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Review

Hydrogen sulfide: Therapeutic or injurious in ischemic stroke?

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ABSTRACT

Hydrogen sulfide (H_2S) has been identified as a vasodilatory, neuromodulatory, and anti-inflammatory gasotransmitter with antioxidant properties. Studies focused in cardiac tissue suggest H_2S functions as a protective agent; however in the central nervous system (CNS) the effects of H_2S during states of stress or injury, such as stroke, remain controversial. Currently, the application of H_2S donors and modulators in stroke depends on the type of H_2S donor and the timing of the therapy.

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1. Background

Stroke remains one of the leading causes of mortality worldwide. Notably, in the United States a stroke occurs every forty seconds and death due to stroke occurs every four minutes. In addition to high mortality, stroke is also a well-known cause of disability, particularly in those above the age of 65. Strokes can be classified as ischemic or hemorrhagic based on the mode of cerebral artery damage. Ischemic stroke, characterized by an occlusion of vessels supplying the brain, accounts for ~87% of stroke incidences worldwide. The remaining ~13% of cases are hemorrhagic strokes, which are caused by rupture of cerebral vessels [1]. In this review, vaso-occlusive stroke and the effects of H_2S on various aspects of stroke are discussed.

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1.1. Vaso-occlusive stroke and risk factors

Vascular occlusive crises in the brain are the primary cause of ischemic stroke [2]. As classified by the Trial of Org 10,172 in Acute Stroke Treatment (TOAST), ischemic stroke can be caused by large-artery atherosclerosis, cardioembolism, small-artery or lacunar occlusions, and other unknown causes. Following vascular blockage, ischemia (mainly in the cerebellum) develops and establishes a highly necrotic, unsalvageable "core" region, or umbra, and a salvageable penumbra. Lack of blood flow in the core region leads to neuronal death by necrotic events and apoptotic mechanisms [3], while the penumbra shows varying degrees of apoptotic cell death, stress, and survival. Rescuing this partially perfused penumbra is the goal of stroke therapy. Experimental models in rodents have shown a strong correlation between an initial decrease in blood flow and the infarct size. For example, a 75% decrease in cerebral blood flow is associated with a 95% higher chance of infarction, while a <50% reduction in cerebral blood flow results in <5% risk of infarct formation [2]. Transient ischemic attack (TIA) occurs as a result of focal and temporary ischemia that leads to reversible neurological loss. TIA is also known to increase the subsequent incidence of stroke [4]. Certain diseases (obesity, diabetes, hypertension and hyperlipidemia) increase risk of cerebral ischemic events, but may be managed by lifestyle changes. However, other risk factors cannot be controlled (gender, age, ethnicity, and family history) [5].

Incidence of ischemic stroke differs between sexes in young adults, with a higher incidence due to intracerebral hemorrhage in young males ages 25–34 [6]. However, the prevalence, severity, and morbidity, due to subarachnoid hemorrhage, is greater in older females (35–45) than in males of the same age [6]. This difference is attributed to the decrease in protective gonadal hormone and estrogen (E2) in post-menopausal women. A similar effect is observed in males with decreased testosterone levels. A decrease in gonadal hormones results in a more inflammatory environment in elderly brains, which leads to increased susceptibility to stroke and a greater rate of morbidity. Estrogen replacement therapy (ERT) is helpful only when it is started immediately after menopause; otherwise, ERT can have adverse effects. Delayed ERT in clinical trials increased infarct volume, severity, and mortality in women. This is also true for the effect of testosterone on stroke in males, suggesting an important role for gonadal hormones in altering vasculature [7].

Ischemic stroke in adults over 65 years old is more common [8]. In young adults ischemic preconditioning of the brain plays an important role in decreasing the probability of a future ischemic event; however, cerebral ischemic preconditioning offers decreased protection in elderly brains due to altered metabolism as well as increased reactive oxygen species (ROS) [9]. A persistent pro-inflammatory environment with elevated levels of tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 has been reported in the elderly population [10,11]. This increase in pro-inflammatory cytokines in elderly brains is termed "inflammaging." It is thought to be a natural adaptive mechanism to protect the aged brain. However, according to Lucke-Wold et al., this pro-inflammatory environment may contribute to the high prevalence of ischemic stroke in the elderly population [11].

1.2. H_2S in stroke

In the following sections we will discuss various aspects of ischemic stroke and the role of H_2S. We will discuss the role of H_2S and ion channels during stroke, the influence of H_2S on brain edema as well as its control of oxidative stress and ROS, and finally H_2S dependent signaling. Before we proceed to the discussion of these topics it is first important to understand how H_2S is synthesized and degraded under physiological conditions.

1.3. H_2S biosynthesis and degradation

(See Fig. 1 for a graphical representation of this section)

1.3.1. Biosynthesis

H_2S has been extensively re-evaluated following the discovery of enzymes that produce endogenous H_2S in the brain. The concentration of H_2S in the brain was estimated to be around 50–160 μM [12] in earlier studies, however these values have been largely debunked as overestimations. Measurement techniques have become more sophisticated and ranges in the brain appear to be lower. H_2S measurements taken from tissue using gas chromatography immediately after death under anoxic conditions showed that mouse brains contain approximately 17 nM of free sulfide [13]. This value decreases with time, showing the volatility of H_2S. Another study using gas chromatography showed approximately 175 nmol/gram of protein of H_2S exists as an acid labile pool in rat brains, the free H_2S was estimated to be less than 9.2 μM, and the bound H_2S to be around 6 μM /gram of protein [14]. Another study demonstrated that accurate biological measurements can be made using a monobromobimane (MBB) method with HPLC [15], despite this methylene blue (MB) is the most commonly used measure, however it results in an overestimation due to both the 670 nm wavelength measured and the liberation of acid labile sulfur species. Ultimately H_2S sensitive probes may provide the best measure of intracellular concentrations of H_2S. There are three major cytosolic enzymes involved in endogenous H_2S production: cystathionine β synthase (CBS), cystathionine γ lyase (CSE) and 3-mercaptosulfur transferase (3-MST).

CBS is a tetrameric protein encoded by the CBS gene that is located on chromosome 21q22.3 in humans. CBS is the only known pyridoxal phosphate (PLP)-dependent enzyme that contains heme. Heme binds reversibly to CBS and can be displaced by carbon monoxide (CO), making CO and H_2S metabolism possibly antagonistically regulated. Heme is important but not essential for CBS enzymatic activity; mutant CBS that cannot bind heme still possesses ~25% activity of the wild-type protein [16]. CBS acts on its substrate homocysteine and L-serine to produce H_2S. Additionally, S-adenosyl methionine (SAM) allosterically activates CBS [17]. CBS is predominantly expressed in the brain, making it relevant to stroke studies [18]. In particular, astrocytes and glial cells are major depots of CBS in the brain. CBS in reactive astrocytes can be triggered by TGF-α, EGF, and cAMP in order to generate H_2S [19].

CSE is another tetrameric [20] PLP-dependent enzyme in the H_2S metabolic pathway. In humans, the CSE gene is found on chromosome 1p31.1. CSE catalyzes the conversion of cystathionine to cysteine, 2-ketobutyrate, and ammonia. L-cysteine and homocysteine can also serve as substrates for CSE [21]. The activity of the CSE enzyme is augmented two-fold by the calcium-calmodulin complex [22]. CSE is the main enzyme producing H_2S in endothelial cells that line blood vessels [19].

3-MST (Mercapto-sulfur transferase) and CAT (cysteine amino transferase) function cooperatively to produce H_2S and are found in both the mitochondria and cytosol [23]. In the aminotransferase reaction catalyzed by CAT, cysteine and α-ketoglutarate are converted to 3-mercaptopyruvate (3-MP;). 3-MP is the substrate for 3-MST in H_2S production via a persulfide intermediate. This reaction requires thioredoxin or dihydrolipoic acid (DHLA) to act as reducing agents. Thiosulfate can also be a substrate for 3-MST; DHLA would then function as the specific physiologic reducing agent for this reaction. Thioredoxin and DHLA can also release H_2S from sulfane-sulfur under physiologic conditions (refer to "Other Sulfur Sources" for a more thorough discussion of sulfane-

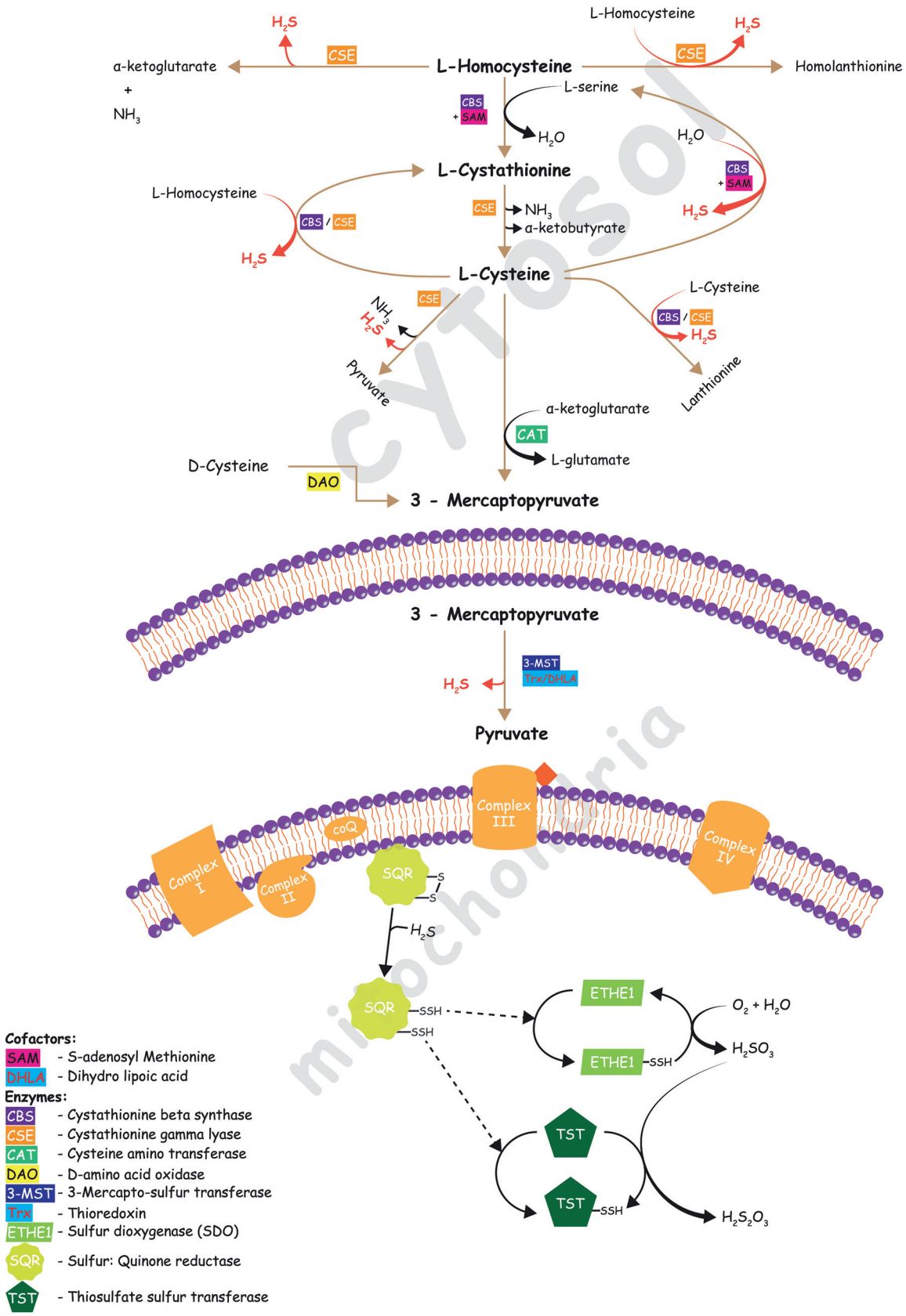


Fig. 1. Graphical representation depicting H_2S biosynthesis and degradation. H_2S biosynthesis pathways of three major cytosolic enzymes involved in endogenous H_2S production: cystathionine β synthase (CBS), cystathionine γ lyase (CSE) and 3-mercaptosulfur transferase (3-MST) have been detailed in the cytosol and mitochondria. Degradation of H_2S by three mitochondrial enzymes: quinone reductase (SQR), sulfur dioxygenase (SDO, ETHE1), and thiosulfate sulfurtransferase (TST) is shown within the mitochondria.

sulfur). The 3-MST/CAT pathway is inhibited by increases in calcium [23]. 3-MST produces bound-sulfur more efficiently than CBS due to its relatively high sulfur carrying capacity. 3-MST plays a major role in producing H_2S in neurons [19].

1.3.2. Degradation

Although H_2S formation is an essential process, H_2S disposal is just as important. H_2S mediates reversible inhibition of cytochrome c oxidase, the terminal electron acceptor in oxidative phosphorylation. The inhibitory constant of hydrogen sulfide that causes inhibition of cytochrome c oxidase, thereby effectively shutting down mitochondrial respiration and ATP synthesis, is 0.45 μm [24]. In order to prevent inhibition, the "sulfide disposal unit" converts H_2S to thiosulfate. There are three mitochondrial enzymes involved in the disposal of H_2S: quinone reductase (SQR), sulfur dioxygenase (SDO, ETHE1), and thiosulfate sulfurtransferase (TST). SQR, an inner mitochondrial flavoprotein, oxidizes H_2S, producing two persulfides (-SSH). Sulfur dioxygenase consumes molecular oxygen and water to convert one persulfide to sulfite. Sulfite then reacts with the other persulfide to produce thiosulfate in a reaction catalyzed by TST [25].

H_2S produced as a result of these enzymatic actions is not stable at physiological pH, and spontaneously dissociates into hydrogen sulfide anions (SH^-). Originally, these anionic sulfide species were considered biologically inactive. However, an anion channel of the formate/nitrate transporter family, FNT3, in *Clostridium difficle* was recently discovered; a human orthologue is yet to be identified [26].

1.3.3. Other sulfur sources

In addition to H_2S, sulfur exists in other forms such as bound sulfur and acid-labile sulfur. Bound sulfur, or sulfane-sulfur, is a term given to sulfur bound to sulfur. H_2S bound to sulfur in proteins, an addition of sulfur to elemental sulfur, or an addition of sulfur to disulfide can generate polysulfides. Bound sulfur acts both as a source of sulfide storage and can exert regulatory control of proteins [19,27]. Bound sulfur is released efficiently from neurons and astrocytes in the presence of glutathione (GSH), a cellular antioxidant. In addition, a slightly alkaline cytosolic environment in astrocytes, created by surrounding neuronal excitation, is sufficient to release bound sulfur from astrocytic cytosol [28]. Acid-labile sulfur is the sulfur that is bound to iron-sulfur clusters; it is primarily of mitochondrial origin [19]. Ogasawara et al. speculated that sulfur may be transported through bonding of proteins, by serum albumin, or by an unknown transport mechanism in vivo [27]. Trisulfides and polysulfides are extra elemental sulfur additions to protein disulfide bridges that require the presence of H_2S and are thought to be rapid-release storehouses for H_2S [29]. A kinetic study by Nagy et al. demonstrated that hypochlorous acid, a product of neutrophil myeloperoxidase, reacts with H_2S at a pH of 7.4 to generate polysulfides [30]. These polysulfides have been observed since 1959 in many proteins in vitro including the heptasulfide superoxide dismutase and the trisulfide glutathione. 3-MST has recently been shown to generate polysulfides in rat brain [31].

1.3.4. H_2S mode of action

H_2S, like nitric oxide (NO), is known to mediate post-translational modifications of proteins by the addition of extra sulfur to reactive cysteine residues. This modification is called S-sulfhydration or sulfhydration. Sulfhydration is thought to be important in activation or inactivation of many classes of proteins, including ion channels such as ATP-dependent potassium channels, TRPV3, TRPV6, TRPM [32], enzymes, and transcription factors (NF-kB, Nrf2) [33]. Modulation of ion channels and inflammatory and antioxidant transcription factors by H_2S post stroke may play a role in attenuation of edema and inflammation.

2. Stroke, ion channels, and role of H_2S

Glucose and oxygen deprivation is detrimental to the brain as a result of ATP depletion that occurs during the ischemic period [2]; glycolysis is a major contributor of reducing equivalents in the ATP-generating oxidative phosphorylation (OXPHOS) pathway. Loss of ATP leads to ion homeostasis imbalance in the cells due to the failure of ATPases, or ATP dependent-ion transporters [34] that regulate the influx of calcium and sodium. This also results in an efflux of potassium due to subsequent ATP depletion and calcium accumulation [35,36]. Increases in intracellular calcium result in the release of glutamate, an excitatory neurotransmitter, which perpetuates calcium overload and the activation of calcium-dependent lipases and proteases [36]. These events result in increased ROS production, the opening of mitochondrial permeability transition pores, inflammation, and neuronal cell death [36,37]. Under normal conditions, astrocytes surrounding the neurons take up the extra glutamate and rescue the neurons from excitotoxicity (Fig. 2) [38]. However, during ischemia, damaged astrocytes may contribute to exacerbation of ischemic reperfusion injury due to the inhibition of a major glutamate transporter (GLT1) [38]. In this section, the role of H_2S in glutamate excitotoxicity and ion channel regulation and its relevance to stroke are discussed.

H_2S is a well-accepted neuronal mediator. Abe and Kimura showed that H_2S (administered as NaHS) activates the NMDA receptor at concentrations between 10 and 130 μM (concentrations of 320 μM and 640 μM appeared to be toxic) resulting in long-term potentiation (LTP), a process important in learning and memory [18]. Neuronal activation results in a release of neurotransmitters, including glutamate that activates NMDA receptors. This in turn leads to an elevation of astrocytic intracellular calcium. The presence of neurons is a requirement for H_2S release from astrocytes and increase in intracellular calcium in astrocytes [39]. Astrocytes produce 7.57 fold more H_2S than microglial cells in vitro [40]. For this study H_2S production was measured to be between 0.24 and 66.89 μmol/g/h using the MB assay. H_2S activates transient receptor potential A1 (TRPA1) channels leading to calcium influx and an activation of astrocytes by calcium wave transmission post-neuronal activation. D-serine is then released from astrocytes, which subsequently activates NMDA channels [30,41]. The ensuing interaction between neurons and astrocytes then facilitates LTP [12]. More recent studies suggest that polysulfides, are 300 times more potent than H_2S in activating TRPA1 receptors, 0.5 μM of Na_2S_3 elicited a response similar to 160 μM NaHS [31]. Polysulfides activate NMDA by sulfhydration that follows H_2S-dependent reduction of the cysteine disulfide in the extracellular domain of the receptor [41]. Although LTP is essential for normal physiological processes such as learning and memory, activation of NMDA channels by H_2S is possibly deleterious in stroke conditions due to glutamate excitotoxicity. As mentioned in the previous paragraph, glutamate, a neurotransmitter, binds to its receptor on neurons triggering calcium influx that in turn leads to neuronal toxicity and death. In contrast, experimental data suggests that glutamate toxicity during ischemia is attenuated by the action of H_2S on K_{ATP} and CFTR Cl^- channels in vitro [42] and by the upregulation of GLT1 transporters [43]. Nevertheless, Cheung et al. provided evidence for H_2S mediated cell death due to excess glutamate in mature mouse cortical neurons (Fig. 2). A concentration of 100 μM extracellular glutamate increased cell death, which was then exacerbated by 25 μM sodium hydrosulfide (NaHS). Co-treatment of NaHS and glutamate activated calpains, calcium-dependent cysteine proteases, rather than caspases. Calpains in turn mediated lysosomal membrane destabilization that lead to protease release and cell death in mature cortical neurons (Fig. 2) [44]. Concentrations of NaHS 200 μM and above induced significant decreases in mature neuron cell viability. The contra-

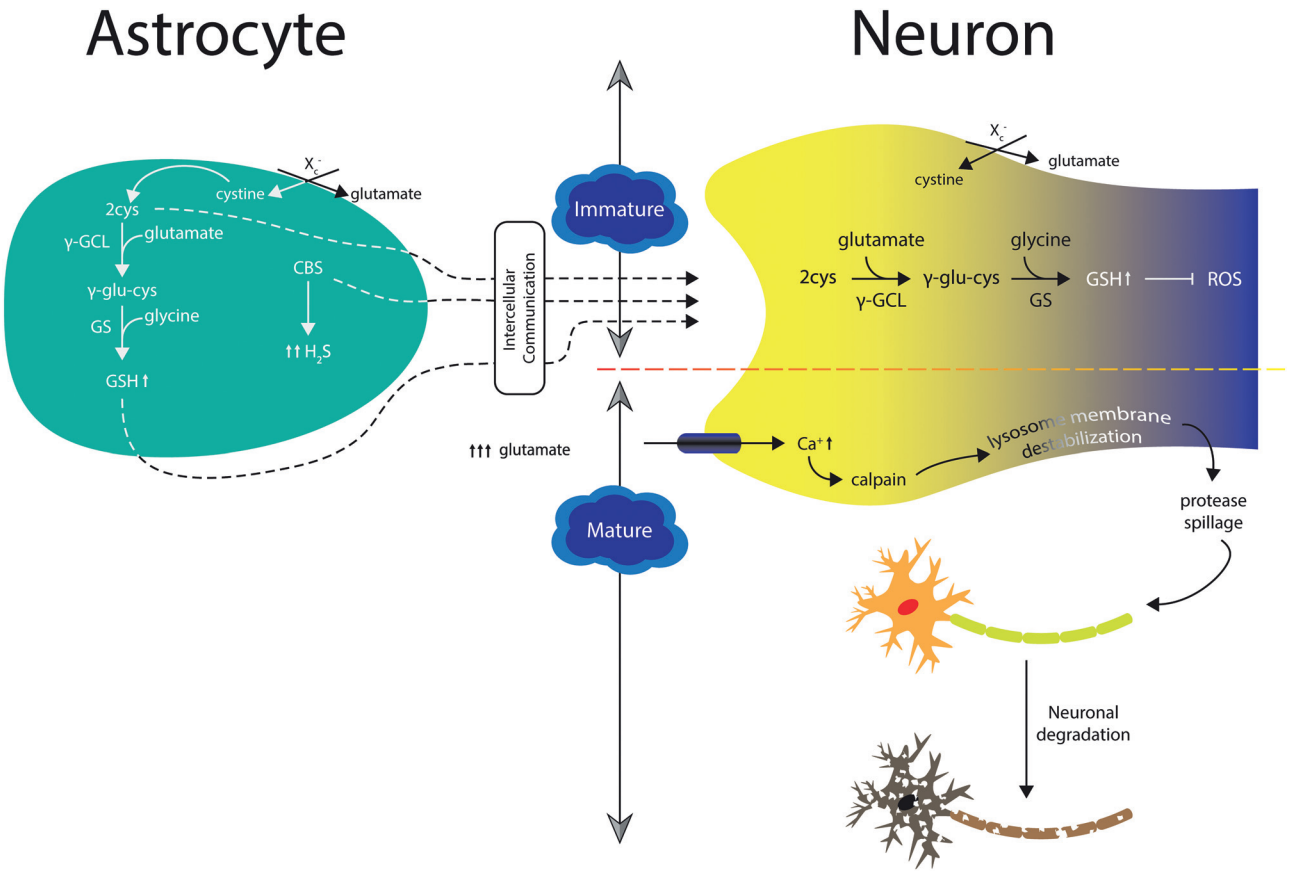


Fig. 2. Interactions between astrocytes and neurons for GSH and H_2S handling. Astrocytes take up excess glutamate surrounding the neurons and provide a cysteine source for neuronal GSH production, both preventing neuronal excitotoxicity. Increased activity of Xc^- antiporters as seen in mature neurons can heighten neuronal toxicity by H_2S mediated cell death with excess glutamate by activating protease release.

dictory role of H_2S in glutamate toxicity in the above-mentioned studies [42,44] is due to the use of mature and immature neurons. Immature neurons do not express all the glutamate receptors of the mature neurons [44]. In addition, astrocyte-neuronal co-culture experiments revealed that the oxidant-induced purinergic receptor (P2YR) increases intracellular calcium, resulting in mitochondrial calcium elevation. This increase in mitochondrial calcium level is sufficient to activate enzymes in the Krebs cycle that donate reducing equivalents to OXPHOS, thereby increasing ATP production and maintaining mitochondrial membrane integrity [45], two important events affected by ischemia. Although Fu et al. utilized vascular smooth muscle cells; they demonstrated stress-induced H_2S production in mitochondria (4–12 nmol/g H_2S as measured by MB) and a subsequent increase in ATP production [46]. Translocation of CSE from the cytosol to the mitochondria involves TOM 20, a mitochondrial outer membrane protein, and calcium. H_2S produced in this manner increases ATP in vascular smooth muscle cells [46]. On the same note, the ability of H_2S to increase ATP production after myocardial infarctions is one of its protective roles against disease progression. Moreover, pretreatment with H_2S donor NaHS (300 μM) also prevented oxygen-glucose deprivation, an in vitro simulation of ischemia, as well as induced ROS-mediated mitochondrial membrane potential changes in cultured cortical neurons after 12 h of reperfusion [47]. Mitochondrial membrane potential changes activate caspase-3 and is then attenuated by NaHS in neuronal cell culture, thus offering protection to cortical neurons from apoptosis [47]. The contradictory role of H_2S in neurons raises questions. Is preserving mitochondrial membrane integrity relevant to glutamate-dependent calcium overload in the stroke-affected brain? Does H_2S cause mature neuronal death in the stroke-affected brain? It is noteworthy to remember that CSE and CBS are calcium-dependent enzymes and elevated intracellular calcium may lead to increased H_2S production.

3. Brain edema, oxidative stress and ROS

3.1. Brain edema

A common characteristic in the pathology of brain ischemia is edema. As mentioned in the previous section, alterations in ion channels results in ion imbalance, stimulating influx of water to maintain osmolarity resulting in edema. Edema increases infarct volume and loss of neurological function, indirectly contributing to stroke-induced disabilities. Water is not freely permeable across the plasma membrane and requires channel proteins called aquaporins (AQP). Aquaporin 4 (AQP4) has been implicated in edema formation after ischemic stroke. Exogenous H_2S administered as a gas (40 and 80 ppm of H_2S) during reperfusion following middle cerebral artery occlusion (MCAO) in rats decreased AQP4 expression by enhancing protein kinase C (PKC) activity. AQP4 inhibition resulted in protection against brain edema by decreasing the edema volume from 21% to 7% as well as improvement in neurological function (motor and sensorimotor), which was inversely related to infarct volume [48]. In addition, to altered ion homeostasis, changes in blood brain barrier (BBB) integrity also lead to brain edema after stroke. Jiang et al, found that inhibition of CSE and 3-MST (H_2S generating enzymes) using topical application of pharmacological agents protected against early BBB dysfunction 3 h after reperfusion. This was confirmed after subjecting CSE $^-$/- mice to MCAO [49]. In a separate study, administration of the H_2S donor ADT

(50 mg/kg/day) or NaHS (25 μmole/kg/day) after 3 h of reperfusion protected BBB integrity 48 h later [50]. Brain concentrations of H_2S for this study were measured to be between 30 and 75 μM using the MB assay. Upregulation of matrix metalloproteinase (MMP) 9 in the ischemic area results in the degradation of tight junction proteins like occludin and zona occludens (ZO-1). Intraperitoneal administration of H_2S in mice prevented the loss of tight junction proteins like occludin and ZO-1 by decreasing NADPH oxidase (Nox) 4 mediated overexpression of MMP9 in the ischemic area, thus decreasing brain edema and preserving BBB integrity [50]. These two studies demonstrate a deleterious effect of H_2S (edema) during reperfusion, but a beneficial effect (protection against loss of barrier function) immediately following reperfusion. New questions arise from these two studies: Will blocking H_2S during reperfusion and supplementing H_2S after 3 h result in full protection? Is the initial edema required for H_2S to play a role following reperfusion? Etc.?

3.2. Oxidative stress and ROS

Following ischemia reperfusion injury, infarct volume correlates with the severity of brain injury. Efforts to decrease this infarct volume have been the subject of numerous investigations. The brain has high metabolic requirements making it vulnerable to oxidative stress [51]. Vascular reperfusion results in excess generation of mitochondrial ROS, primarily through electron leakage at complexes I and III in the electron transport chain, triggering apoptosis. Sources of ROS generation in the cell include mitochondria, superoxide-producing enzymes like xanthine oxide, NADPH oxidase and hydrogen peroxide-producing enzymes like superoxide dismutase, etc. ROS scavengers include antioxidants such as glutathione, as well as enzymes such as superoxide disumutase (scavenges superoxide), and catalase (scavenges hydrogen peroxide). In this section, we will discuss the role of H_2S in combating superoxide anion and enhancing GSH in brain cells.

In cultured brain endothelial cells and hippocampal neuronal cell lines, Na_2S (50 μM) or NaHS (50 and 250 μM) is reported to increase activity of antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase [52,53]. A similar effect was observed when NaHS (5 mg/kg) was administered 2 h after MCAO in Sprague–Dawley rats [53]. H_2S (5 μm NaHS) reversibly inhibits NOx in vesicles released by cortical neurons at synapses by the direct reduction of membrane-bound intravesicular cytochrome b5, thus decreasing superoxide production [54]. Aside from these indirect effects on ROS, H_2S is known to react with nitric oxide and neutralize peroxynitrite [55]. Chan et al. demonstrated that 300 μM NaHS was cytotoxic to primary cortical astrocytes when given prior to 8 h of oxygen glucose deprivation [56]. In addition, permanent ischemia in transgenic mice overexpressing superoxide dismutase 1 (SOD1) decreases infarct volume and edema. Furthermore, SOD 1 overexpression increases cellular antioxidants such as GSH and ascorbate [57]. in vivo experiments provide evidence for the role of H_2S in many pathways that combat oxidative stress, including the glutathione cycle, enzyme activation, and transcription factors pertinent to redox balance. In ischemic disease such as critical limb ischemia sulfide levels are decreased in skeletal muscle which correlates to a decrease in Nrf2 and its downstream targets such as SOD1, catalase and glutathione peroxidase, all important regulators of ROS homeostasis in the cell [58]. This investigation by Islam et al, showed that tissue ischemia decreases sulfide levels that may be critical in maintaining redox homeostasis suggesting that exogenous supplementation of H_2S maybe beneficial in ischemic stroke; however these experiments have yet to be performed in the brain.

H_2S (NaHS 100–150 μM) is known to increase intracellular GSH levels [59,60] by increasing the transport of cystine, an amino acid formed from two cysteine subunits, into the cells through the glutamate/cystine antiporter, Xc^-. The Xc^- antiporter allows glutamate efflux and cystine influx. Both glutamate and cystine act as competitive inhibitors for each other. Thus, excess extracellular glutamate inhibits cystine uptake. This process thereby decreases intracellular cysteine, one of the substrates for γ-glutamyl cysteinyl ligase, a rate-limiting enzyme in GSH biosynthesis. Cysteine provided by astrocytes serves as an important source of neuronal GSH production [61]. Accordingly, H_2S supplementation by NaSH (100 μM), with or without glutamate, attenuated excitotoxicity and increased intracellular GSH levels in a dose-dependent manner in primary neuronal cultures [62]. Notably, this increase in GSH can be attributed to H_2S-dependent increased activity of Xc- antiporters, which increased intracellular cysteine as much as six-fold under glutamate overload in the extracellular space. Kimura et al. attribute this increase in intracellular cysteine to the direct role of H_2S in reducing cystine to cysteine [62] suggesting that H_2S may contribute to both the import of cystine and its subsequent conversion to cysteine. In addition, H_2S increases the activity of the rate-limiting enzyme, γ-glutamycysteinyl synthetase, in GSH synthesis and activates the nuclear transcription factor Nrf2. Nrf2 promotes GSH synthesis and transport while decreasing GSH catabolism [63]. Furthermore, metabolic radiolabelling confirms the incorporation of cysteine generated by transsulfuration pathway, such as by CBS and CSE, into glutathione in astrocytes and neurons [64]. These studies imply that H_2S may be beneficial in boosting the cellular antioxidant defense mechanism in stroke brain.

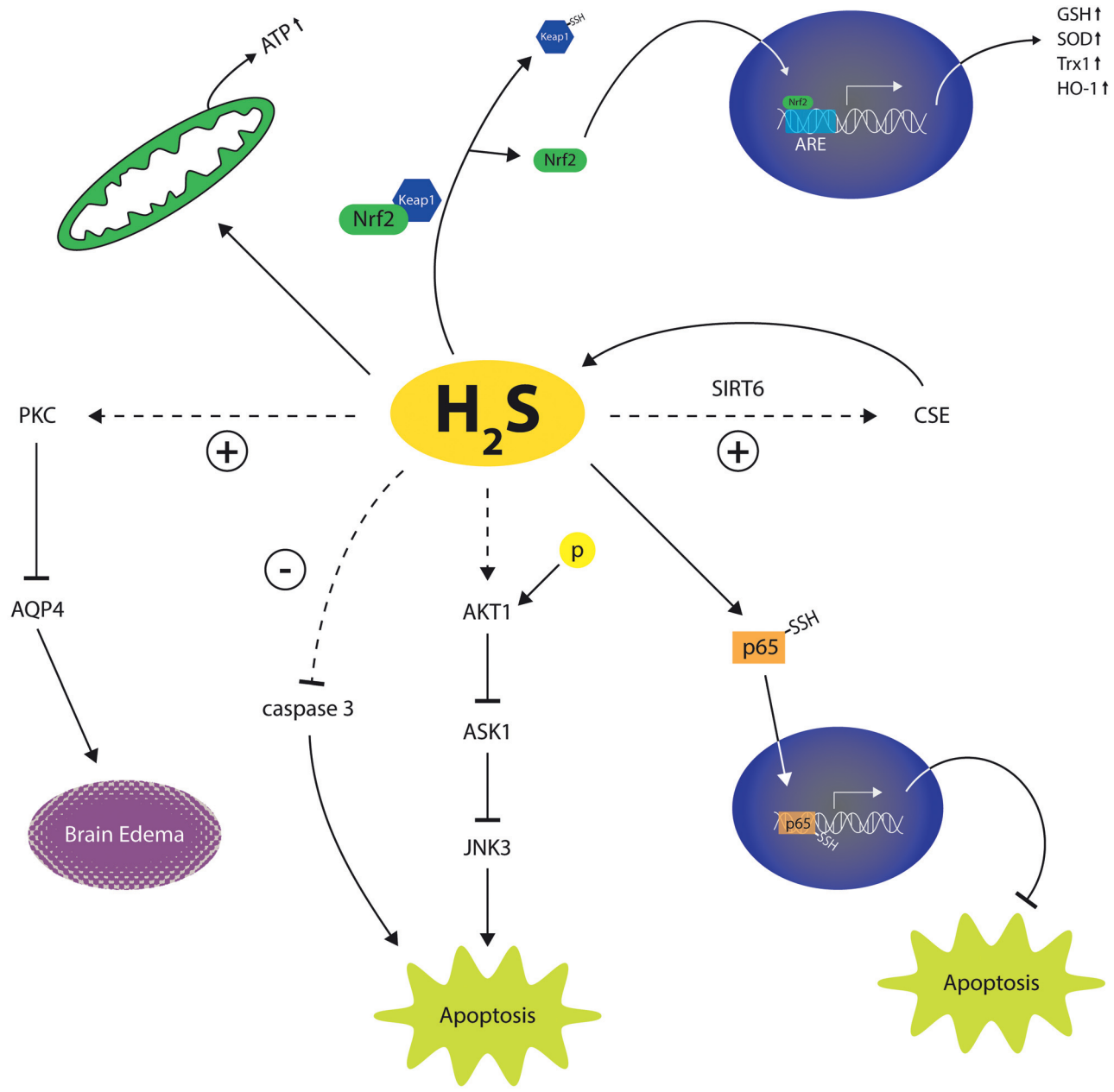
4. H_2S-dependent signal transduction

(see Fig. 2 for a reference to signaling and Fig. 3 for astrocyte and neuronal specific signaling detailed in this section)

4.1. NF-κB signaling and role of H_2S

Nuclear factor κB (NF-κB) is a heterodimeric transcription factor comprised of DNA binding and transcription activator partners. Of the many associated partners, p50 (DNA binding) and p65 (transcriptional regulator) are commonly studied in stroke. Many agents, including inflammatory cytokines and oxidants, activate NF-κB. In stroke-affected brains, NF-κB expression correlates with the severity of damage and neuronal apoptosis. In the forebrain of rats that underwent global brain ischemia, NF-κB translocation (both p50 and p65 subunit) to the nucleus was observed at 24 h. The degenerating or apoptotic neurons in the hippocampus showed persistent activation until 72 h post-ischemia suggesting a dual role of this transcription factor in apoptotic and non-apoptotic neurons [65]. In a rat transient MCAO model, Gabriel et al. reported a similar period for p65 induction due to reactive astrocyte and microglial/macrophage activity [66]. In addition, excitotoxicity of rat striatal neurons triggered p65 activation and apoptosis by upregulation of c-myc and p53 gene products leading to apoptosis [67]. Of note, heat shock protein 70 (HSP70) - expressing neurons did not undergo apoptosis, possibly by inhibiting the NF-κB pathway [68]. Taken together, these findings highlight the importance of studying NF-κB signaling pathway in cerebral ischemia. The important relationship between NF-κB signaling and H_2S has been the subject of several studies discussed in the following section.

Binding of tumor necrosis factor (TNF)-α to its receptor releases NF-κB from its inhibitor (IκBα), which stabilizes NF-κB in the cytosol, allowing NF-κB to translocate to the nucleus and activate the target gene. TNF – α increases CSE expression by its action on SP1, another transcription factor, increasing cellular H_2S levels. H_2S enhances activity of the NF-κB p65 subunit by sulfhydrating cys38 residue, a post-translational modification that enhances p65 and ribosomal protein S3 (RPS3) binding, leading to anti-



Akt1- RAC-alpha serine/threonine-protein kinase
AQP4- aquaporin 4
ARE - Antioxidant Response Element
ASK1- apoptosis signal-regulating kinase 1
ATP- adenosine triphosphate
CSE- cystathionine gamma-lyase
GSH - glutathione
H2S- hydrogen sulfide
HO-1 - heme oxygenase
JNK3- c-Jun N-terminal kinase 3
Keap1- Kelch-like ECH-associated protein 1
Nrf2- nuclear factor (erythroid-derived 2)-like 2
p65- nuclear factor NF-kappa-B p65 subunit
PKC- protein kinase c
SiRT6- Sirtuin-6
SOD - superoxide dismutase
Trx1 - thioredoxin
– – – – Mechanism Unknown

Fig. 3. H_2S-dependent signal transduction affecting stroke related events such as edema and inflammation with associated cellular events - apoptosis and gene transcription, and important stroke mediators including NfKB, Nrf2, Akt and caspase pathway.

apoptotic gene transcription in hepatic tissues and cells including macrophages [69]. It is unclear if the same mechanism exists in the ischemic brain. Additionally, H_2S (28 mmole/kg NaHS for 7 days prior to infarct) inhibits hippocampal CA1 neuron (more sensitive to oxidative stress) apoptosis by activating Akt1, thereby inhibiting ASK1/JNK3 dependent caspase-3 activation in a rat MCAO model [70]. Conversely, H_2S is known to mediate the upregulation of pro-inflammatory cytokines in a NF-kB independent fashion. Cyclooxygenase (COX)-2, interleukin (IL)-1α, IL-1β, TNF – α, IL-8 were all shown to increase with <10 ppm of H_2S in synoviocytes (fibroblast-like mesenchymal cells). Short-term H_2S stimulation of these cells resulted in compromised mitochondrial function and increased ERK1/2, c-JNK and p38 MAPKs [71]. The presence of inflammatory cytokines seems to alter the protective role of H_2S

in preserving mitochondrial function, at least in synoviocytes. This raises the question whether H_2S would protect or exacerbate mitochondrial function in the inflammatory milieu of an ischemic brain. In endothelial cells, cerebral ischemic/reperfusion injury results in apoptosis-inducing factor (mitochondrial antioxidant protein) and PARP-1 translocation to the nuclear compartment. However, NaHS (250 μM) treatment prevented both of the above-mentioned events in vitro [53].

One of the target genes of NF-κB is IL-6, a pro-inflammatory cytokine, which is elevated in the serum and cerebrospinal fluid of stroke patients. IL-6 upregulation in cerebral ischemia turns on the JAK/STAT3 cascade, a pro-survival pathway. STAT3 phosphorylation was increased in neurons and endothelial cells in the ischemic core but not in astrocytes or microglia after reperfusion. Activated STAT3 translocates to the nucleus and triggers anti-apoptotic gene expression and is believed to be neuroprotective during cerebral ischemic injury [72]. In addition, axonal regeneration by stimulation of JAK/STAT3 pathway is shown in retinal ganglion cells [73]. Interestingly, NMDA-induced excitotoxicity is antagonized by IL-6 via the STAT3 mediated anti-apoptotic pathway in cerebellar granule neurons [74]. Post-conditioning with the H_2S donor NaHS (10 μm for 15 s every 30 s 4 times) has been shown to protect isolated hearts from ischemic-reperfusion injury via activation of the JAK2/STAT3 pathway [75]. In isolated astrocyte culture, TNF – α and IL-6 reduced the expression of CBS by 2.5–3 fold and decreased H_2S via a p38 MAPK – NF-kB dependent mechanism. However, exogenous supplementation of H_2S (10 μm NaHS) abolished this effect in cultured astrocytes [40]. More in vivo studies are necessary to understand H_2S-dependent signaling under cerebral ischemic injury.

4.2. Nrf2 signaling and role of H_2S

Nrf2 is a member of the cap-n-collar proteins containing a basic leucine zipper motif, which participates in the oxidative stress response by promoting antioxidant gene transcription [76]. Keap1 is a stress sensor and acts as a negative regulator of Nrf2 by trapping Nrf2 in the cytosol [77]. Keap1 is also a substrate adapter for E3 ubiquitin ligase, cul3, which targets Nrf2 for degradation [76–78]. Dissociation of Nrf2 from Keap1 is important during oxidative stress conditions, which allows Nrf2 to bind to antioxidant/ electrophile responsive elements (ARE) in the DNA. ARE/Nrf2 binding results in the expression of GSH synthetic machinery, H_2S biosynthetic enzymes, SQR of sulfide disposal unit and anti-apoptotic proteins [76,78]. Nrf2 signaling in ischemic tissue and the relationship between H_2S and Nrf2 are discussed in the following paragraph.

In ischemic cardiac tissue, anti-apoptotic activity of H_2S (100 μg/kg Na_2S) was mediated by increased Nrf2 translocation to nucleus. Nrf2 translocation was shown to be associated with an increase in its downstream targets thioredoxin-1 (Trx1) and heme oxygenase-1 after 24 h [79]. Txr1 is an important physiologic reducing agent involved in H_2S generation, suggesting a possible feedback regulation of H_2S production in vivo [23]. H_2S also increases BAD (Bcl-2 associated death promoter) and PKCε phosporylation in ischemic cardiac tissue. BAD phosphorylation inactivates this pro-apoptotic factor while PKCε phosphorylation is necessary for its activation. PKCε in turn signals via its downstream target p44/42 to cause STAT3 nuclear translocation and transcription of anti-apoptotic genes like heat shock proteins, Bcl-2, COX-2 and Bcl-xl [79]. In addition, H_2S directly promotes Nrf2 nuclear translocation by Keap1 S-sulfhydration. Keap1 is modified by H_2S at cys 151 in the BTB domain, which is essential in the formation of functional E3 ubiquitin ligase complex [80]. The cys 151 S-sulfydration results in the dissociation of Nrf2 from Keap1 facilitating Nrf2 signaling. This pathway is known to protect mouse embryonic fibroblasts in culture against cellular senescence [80]. Interestingly, Satoh et al. has proposed Keap1 as an oxidative stress regulator; they believe depletion of Keap1 protects cortical neurons against oxidative stress [78]. It has also been elucidated that Keap1 inhibition by H_2S involves two other cysteine moieties at 226 and 613. H_2O_2-dependent oxidation is thought to precede S-sulfhydration by H_2S of these amino acids, leading to Keap1 inhibition. This inhibition can be reversed by the Trx system, which is also a downstream target of Nrf2 suggesting a feedback regulation loop [76]. From these studies it can be inferred that H_2S mediated Nrf2 activation by Keap1 inhibition may be protective in cerebral ischemia by preventing neuronal death during oxidative stress, however this is yet to be reported in the brain.

5. Conclusion

The toxicity and therapeutic value of hydrogen sulfide depends on the concentration, with lower concentrations playing a physiologic role and very high concentrations causing death [81]. While hydrogen sulfide is widely considered a gasotransmitter, there is uncertainty about the total concentration of this volatile gas or the highly reactive anionic species (SH^-) in both plasma and tissues [82]. Adding to the complexity, polysulfides, a recently characterized addition to sulfide biology, are more potent than H_2S and mediate sulfhydration of proteins. The literature has thoroughly documented the reactions of H_2S with many signal mediators, transcription factors, and channel proteins in neurons and glial cells both in vivo and in vitro. However, little is known about the interaction of H_2S intercellular communication and its consequence in ischemic stroke. Such information will serve as an invaluable tool to determine whether H_2S is cytoprotective or cytotoxic in cerebral ischemia. An important caveat to this research is that measured ranges in the literature are variable based on the detection method. Methylene blue overestimates the amount of free hydrogen sulfide but gives an approximation of total hydrogen sulfide that may or may not be available depending on the acidity of the surrounding environment. The toxicity and therapeutic value of hydrogen sulfide depends on the concentration, with lower concentrations playing a physiologic role and very high concentrations causing death. Overall there is still much work to be done in the field to form a more complete picture of the benefit of sulfide during and after ischemia.

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