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Hydrogen sulfide regulates cardiac mitochondrial biogenesis via the activation of AMPK

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ABSTRACT

Background: Hydrogen sulfide (H_2S) is an important regulator of mitochondrial bioenergetics, but its role in regulating mitochondrial biogenesis is not well understood. Using both genetic and pharmacological approaches, we sought to determine if H_2S levels directly influenced cardiac mitochondrial content.

Results: Mice deficient in the H_2S-producing enzyme, cystathionine γ-lyase (CSE KO) displayed diminished cardiac mitochondrial content when compared to wild-type hearts. In contrast, mice overexpressing CSE (CSE Tg) and mice supplemented with the orally active H_2S-releasing prodrug, SG-1002, displayed enhanced cardiac mitochondrial content. Additional analysis revealed that cardiac H_2S levels influenced the nuclear localization and transcriptional activity of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) with higher levels having a positive influence and lower levels having a negative influence. Studies aimed at evaluating the underlying mechanisms found that H_2S required AMP-activated protein kinase (AMPK) to induce PGC1α signaling and mitochondrial biogenesis. Finally, we found that restoring H_2S levels with SG-1002 in the setting of heart failure increased cardiac mitochondrial content, improved mitochondrial respiration, improved ATP production efficiency, and improved cardiac function.

Conclusions: Together, these results suggest that hydrogen sulfide is an important regulator of cardiac mitochondrial content and establishes that exogenous hydrogen sulfide can induce mitochondrial biogenesis via an AMPK-PGC1α signaling cascade.

1. Introduction

Mitochondria occupy an important position as mediators of cellular homeostasis due to their role in the regulation of fuel utilization, calcium storage, intracellular signaling, and cell death [1,2]. As such, impairments in mitochondrial function lead to the development of various disorders, such as neurodegenerative disease, cancer, aging, diabetes, and heart failure [1]. The heart is particularly susceptible to impairments in mitochondrial function given its limited regenerative capacity and persistent energy requirements [2]. As a result, the mitochondrial quality control system, consisting of mitophagy, fission and fusion, and biogenesis, is critically important in maintaining the fidelity of the heart under physiological and pathological conditions [1–3].

The mitochondrial biogenic response in the heart is tightly regulated by a complex network orchestrating both nuclear and mitochondrial genome transcription and replication [3]. This system coordinates both genomes during cardiac development and in response to physiological stimuli when there are changes in substrate availability and energetic demands [3]. Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is a master regulator of mitochondrial biogenesis and energy expenditure [4,5]. Cardiac PGC-1α is induced at birth when the heart undergoes a dramatic shift in fuel preference from relying on glucose and lactate during the fetal period to the use of fatty acids (FA) after birth [6]. PGC-1α regulates the activity of a number of

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transcription factors, including, peroxisome proliferator-activated receptor-α (PPARα), estrogen receptor–related α (ERRα) and nuclear respiratory factor 1 (NRF1) [5]. By regulating the transcriptional activities of these proteins, PGC-1α modulates genes involved in mitochondrial biogenesis and metabolic pathways. Mitochondrial content is significantly reduced in the failing hearts of both rodents and humans [7,8]. Furthermore, downregulation of PGC-1α signaling has also been observed in the setting of experimental heart failure [9]. As such, understanding the mechanisms by which PGC-1α signaling is regulated in the heart could lead to the development of therapies aimed at inducing mitochondrial biogenesis and augmenting energy production in the setting of increased contractile demand [8].

Hydrogen sulfide (H_2S) is a critically important physiological gaseous signaling molecule that regulates a multitude of biological processes, including angiogenesis, proliferation, redox balance, inflammation, and cell death [10]. It is produced enzymatically in all mammalian species via the actions of cysteine metabolic enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfutransferase (3MST) [10,11]. Although all three enzymes are expressed in the cardiovascular system, prevailing data indicates that CSE plays the foremost role in cardiovascular physiology [10]. Numerous proteins and pathways have been identified as cellular targets of H_2S. However, a common cellular target for many studies aimed at understanding the biology and therapeutic potential of H_2S has been the mitochondria. H_2S has a dual affect on mitochondrial bioenergetics with low concentrations serving as electron donors to the electron transport chain and higher concentrations serving as inhibitors of cytochrome *c* oxidase [12]. H_2S also influences the levels/activation of a number of proteins related to mitochondrial biogenesis (PGC1α [13,14]; AMP-activated protein kinase (AMPK) [15,16]; endothelial nitric oxide synthase (eNOS) [11,17,18]) and there is evidence that mitochondrial content is higher in brains [13] and hearts [19] treated with exogenous H_2S. While these studies provided evidence for elevated mitochondrial levels in response to H_2S treatment, it was not clear if the observed increase was due to a direct effect of H_2S or was simply an indirect consequence of H_2S altering injury. Therefore, the main goal of the current study was to address this issue by determining if H_2S levels directly influence cardiac mitochondrial content under non-stressed conditions. Additionally, we sought to gain insights into the mechanisms by which H_2S induces mitochondrial biogenesis in the setting of myocardial ischemia-reperfusion.

2. Materials and methods

2.1. Animals

The following strains of mice on a C57BL/6J background were utilized in this study: (1) C57BL/6J (Jackson Labs, Bar Harbor, ME), (2) Cardiac specific cystathionase-γ-lyase transgenic (CSE Tg^+), (3) Cystathionase-γ-lyase deficient (CSE KO), (4) AMPKα2 floxed (Stock#: 014142, Jackson Labs, Bar Harbor, ME), (5) αMHC-Cre transgenic (Stock#: 011038, Jackson Labs, Bar Harbor, ME), and (6) endothelial nitric oxide synthase deficient mice (eNOS KO; Stock# 002684, Jackson Labs, Bar Harbor, ME). CSE Tg^+ were generated by ligating the full-length *Mus musculus* cystathionine γ-lyase cDNA to the murine α-myosin heavy chain promoter, followed by injection of the DNA into newly fertilized mouse embryos (FVB/n background) [20]. The mice were then backcrossed to C57BL/6J for 9 generations. Global CSE KO knockout mice were generated by replacing exon 1 (including the ATG start codon), exon 2, and exon 3 with a neomycin selection cassette [11]. The mice were then backcrossed to C57BL/6J for 9 generations. Cardiac specific AMPKα2 deficient mice (αMHC-Cre^+ x $AMPK^{fl/fl}$) were generated by breeding $AMPK^{f/f}$ mice with αMHC-Cre^+ mice. In all experiments, Wild-Type (WT) littermates were used as controls. Male mice between the ages of 8–10 weeks were utilized. All experimental protocols were approved by the Institute for Animal Care and Use Committee at T3 Laboratories and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996), and with federal and state regulations.

2.2. Patient samples

Left ventricular samples were procured from patients with advanced ischemic heart failure undergoing a heart transplant at Emory University in accordance with Institution Review Board protocols. Additional non-failing heart failure samples were obtained from LifeLink. All patient identifiers were removed to strictly maintain donor confidentiality and anonymity. Both sample sets included male and female patients (Supplemental Table 1).

2.3. Materials

The orally active H_2S-releasing prodrug, SG-1002, was provided by Sulfagenix (Cleveland, OH). SG-1002 was administered to mice in the diet (Purina 5001; Research Diets Inc., New Brunswick, NJ) to achieve a dose of 20 mg/kg/day [11]. Mice received the diet for 4 weeks. Control mice received standard chow (Chow; Purina 5001) for the same duration. For the in vitro experiments, H_2S was administered as sodium sulfide (Na_2S; Sigma Aldrich).

2.4. In vitro cell culture

H9c2 cardiomyocytes were purchased from ATCC (Rockville, MD, USA). Cells were grown in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM; catalog# 30-2002) with 10% fetal bovine serum (FBS). Cells were maintained in this media until 80% confluent. Cells were then maintained in DMEM with 0.5% FBS for 12 h. Cells were then exposed to 100 μM of Na_2S for 10, 20, 40, or 60 min. Additional groups of cells were exposed to 100 μM of Na_2S for 3 consecutive days.

2.5. Cellular fractionation and Western blot analysis

Whole cell, cytosolic, and nuclear fractions were obtained from heart homogenates as previously described [21]. Protein concentrations were measured with the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were loaded into lanes of Criterion™ TGX (Tris-Glycine eXtended) Stain-Free PAGE gels (BioRad). The gels were electrophoresed and activated using a ChemiDoc MP Visualization System (BioRad). The protein was then transferred to a PVDF membrane. The membranes were then imaged using a ChemiDoc MP Visualization System to obtain an assessment of proper transfer and to obtain total protein loads. The membranes were then blocked and probed with primary antibodies (Supplemental Table 2) overnight at 4 °C. Immunoblots were next processed with secondary antibodies (Cell Signaling) for 1 h at room temperature. Immunoblots were then probed with a Super Signal West Dura kit (Thermo Fisher Scientific) to visualize signal, followed by visualization using a ChemiDoc MP Visualization System (BioRad). Data was analyzed using Image Lab (BioRad). The total protein images were used as loading controls. For each protein of interest, the portion of the protein load image corresponding to the molecular weight of the protein of interest was used as the loading control [16].

2.6. mRNA and qPCR

RNA was isolated using the RiboPure kit according to the manufacturer's instructions (Ambion). Reverse transcription was performed in a standard fashion with QuantiTect Reverse Transcription Kit (QIAGEN) supplemented with DNase treatment. Taqman qPCR was carried out according to the manufacturer's instructions using probe