CURRENT REVIEW ON H_2S SIGNALING

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Abstract—Hydrogen sulfide, a pungent gas traditionally thought to be toxic to the human body, is now emerging as one of the most important endogenously synthesized gaseous transmitters. Over the past decade, we have witnessed better characterization of its synthetic pathways, discovered how it affects its targets at the molecular level by modifying cysteine residues, a process termed sulfhydration, as well as begun to identify the critical role it plays in the modulation of various cellular processes. In this article, we will review some of these findings.

Keywords—Hydrogen Sulfide (H_2S; Sulfhydratio; Gasotransmitt; Vasorelaxation

INTRODUCTION

The gas hydrogen sulfide (H_2 S) had been historically considered a poison and associated with various mass extinction events throughout Earth's history. For example, it is thought that the release of enormous quantities of H_2S in the atmosphere following the collision of an asteroid with Earth around 250 million years ago led to the death of most living organisms on the planet, an event known as the Permian-Triassic Extinction (Kump *et al.* 2005 and Grice *et al.* 2005). As such, the possibility that H_2 S could play a beneficial role in biology had not been seriously considered until the discovery of enzymes that endogenously synthesize H_2S in the body. Today, we know of three independent pathways through which H_2 S is generated (Paul *et al.* 2012).

In addition, analogous to another well-studied gaseous transmitter nitric oxide (NO), H_2S has been shown to modify cysteine residues on target proteins by adding an extra –SH group leading to the formation of –SSH in a process termed *S*-sulfhydration (Mustafa *et al.* 2009a). Since the discovery in 2009 of this novel post-translational modification, numerous targets have been identified, some of which we will elaborate on here. Over these past few years, it has become clear that H_2S plays a critical role in the modulation of various cellular processes with an overarching impact on both human physiology and pathophysiology.

1. H_2S Synthesis

Being a gaseous transmitter, H_2S cannot be stored in vesicles like most neurotransmitters. It is rather synthesized on demand in a tightly regulated manner. As mentioned before, H_2S is generated by three specific enzyme pathways, cystathionase (CSE), cystathionine β-synthase (CBS) and the aspartate aminotransferase (AAT)/3-mercaptopyruvate sulfurtransferase (3-MST) system (Paul *et al.* 2012). While CSE and CBS are well-established *in vivo* H_2S generators, the AAT/3-MST system has yet to be shown to play a critical role in endogenous H_2S formation (Paul *et al.* 2012). CSE is mainly located in peripheral tissues including the liver, kidneys, heart and endothelial cells of blood vessels, while CBS is mainly found in the brain (Mustafa *et al.* 2009b, Paul *et al.* 2012). CSE is also responsible for the production of cysteine from cystathionine, while CBS also serves as the homocysteine degrading enzyme (Paul *et al.* 2012). In patients with hyperhomocysteinemia, which leads to major cardiovascular abnormalities, CBS dysregulation is present (Beard *et al.* 2011). Mouse models of CBS -/- die *in utero* from cardiovascular abnormalities (Watanabe *et al.* 1995). CBS has also been reported to be modulated by carbon monoxide (CO) as well as *S*-adenosylmethionine (SAM) (Kabil *et al.* 2011, Finkelstein *et al.* 1975). Alternatively, CSE regulation has not been studied in depth.

2. Protein S-Sulfhydration

2.1. Evidence for Sulfhydration

NO, the best studied gasotransmitter to

date, had for long been known to chemically modify reactive cysteine residues on proteins leading to the formation of the –SNO moiety (Mustafa *et al.* 2009b). However, the first and most reliable biochemical assay for *S*-nitrosylation was not developed until 2001. Known as the biotin switch method, the assay essentially worked by blocking all free cysteine residues with the oxidizing agent methyl methanethiosulfonate (MMTS), followed by the reduction of nitrosylated cysteines with ascorbic acid and their subsequent biotinylation (Jaffrey *et al.* 2001). Using avidin beads, the biotinylated, and therefore nitrosylated, proteins could then be pulled-down and identified using Western Blotting.

Long thought to act as a reducing agent, the effects of H_2 S on potential denitrosylation of proteins was tested using the biotin switch method, and surprisingly it was discovered that H_2S in fact enhanced the signal (Mustafa *et al.* 2009a). It was later discovered that after MMTS modified all free cysteines, the H_2S modified cysteines were able to react with biotin without having to be reduced by ascorbic acid (Mustafa *et al.* 2009a). This was termed the modified biotin switch method. H_2S was thus found to be modifying cysteine residues by adding an extra –SH group leading to the formation of –SSH cysteines. This sulfhydration of cysteine residues was further confirmed by mutational analysis, radioactive cysteine assays, as well as mass spectrometry (Mustafa *et al.* 2009a). Other permutations to the modified biotin switch assay were also later developed and confirmed the detection of sulfhydrated proteins (Sen *et al.* 2012).

2.2. Selected List of Sulfhydration Targets

Much like nitrosylation, sulfhydration of proteins is a ubiquitous post-translational modification affecting an estimated 10-25% of all proteins (Mustafa *et al.* 2009a). Since the advent of the modified biotin switch method and the discovery of sulfhydration, numerous high impact targets have been identified. Table 1 lists a few that have recently been characterized.

Target Protein	Cysteine(s)	Cellular Pathway	Ref
K_{ATP} channels	C43 of Kir6.1 subunit	Vasorelaxation	Mustafa *et al.* 2011
SK_{Ca} channels	Unknown	Vasorelaxation	Mustafa *et al.* 2011
IK_{Ca} channels	Unknown	Vasorelaxation	Mustafa *et al.* 2011
eNOS	C443	Vasorelaxation	Altaany *et al.* 2014
GAPDH	C150	Glucose Metabolism & Memory Formation	Mustafa *et al.* 2009a, Mir *et al.* 2014
Parkin	C95	Neuroprotection	Vandiver *et al.* 2013
Androgen Receptor	C611, C614	Androgen Signaling	Zhao *et al.* 2014
TRP_{Ca} channels	C172, C329 of TRPV6	Mesenchymal Stem Cell Function and Bone Homeostasis	Liu *et al.* 2014
NF-κB	C38 of p65 subunit	Transcription regulation & Anti-Apoptosis	Sen *et al.* 2012
Keap1	C151	Anti-Cellular Aging & Gastric Mucosal Protection	Yang *et al.* 2013, Guo *et al.* 2014
PTP1B	C215	Endoplasmic Reticular Stress Response	Krishnan *et al.* 2011

Table 1 . Selected List of Sulfhydration Targets to date. K_{ATP} – Potassium ATP channel, SK_{Ca} – Small Conductance Potassium channel, IK_{Ca} – Intermediate Conductance Potassium channel, eNOS – endothelial Nitric Oxide Synthase, GAPDH – Glyceraldehyde 3-Phosphate Dehydrogenase, TRP_{Ca} – Transient Receptor Potential channel, NF-κB – Nuclear Factor-κB, PTP1B – Protein Tyrosine Phosphatase 1B.

3. *Physiologic and Pathophysiologic Impact of H_2S*

3.1. H_2S in Vasoregulation

The first clue for a physiologic role of H_2S emerged from its regulation of vascular tone. Following the discovery that exogenous H_2 S was able to relax the vasculature in mice, it was noted that mice knockout for CSE were significantly hypertensive to a similar extent as eNOS knockout animals (Yang *et al.* 2008). Acetylcholine-mediated relaxation was heavily impaired in the CSE -/- (Yang *et al.* 2008). It was further found that CSE was expressed specifically in arterial endothelial cells (Yang *et al.* 2008). Despite these major steps forward in understanding the role of H_2S in blood vessels, the mechanism by which H_2S relaxed smooth muscle remained unclear.

It had been known since the 1980s and early 1990s that there were three major components to physiologic vasorelaxation (Cohen 2005). The first is carried out through activation of cyclic GMP by a diffusible factor termed endothelial derived relaxing factor (EDRF) (Mustafa *et al.* 2009b). It was later discovered that NO fulfilled this role particularly in large conductance vessels such as the aorta (Mustafa *et al.* 2011), earning the discoverers the 1998 Nobel Prize in Physiology and Medicine. The second vasorelaxation pathway is linked to prostacyclins and plays a significant intra-uterine role in relaxing the ductus arteriosus (Schneider *et al.* 2006). The third pathway involves another diffusible component that modulates the tones of smaller resistance vessels such as the mesenteric arteries and is thought to be the primary regulator of blood pressure (Cohen 2005). This entity shares many characteristics with NO including its stimulation by acetylcholine and synthesis in endothelial cells (Cohen 2005). However, unlike NO, it functions mainly by hyperpolarizing both endothelial as well as smooth muscle cells via calcium and potassium channel signaling earning it the title endothelial derived hyperpolarizing factor (EDHF) (Cohen 2005). Given that H_2S is a diffusible molecule synthesized by CSE in endothelial cells following acetylcholine stimulation, it was surmised that H_2 S might potentially be the missing EDHF. Upon further investigation, it was indeed noted that exogenous H_2 S was able to hyperpolarize both endothelial as well as the smooth muscle cells (Mustafa *et al.* 2011). Moreover, blocking intermediate and small conductance potassium channels (IK_{Ca} and SK_{Ca} respectively) in endothelial cells as well as potassium sensitive ATP channels (K_{ATP}) in smooth muscle cells abolished the H_2S effect (Mustafa *et al.* 2011). Further, mice knockout for CSE were unable to hyperpolarize their endothelial and smooth muscle cells upon acetylcholine stimulation (Mustafa *et al.* 2011). It was also noted that H_2S specifically carried out its activation of K_{ATP} channels via sulfhydration of its Kir6.1 subunit cysteine 43 (Mustafa *et al.* 2011). Further investigation into the role of H_2S in activating the IK_{Ca} and SK_{Ca} channels are ongoing.

3.2. H_2S in Cardioprotection

Besides vasoregulation, H_2 S has also been noted to play a role in heart physiology. Administration of exogenous H_2S, as well as over-expression of CSE and thus over-production of endogenous H_2 S prior to cardiac ischemia protects cadiomyocytes from reperfusion injury (Polhemus *et al.* 2014). Moreover, CSE -/- mice show a 48% increase in infarct size compared to wild-type animals (Polhemus *et al.* 2014). These cytoprotective actions of H_2S are thought to result from its anti-apoptotic, anti-inflammatory and anti-oxidant effects, as

well as its influence on mitochondrial function (Polhemus *et al.* 2014). It has been further noted that H_2S over-production in the hearts of mice improves their left ventricular function and overall survival in ischemic heart failure (Calvert *et al.* 2010). It is thought that this effect of H_2S is mediated by up-regulation of the matrix metalloproteinase-2 (MMP -2) (Givvimani *et al.* 2011). Whether this is related to direct sulfhydration of the enzyme is not known. H_2S has also been shown to play an important role in prevention of atherosclerosis. Specifically, exogenous H_2S inhibited the formation of foam cells from macrophages by preventing the production of the pro-atherogenic oxidized low-density lipoprotein (LDL) (Wang *et al.* 2009) and
suppressed inflammatory cell migration into vessels (Zanardo *et al.* 2006). On the other hand, CSE -/- animals demonstrated greater inflammatory cell migration (Zanardo *et al.* 2006). Research is currently underway to employ H_2 S-releasing compounds to serve as cardioprotectants.

3.3. H_2S in Cellular Transcription

Nuclear Factor-κB (NF-κB) is a well-studied transcription factor that has been shown to mediate the up-regulation of specific anti-apoptotic pathways under various conditions of cellular stress (Sen *et al.* 2012). Mice with deletion of the p65 subunit die *in utero* from extensive apoptosis of hepatocytes (Beg *et al.* 1995), and mouse embryonic fibroblast are more sensitive to tumor necrosis factor-α (TNF-α)-mediated cell death (Beg *et al.* 1996). Given the anti-inflammatory properties of H2S, it was postulated that it might do so through NF-κB. Indeed, following stimulation by TNF-α, the transcription factor specificity protein (SP1) binds to the CSE promoter up-regulating its production (Sen *et al.* 2012). This leads to the enhanced production of H2S which appears to sulfhydrate cysteine 38 of the p65 subunit of NF-κB (Sen *et al.* 2012). This allows for enhanced binding of its co-activator ribosomal protein S3 (RPS3) therefore increasing NF-κB anti-apoptotic activity (Sen *et al.* 2012). Further, CSE -/- mice show a drastic reduction in NF-κB function as a result of its inability to interact with RPS3 and hence demonstrate diminished anti-apoptotic activity (Sen *et al.* 2012). Investigations are underway on the involvement of H2S-NF-κB-mediated anti-apoptosis in cancer.

3.4. H2S in Glucose Metabolism

Glucose, one of the major fuels for cells, serves as the starting point for glycolysis leading to the production of adenosine triphosphate (ATP) (Voet *et al.* 1995). The reverse process takes place during cellular starvation termed gluconeogenesis.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serves as a key enzyme in both the forward as well as the reverse pathway. H_2S was noted to enhance the activity of GAPDH by 7-folds in *in vitro* experiments (Mustafa *et al.* 2009a), and CSE -/- livers demonstrate a 25-30% decrease in GAPDH activity (Mustafa *et al.* 2009a). This effect is mediated by the sulfhydration of cysteine 150 (Mustafa *et al.* 2009a).

3.5. H_2S in Insulin Homeostasis

A further implication of H2 S in glucose metabolism relates to its effects on KATP

channels. The latter are present on the membranes of insulin secreting pancreatic beta cells (Ashcroft *et al.* 2013). At rest, these channels are open allowing potassium ions to exit the cells, but in the presence of high glucose metabolism and greater ATP production, these channels are inhibited by the binding to ATP leading to the depolarization of beta cells and subsequent release of insulin (Ashcroft *et al.* 2013). H2 S has been shown to activate KATP channels in pancreatic beta cells suppressing insulin release (Yang *et al.* 2005). Whether H2 S affects the glycolytic pathway in these cells remains to be determined.

3.6. H_2S in Mesenchymal Stem Cell

Function and Bone Homeostasis CBS deficiency is a known autosomal recessive disorder in which, among other things, patients suffer from osteoporosis (Maron *et al.* 2009). Therefore, the effects of H_2S on bone homeostasis were studied in bone marrow mesenchymal stem cells (BMMSC), which express both CBS and CSE (Liu *et al.* 2014). It was noted that H_2S deficiency causes defects in BMMSC differentiation (Liu *et al.* 2014). H_2 S was found to sulfhydrate various cysteine residues on transient receptor potential (TRP) channels, in particular cysteines 172 and 329 of TRPV6, and promote intracellular calcium influx (Liu *et al.* 2014). H_2S deficient cells demonstrate impaired calcium influx as a result of absent TRP channel sulfhydration, down-regulation of the PKC/Erk kinase-mediated Wnt/β-catenin signaling and therefore osteogenic BMMSC differentiation (Liu *et al.* 2014). This can be rescued with exogenous H_2S in CBS +/- animals, which at baseline demonstrate an osteopenic phenotype and BMMSC impairment (Liu *et al.* 2014). As such, H_2S treatment might potentially benefit patients suffering from osteoporosis.

3.7. H_2S in Memory Formation

Detrimental effects on postsynaptic density 95 (PSD95), a scaffold protein, has been associated with various neurological deficits including loss of synapse maturation and synaptic plasticity leading to the impairment of memory formation in the brain (El-Husseini *et al.* 2000). It was recently discovered that the pro-inflammatory molecule Interleukin-1β (IL-1β) enhances the synthesis of CBS in brains of mice. This leads to greater production of H2S, which in turn sulfhydrates GAPDH at cysteine 150 allowing for its binding to the E3 ubiquitin ligase siah (Mir *et al.* 2014). Siah in turn binds to PSD95 and degrades it via ubiquitination (Mir *et al.* 2014). In CBS +/- mice, IL-1β-mediated GAPDH sulfhydration and the subsequent degradation of PSD95 could be rescued (Mir *et al.* 2014). In neurodegenerative diseases leading to memory impairment whereby IL-1β plays a critical role, inhibition of H2S production might be of clinical benefit.

3.8. H_2S in Huntington's Disease

Inherited in an autosomal dominant manner, Huntington's disease results from a mutation in the gene huntingtin (Htt) causing it to harbor extra glutamine repeats

(HDCRG 1993). This leads to neurotoxicity, oxidative stress, as well as motor and behavioral changes in affected individuals (HDCRG 1993). CSE -/- mice were noted to produce hind limb clasping and clenching in a manner akin to mouse models of Huntington's (Paul *et al.* 2014). This prompted researchers to look into the expression of CSE in these mice. It was indeed noted that CSE was significantly down-regulated and supplementing tissues and mouse models of Huntington's disease with cysteine, the primary synthetic enzyme for which is CSE, alleviated the characteristic abnormalities of the disease (Paul *et al.* 2014). It was also determined that CSE down-regulation takes place at the transcriptional level with mutant Htt binding to the CSE transcription activator SP1 and inhibiting its activity (Paul *et al.* 2014). Whether sulfhydration has any particular role in Huntington's disease remains to be determined.

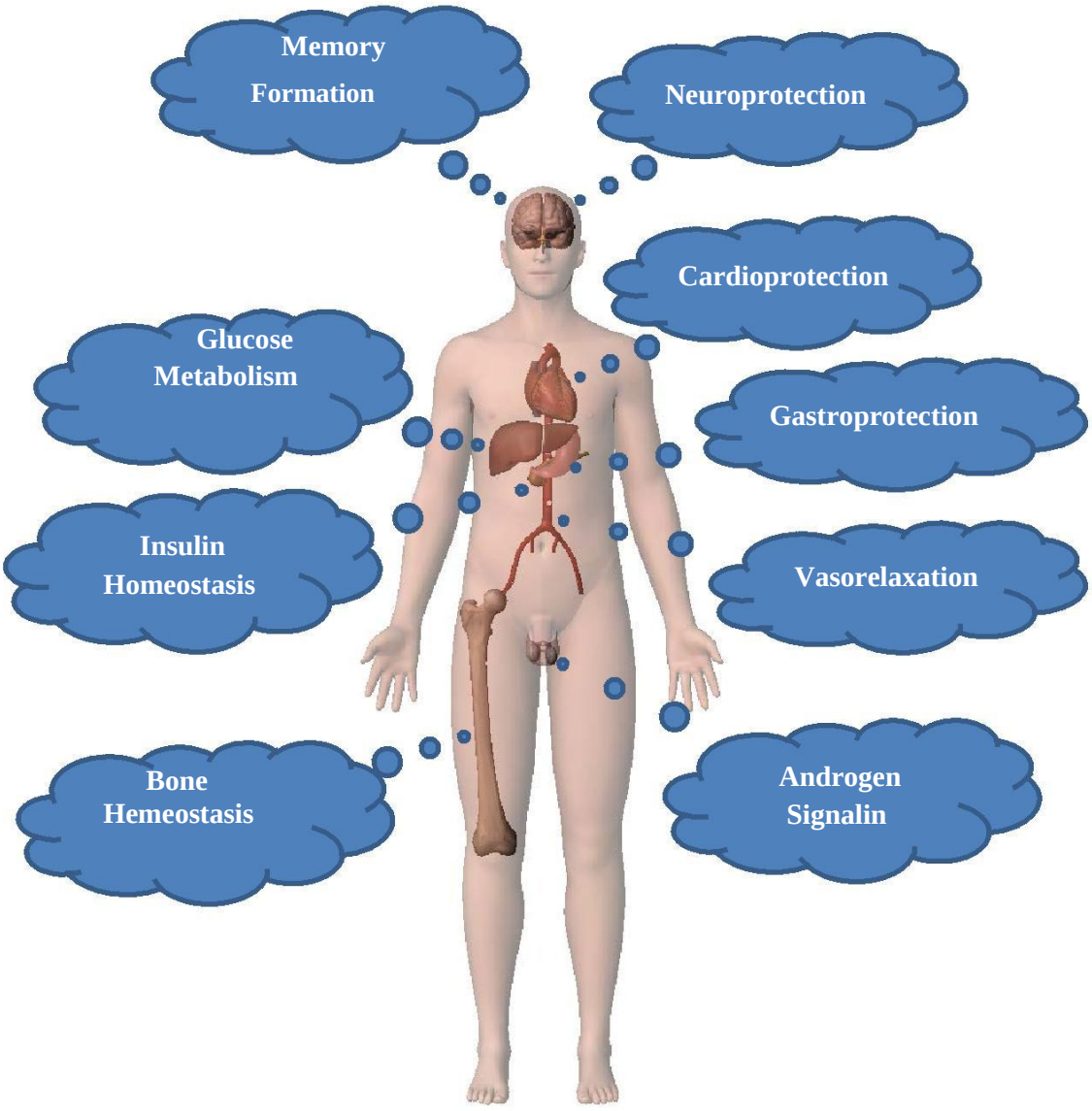
3.9. H_2S in Parkinson's Disease

Unlike Huntington's, protein sulfhydration has been linked to the pathogenesis of Parkinson's (Vandiver *et al.* 2013). Parkinson's disease, with its characteristic motor abnormalities and destruction of the basal ganglia, is thought to result from the *S*-nitrosylation and inactivation of the E3 ubiquitin ligase parkin (Chung *et al.* 2004). It was discovered that parkin can also be sulfhydrated, but unlike nitrosylation, sulfhydration leads to an increase in parkin activity as well as its neuroprotective effects (Vandiver *et al.* 2013). Cysteine 95 appears to be the main target of H_2 S (Vandiver *et al.* 2013). Additionally, in brain tissue from Parkinson's patients, concurrent increase in parkin nitrosylation and a nearly 60% decrease in sulfhydration were observed (Vandiver *et al.* 2013). This indicates that methods to augment H_2S activity in Parkinson's sufferers may therefore be of benefit.

CONCLUSION

In sum, since the discovery of H_2S as a physiologic vasorelaxant, huge strides have been made in understanding how it's regulated and in turn affects various signaling pathways. Also, the critical discovery of sulfhydration as the molecular mechanism by which H_2S works has undoubtedly accelerated the identification of its targets. Today we know that H_2S not only functions in the vasculature to modulate blood pressure, but also plays a significant role in various physiologic and pathologic processes of which only a select few have been presented in this review. Research in the upcoming years will hopefully unlock yet more secrets of this noxious yet clinically important gas and drugs that modulate its levels *in vivo* will be highly sought after.

Figure 1. Selected List of Organ Systems physiologically regulated by H_2S



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