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Broad spectrum proteomics analysis of the inferior colliculus following acute hydrogen sulfide exposure

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ABSTRACT

Acute exposure to high concentrations of H_2S causes severe brain injury and long-term neurological disorders, but the mechanisms involved are not known. To better understand the cellular and molecular mechanisms involved in acute H_2S-induced neurodegeneration we used a broad-spectrum proteomic analysis approach to identify key molecules and molecular pathways involved in the pathogenesis of acute H_2S-induced neurotoxicity and neurodegeneration. Mice were subjected to acute inhalation exposure of up to750 ppm of H_2S. H_2S induced behavioral deficits and severe lesions including hemorrhage in the inferior colliculus (IC). The IC was microdissected for proteomic analysis. Tandem mass tags (TMT) liquid chromatography mass spectrometry (LC-MS/MS)-based quantitative proteomics was applied for protein identification and quantitation. LC-MS/MS identified 598, 562, and 546 altered proteomic changes at 2 h, and on days 2 and 4 post-H_2S exposure, respectively. Of these, 77 proteomic changes were statistically significant at any of the 3 time points. Mass spectrometry data were subjected to Perseus 1.5.5.3 statistical analysis, and gene ontology heat map clustering. Expressions of several key molecules were verified to confirm H_2S-dependent proteomics changes. Webgestalt pathway over-representation enrichment analysis with Panther engine revealed H_2S exposure disrupted several biological processes including metabotropic glutamate receptor group 1 and inflammation mediated by chemokine and cytokine signaling pathways among others. Further analysis showed that energy metabolism, integrity of blood-brain barrier, hypoxic, and oxidative stress signaling pathways were also implicated. Collectively, this broad-spectrum proteomics data has provided important clues to follow up in future studies to further elucidate mechanisms of H_2S-induced neurotoxicity.

1. Introduction

Hydrogen sulfide (H_2S) is a highly neurotoxic colorless gas with a "rotten egg" odor (Chou et al., 2016). It is as an environmental pollutant and an occupational hazard in a variety of industries including the oil and gas industry, intensive animal farming operations, sewer and waste water treatment plants, pulp and paper plants, and gas storage facilities, among several others (Chou et al., 2016). It is the second leading cause of fatal gas exposure in the workplace after carbon monoxide (Greenberg and Hamilton, 1998). It is estimated that there are > 1000 reports of human exposures to H_2S each year in the United States (Chou et al., 2016). Besides exposures under environmental and industrial settings, intentional H_2Spoisoning for suicide has recently increased in Western and Asian societies (Morii et al., 2010; Reedy et al., 2011). This is possible because raw chemical ingredients used to generate H_2S for suicide are readily accessible in local stores (Morii et al., 2010). Also law enforcement and first responders called to help, as well as innocent bystanders are at risk of acute exposure to H_2S (Sams et al., 2013). H_2S was previously developed as a chemical weapon (Foulkes, 2009). There are concerns of potential nefarious use of this gas by terrorists, particularly in confined spaces, such as underground transit systems. For this reason, H_2S has been identified as a priority chemical for research by the US Department of Homeland security (Security, 2017). The central nervous system (CNS) is the

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primary target organ of acute H_2S-intoxication and neurotoxicity is the primary cause of death (Tvedt et al., 1991a; Tvedt et al., 1991b; Snyder et al., 1995; Guidotti, 2015). Short-term effects of acute H_2S poisoning by inhalation include knockdown, seizures, dyspnea, comma, and death. Currently, there is consensus that survivors of acute H_2S poisoning develop neurological sequelae, which can be incapacitating and last many years often leading to disability (Tvedt et al., 1991a; Snyder et al., 1995; Guidotti, 2015). A number of neurological sequelae have been reported. These include dizziness, vertigo, ataxia with tendency to fall, insomnia, fatigue, anxiety, learning and cognition deficits, hearing impairment, recurrent seizures, lack of libido, and increased sensitivity to H_2S, among others (Tvedt et al., 1991a; Tvedt et al., 1991b; Snyder et al., 1995). Extreme cases progress to permanent vegetative states. Neurotoxicity, is manifested in individuals acutely exposed to H_2S at concentrations > 500 ppm (Snyder et al., 1995; Guidotti, 2015; Rumbeiha et al., 2016).

Currently, there are no FDA approved drugs for treatment of either short-term or long-term effects of acute H_2S-induced neurotoxicity. The development of effective therapeutics requires a good understanding of the molecular mechanisms and pathways of acute H_2S-inducedneurotoxicity. These mechanisms remain largely unknown. There is an acute need for countermeasures for treatment of mass civilian casualties of acute H_2S poisoning in the field, such as following catastrophic industrial meltdowns or intentional terrorist activities. Elucidating molecular mechanisms underlying H_2S-induced neurotoxicity is essential in identifying suitable therapeutic targets to counter both immediate and delayed neurotoxic effects of acute H_2S poisoning in humans.

We recently developed an inhalation mouse model of acute H_2S intoxication that exhibits key phenotypes of short- and long-term effects of acute H_2S poisoning in victims and survivors (Anantharam et al., 2017). Briefly, this model is unique because normal walking mice without anesthesia are exposed to H_2S by inhalation, recapitulating the typical exposure scenario, following accidents, or during suicide. Clinically, mice suddenly collapse, have seizures, and are dyspneic during exposure. Surviving mice manifest motor deficits on rotarod and open field tests, and developed neurodegeneration, faithfully recapitulating the human condition. The objective of this study was to use this mouse model to investigate proteomic changes following acute H_2S-exposure to identify novel toxic mechanisms that could potentially be targeted for therapeutic intervention. These studies focused on the central inferior colliculus (IC), part of the brainstem, because our previous studies revealed this to be the most sensitive brain region to H_2S-induced neurodegeneration (Anantharam et al., 2017). However, we also previously observed histopathological changes in other parts of the brain, especially the thalamus and cortex (Anantharam et al., 2017). This ground breaking H_2S-study demonstrated, for the first time, that H_2S exposure induces significant proteomic changes in the IC, including albumin (Alb) leakage, neuroinflammation, and oxidative stress, which collectively play an important role in the execution of H_2S-induced neurotoxicity and neurodegeneration.

2. Materials and methods

2.1. Materials

H_2S gas was purchased from Airgas (La Porte, TX). RNeasy mini kit was purchased from Qiagen (Germantown, MD). High Capacity cDNA RT kit were purchased from ThermoFisher Scientific (Waltham, MA). RT^2 SYBR Green ROX qPCR Mastermix and primers for Gapdh were purchased from Qiagen (Valencia, CA). Primary antibodies against Alb, hypoxia inducing factor 1 alpha (Hif-1α), and Vimentin (Vim) were purchased from Cell signaling (Danvers, MA). Primary antibodies against nuclear factor-like 2 (Nrf2) and 3-Oxoacid CoA Transferase 1 (Oxct1) were purchased from Abcam (Cambridge, MA). Primary antibody against Fas was purchased from SantaCruz Biotechnology (SantaCruz, CA). Primary antibody against NeuN was purchased from Millipore (Billerica, MA). U-PLEX combo kit against TNF-α was purchased from Meso Scale Diagnostics (Rockville, MD).

2.2. Animals and treatment

This study was approved by Iowa State University Animal Care and Use Committee. Seven- to eight-week-old male C57 BL/6 J mice were used because previous studies from our lab showed that males were more sensitive than females. The mice were housed at room temperature of 20–22 °C under a 12-h light cycle, and a relative humidity of 35–50%. Protein rodent maintenance diet (Teklad HSD Inc., WI, US) and water were provided ad libitum. Prior to H_2S exposure on day 1, all mice were acclimated to breathing air for 40 min for two consecutive days. Freely moving unanesthetized mice were exposed either to normal breathing air (Control) or to 650–750 ppm H_2S (H_2S-treated) either once or to 2–7 short-term exposures. Both breathing air and H_2S were supplied from compressed gas cylinders. The inhalation dosage of H_2S was selected basing on published literature indicating concentrations of H_2S > 500 ppm are associated with knockdown and neurotoxicity in human (Snyder et al., 1995; Guidotti, 2015; Rumbeiha et al., 2016). Typically, humans are exposed once, but repeated exposures have been reported (Ahlborg, 1951). For purposes of studying the progression of neurotoxicity, separate groups of mice in this study were euthanized after receiving either only one or 2–7 acute exposures. For proteomic studies, the first batch of mice was terminated on the first day, day 1, only 2 h post exposure. Others were terminated on days 2 and 4. Negative controls were exposed to normal breathing air daily and euthanized on day 4. The gas exposure paradigm is summarized in Fig. 1. Animals were cared for in accordance with the Institutional Animal Care and Use committee guidelines.

2.3. Behavioral assessment

The VersaMax open field test was used to assess motor deficits induced by H_2S. We used this test because previous studies in the lab had indicated it to be sensitive to acute H_2S intoxication in this mouse model (Anantharam et al., 2017). Ataxia and other movement disorders are frequently reported neurological sequelae in survivors of acute H_2S poisoning (Ahlborg, 1951; Tvedt et al., 1991a; Tvedt et al., 1991b). Spontaneous activity was measured using an automated computer

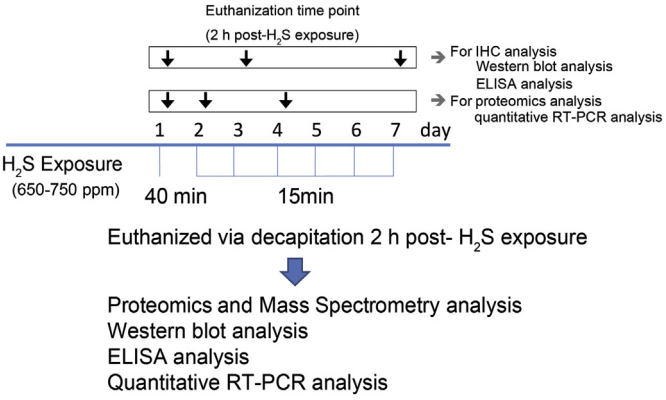

Fig. 1. Acute exposure paradigm of hydrogen sulfide in C57 black mice. Mice were exposed to 765 ppm of H_2S in a chamber for 40 min either once only (day 1) and for 15 min on the subsequent days up to day 7. Mice were sacrificed 2 h post-H_2S exposure on specified days of the study. Negative control mice were exposed to breathing air from a cylinder daily up to day 7. Separate groups of mice were sacrificed on days 1 (2 h post-exposure), 3, and 7 for immunohistochemistry, Western blot assay, and ELISA analysis. Groups of mice for proteomics studies and quantitative RT-PCR analysis were sacrificed on days 1 (2 h post-exposure), 2 and 4.