-1 is increased and leads to the

Abbreviations: eNOS, endothelial nitric oxide synthase; CSE, cystathionine-γ-lyase; CBS, cystathionine-β-synthase; 3-MST, 3-mercapto-sulfurtransferase; NBD-SCN, 4-nitro-7-thiocyanatobenz-2-oxa-1,3-diazole; Akt, protein kinase B; ERK, extracellular signal-regulated kinase.

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formation of inflammatory signaling in ECs [8]. The mechanism of how NO/ROS modifies proteins and thus modulates the function of these proteins and the physiological responses of ECs, is still an important issue of research [9–11].

In addition to NO, the toxic gas hydrogen sulfide (H_2S) has been recognized as a novel vasodilator for several years [12]. Cellular H_2S can be produced by the conversion of cysteine with three enzymes: cystathionine-γ-lyase (CSE), cystathionine-β-synthetase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) [13]. Mutation of H_2S-producing enzymes result in some disorders such as neural degeneration, skeletal abnormalities, increased risks of thromboembolism, and early onset of atherosclerosis [14]. With H_2S treatment, the production of adhesion molecules and the proliferation of vascular smooth muscle cells (VSMCs) can be suppressed and then prevents the formation of atherosclerotic lesions [15]. Increasing evidence demonstrates that hydrogen sulfide (H_2S) decreases the generation of ROS/RNS and leads to the proliferation and migration of ECs, indicating that H_2S is a ROS scavenger that protects ECs from hydrogen peroxide-induced injury [16,17]. H_2S would increase the expression of microRNA-21 (miR-21) and then attenuates myocardial injury through the suppression of the inflammasome, a macromolecular assembly that is implicated in many pathogenic processes [18]. As a result, H_2S has been a potent target in preventing heart failure, atherosclerosis, neuron degeneration, and cell aging [19,20].

A crosstalk between NO and H_2S in the cardiovascular system has been proposed [21,22]. The signaling responses after H_2S treatment shares a high similarity with laminar shear flow in cases of anti-oxidative stress, anti-inflammation and anti-atherosclerosis [12]. H_2S might, therefore, be implicated as a shear flow-triggered signaling molecule. To elucidate this issue, the measurement of endogenous levels of H_2S is important. Even though several approaches were designed to measure H_2S, the accuracy and a long operation time were unsatisfying [23,24]. Hence, a fluorescence probe was applied, using the reaction of 4-nitro-7-thiocyanatobenz-2-oxa-1,3-diazole (NBD-SCN) with cysteine and homocysteine [22,25]. The cysteine/homocysteine then can be converted to H_2S *via* CSE, CBS and 3-MST [14].

Protein kinase B (Akt), an upstream enzyme activating eNOS can prevent cell apoptosis by inhibiting the action of caspase-9 and forkhead box O3 (FOXO3) transcription factor [26]. Interestingly, the Akt/FOXO3 pathway is also implicated in a H_2S-mediated vascular homeostasis [27]. Extracellular signal-regulated kinase (ERK) is a member of mitogen-activated kinase (MAPK) that functions in the p38 MAPK/ERK pathway and its regulation either by mechanical shear flow or hydrogen sulfide have been broadly discussed [28,29].

Our particular objectives were to study shear flow-induced H_2S by using fluorescent probes that can bind to cysteine/homocysteine and the possible implications of Akt, ERK and eNOS in the downstream signaling by siRNA silencing of CBS, CSE and 3-MST enzymes.

2. Materials and methods

2.1. Cell culture and shear flow treatment

Human umbilical vein endothelial cells (HUVECs) were obtained from Cell Applications, Inc. (San Diego, CA, USA). HUVECs were cultured in M199 medium (Gibco, Thermo Fisher Scientific, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin/streptomycin, 2.5 mg/ml amphotericin B, 20% fetal bovine serum (FBS), and 20% endothelial cell growth medium (Lonza, MD, USA). HUVECs from passages 3–4 were cultured on glass slides and exposed to shear flow (12 dyn/cm^2) for 30 min in a well-defined parallel plate flow chamber system [10].

2.2. Applications of fluorescent probe and flow cytometry

Under shear flow, the ECs were coincubated with 5 μM of 4-nitro-7-thiocyanatobenz-2-oxa-1,3-diazole (NBD-SCN) for 30 min. Cysteine/homocysteine, that represents the level of H_2S were observed by fluorescence microscopy (λex 460 nm, λem 550 nm; Axiovert 40 CFL, Zeiss, Göttingen, Germany) [25]. After fluorescence microscopic observations, the ECs were washed twice with PBS buffer and detached by tryptic reaction. ECs were collected by centrifugation and then resuspended in PBS buffer. The fluorescence was immediately measured by the Accuri C6 flow cytometer (BD Bioscience, San Diego, CA, USA) with excitation and emission settings of 488 and 530 nm, respectively. The fluorescence strength was obtained from 1×10^4 cells and statistically calculated from three repeats.

2.3. Cell lysis and protein extraction

After treatment, ECs were washed with cord buffer: [NaCl (0.14 M), KCl (4 mM), glucose (11 mM), HEPES (10 mM, pH 7.4)] and then lysed with 100 μl of lysis buffer: [Hepes (250 mM, pH 7.7), EDTA (1 mM), neocuproine (0.1 mM) and CHAPS (0.4%, w/v)]. After centrifugation, proteinaceous supernatant was collected and protein concentrations were determined with BCA assay reagents (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Western blot analysis

Forty micrograms of cell lysate with various treatments were mixed with equal volumes of sample buffer [Tris–HCl (62.5 mM, pH 6.8), SDS (3%, w/v), 2-mercaptoethanol (5%, v/v), glycerol (10%, v/v)], and then separated by SDS-PAGE. The gel was transferred to PVDF membranes (Millipore, Billerica, MA, USA) and immunoblotted with antibodies: CBS (1:1000, Abnova, Taipei, Taiwan), CSE (1:1000, Abnova), 3-MST (1:1000, Abcam, Cambridge, UK), Akt (1:2000, BD Biosciences), pAkt^{S473} (1:3000, Cell Signaling Tech., Lane Danvers, MA, USA), ERK (1:2000, BD Biosciences), pERK$^{T202/Y204}$ (1:1500, Millipore), eNOS (1:3000, Cell Signaling Tech.), peNOS^{S1177} (1:2000, Cell Signaling Tech.). The membranes were visualized with the SuperSignal West Femto reagent (Thermo Fisher Scientific) on X-ray films. The images on X-ray films were scanned using a digital scanner (Microtek International Inc., Hsinchu, Taiwan) and the density was calculated by the Progenesis Samespots v2.0 software (NonLinear Dynamics, Newcastle, UK).

2.5. siRNA transfection

The CBS and 3-MST siRNA were generated by Dharmacon ON-TARGET plus SMART pool human CBS #L-008617-00 and #L-010119-00 (Thermo Fisher Scientific). The CSE siRNA sequence was designed as sense strand 5′-GGUUAUUUAUCCUGGGCUGdTdT-3′ and anti-sense strand 5′-CAGCCCAGGAUAAAUAACCdTdT-3′ (MDBio Inc., Taipei, Taiwan). ECs were co-transfected with siRNA using TurboFect™ (Thermo Fisher Scientific) and then cultured on glass slides for subsequent shear flow treatment.

2.6. Data analysis

All the data were collected and statistically calculated from three repeats. Statistical significance with increased level (shown by $^*p < 0.05$, $^{**}p < 0.01$) or decreased level (#$p < 0.05$, ##$p < 0.01$) was evaluated by using ANOVA with post-hoc Tukey HSD test. Since the exposure time would affect the visibility of resulting images on

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