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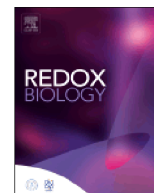

Research Paper

Total sulfane sulfur bioavailability reflects ethnic and gender disparities in cardiovascular disease

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ABSTRACT

Hydrogen sulfide (H_2S) has emerged as an important physiological and pathophysiological signaling molecule in the cardiovascular system influencing vascular tone, cytoprotective responses, redox reactions, vascular adaptation, and mitochondrial respiration. However, bioavailable levels of H_2S in its various biochemical metabolite forms during clinical cardiovascular disease remain poorly understood. We performed a case-controlled study to quantify and compare the bioavailability of various biochemical forms of H_2S in patients with and without cardiovascular disease (CVD). In our study, we used the reverse-phase high performance liquid chromatography monobromobimane assay to analytically measure bioavailable pools of H_2S. Single nucleotide polymorphisms (SNPs) were also identified using DNA Pyrosequencing. We found that plasma acid labile sulfide levels were significantly reduced in Caucasian females with CVD compared with those without the disease. Conversely, plasma bound sulfane sulfur levels were significantly reduced in Caucasian males with CVD compared with those without the disease. Surprisingly, gender differences of H_2S bioavailability were not observed in African Americans, although H_2S bioavailability was significantly lower overall in this ethnic group compared to Caucasians. We also performed SNP analysis of H_2S synthesizing enzymes and found a significant increase in cystathionine gamma-lyase (CTH) 1364 G-T allele frequency in patients with CVD compared to controls. Lastly, plasma H_2S bioavailability was found to be predictive for cardiovascular disease in Caucasian subjects as determined by receiver operator characteristic analysis. These findings reveal that plasma H_2S bioavailability could be considered a biomarker for CVD in an ethnic and gender manner. Cystathionine gamma-lyase 1346 G-T SNP might also contribute to the risk of cardiovascular disease development.

1. Introduction

With recognition and definition of physiologic effects of nitric oxide, there has been increasing interest in the biological activity of the "other" gaseous signaling molecules, namely hydrogen sulfide (H_2S) and carbon monoxide (CO). H_2S is produced endogenously via enzymes of the transsulfuration pathway including cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL, CTH or CSE), as well as the mitochondrial enzyme 3-mercaptopyruvate sulfurtransferase (MST). H_2S may also be generated through a non-enzymatic process from glucose (via glycolysis, NADPH oxidase), glutathione (direct reduction), inorganic and organic polysulfides (present in foods) or through elemental sulfur (direct reduction) [1,2]. Alteration of H_2S bioavailability and metabolism through many of these pathways are known to influence cardiovascular function and health in experimental models [3]. Unfortunately, the relationship of H_2S bioavailability with clinical cardiovascular disease conditions remains poorly defined [4,5].

Atherosclerotic cardiovascular disease is still the most common and costly cause of death in the United States and much of the world [6,7]. Chronic vascular inflammation and sub-endothelial accumulation of foam cells stimulate occlusion and stenosis of blood vessels, which is a common culprit underlying peripheral and coronary arterial disease [8]. Studies have reported that metabolic dysfunction involving reduced production of cellular H_2S may be a critical factor in the

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progression of experimental cardiovascular disease [2]. Endogenous H_2S production is significantly reduced in the CTH (CSE) knockout mouse model, which is associated with impaired endothelial vasodilation and hypertension, increased production of reactive oxygen species, increased vascular inflammatory responses, and enhanced vascular atherosclerosis [9]. Further, H_2S therapy using sulfide donors has been reported to diminish vascular inflammatory responses, decrease reactive oxygen species, and promote ischemic vascular remodeling/angiogenesis involving increased NO production [1].

Congruent with the current literature, H_2S exists in different biochemical forms, including free or unbound sulfide (S^{2-}, HS^- or H_2S), acid labile sulfide (ALS), and bound sulfane sulfur (BSS) [10–12]. These sulfur pools are crucial in regulating the total amount of bioavailable sulfide. ALS exists primarily in the form of iron–sulfur (Fe–S) complexes that modulate cellular functions including mitochondrial respiration and cytoplasmic redox reactions. BSS includes various compounds such as persulfides, polysulfides, thiosulfate, polythionates, thiosulfonates, bisorganylpolysulfanes or monoarylthiosulfonates, elemental sulfur, and many others. BSS compounds such as per/polysulfides can release H_2S under reducing conditions suggesting that the cellular redox state is important for regulating its bioavailability. The precise chemistry through which these different biological pools of H_2S interact to affect their pathophysiological functions is an area of active research. However, differences in bioavailability of these biochemical pools of sulfide remain largely unknown in part due to difficulties in measuring them. Overall, the sulfide field has been limited by controversies related to measurements of H_2S in various biological systems. Our lab has established and validated analytical chemistry methods to accurately detect and quantify discrete H_2S pools using a monobromobimane (MBB) assay coupled with reverse-phase high performance liquid chromatography (RP-HPLC), which was verified by electrospray ionization mass spectrometry [11,13,14].

In this study, we report findings of a clinical case-control study to accurately measure the amounts of different sulfide biochemical pools, namely ALS (with free sulfide combined), BSS, and total sulfide in subjects with coronary artery disease (CAD) or peripheral artery disease (PAD) compared to those without disease (controls). By combining clinically validated diagnoses with thoroughly established analytical chemistry techniques, these data provide important new insight regarding variations in bioavailability of sulfide biochemical pools and their association with cardiovascular disease states.

2. Materials & methods

2.1. Study design

This was a case-control study approved by the Institutional Review Board (IRB) of Louisiana State University Health Sciences Center at Shreveport (LSUHSC-S). Patients over 40 years of age who presented to the cardiac catheterization laboratory at LSUHSC-S for coronary or peripheral angiography were recruited for this study. Healthy, age-matched volunteers were also enrolled as controls. Each patient's ankle brachial index (ABI) was measured as we previously described [6] and each patient was also administered the San Diego Claudication Questionnaire prior to angiography. Following exclusion criteria, the total study population consisted of 278 Caucasian and African American (AA) subjects categorized into three basic subgroups (Fig. 1):

Healthy controls: healthy volunteers and patients with less than 50% occlusion of all major coronary or peripheral arteries and a normal ABI ($1.4 > ABI > 0.9$).

Coronary arterial disease (CAD): patients with greater than or equal to 50% occlusion of any major coronary artery and a normal ABI ($1.4 > ABI > 0.9$).

Peripheral arterial disease (PAD): patients with greater than 50% occlusion of a major limb artery and/or an abnormal ABI ($ABI < 0.9$).

2.2. Exclusion criteria

Volunteers who were excluded from this study were those who could not provide informed consent, were participating in another clinical trial involving experimental therapeutics, or were pregnant or nursing. Patients with ST elevated myocardial infarction or cardiogenic shock were not included to avoid interference with time-sensitive revascularization and confusion of pathophysiological events. Additional exclusion criteria included patients with an $ABI > 1.4$ (due to noncompressible arteries) or patients with Buerger's disease (non-atherosclerotic PAD).

2.3. History and blood collection

Patients were interviewed and medical record data were collected for analysis of typical cardiovascular risk factors such as hypertension, diabetes, obesity, tobacco use, and dyslipidemia. Blood samples were collected from already-established catheterization into 6 mL BD vacutainer tubes with lithium heparin. Samples were transported to the lab within 15 min on ice and were centrifuged at 1500 RCF for 4 min at 4 °C.

2.4. Measurement of biological pools of H_2S

Plasma samples were analyzed for free sulfide, ALS, BSS, and total sulfide levels as we have previously reported [11,13]. Free sulfide was measured using the MBB method as previously reported [11]. For detection of ALS and BSS, 50 µl of plasma was added separately into two sets of 4 mL BD vacutainer tubes. Four hundred fifty microliters of 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA) was added to one tube [acid labile reaction] and 450 µl of 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA) plus 1 mM TCEP was added to the second tube [total sulfide reaction]. Following a 30-min incubation on a nutator, the reaction liquid was removed and sulfide gas subsequently trapped by adding 500 µl of 100 mM Tris-HCl buffer (pH 9.5, 0.1 mM DTPA) into the BD vacutainer tube and incubated again for 30 min on a nutator mixer. The trapping solutions were removed and sulfide levels measured using the MBB method as we have previously reported [13]. Determination of ALS was made by reacting plasma samples with acidic phosphate buffer alone and subsequent trapping of evolved sulfide. Measurement of BSS was determined by subtracting the acid labile value from the total sulfide protocol containing TCEP reductant treatment under acidic conditions. Total sulfide levels were directly obtained from the total sulfide reaction.

2.5. MBB assay and RP-HPLC detection

Thirty microliters of reaction buffer with trapped sulfide was transferred to a PCR tube and mixed with 70 µl of H_2S stabilization buffer (100 mM Tris-HCl, 0.1 mM DTPA, pH 9.5) and 50 µl MBB solution (10 mM). Samples were then incubated in a hypoxic chamber (1% O_2) for 30 min at room temperature. The reaction was stopped by adding 50 µl of 200 mM sulfosalicylic acid, followed by centrifugation at 12,000 rpm for 10 min at 4 °C. One hundred microliters of supernatant was collected for RP-HPLC. Ten microliters of the supernatant was transferred into the RP-HPLC system with an Agilent Eclipse XDB-C18 column (5 µm, 80 Å, 4.6 mm × 250 mm) equilibrated with 15% CH_3CN in water containing 0.1% (v/v) TFA for fluorescence detection (excitation: 390 nm; emission: 475 nm).

MBB and sulfide-dibimane were separated using the gradient of two mobile phases: (A) water containing 0.1% (v/v) TFA and (B) 99.9% CH_3CN, 0.1% (v/v) TFA at a flow rate of 0.6 mL/min. Retention time for sulfide-dibimane is 16.5 min and MBB is 17.6 min. The amount of H_2S was measured from linear plots of the HPLC peak areas of sulfide-

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