

# H<sub>2</sub>S-based therapies for ischaemic stroke: opportunities and challenges

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## ABSTRACT

Stroke is a cerebrovascular disease displaying high mortality and morbidity. Despite extensive efforts, only very few therapies are available for stroke patients as yet. Hydrogen sulfide (H<sub>2</sub>S) is thought to be a signalling molecule that is endogenously produced and plays functional roles in the central nervous system. Currently, numerous studies show that H<sub>2</sub>S impacts stroke outcomes in animal and cellular models. Here, we review the recent research regarding the effects of endogenously produced H<sub>2</sub>S as well as exogenous H<sub>2</sub>S donors on stroke pathology, focusing on the potential of H<sub>2</sub>S-based therapies in treating ischaemic stroke. We also discuss the several issues that hinder the clinical translation of H<sub>2</sub>S-based therapies from the bench. Taken together, we think that H<sub>2</sub>S-based therapies are promising strategies for treating cerebral ischaemia if we successfully address these issues.

## INTRODUCTION

Stroke is the second leading cause of death and leading cause of adult disability worldwide, with cerebral ischaemia as the major form of stroke, which approximately accounts for more than 85% of all strokes.<sup>1</sup> Currently, the therapies for ischaemic stroke are rather limited, with tissue plasminogen activator (tPA) as the only drug treatment approved by the US Food and Drug Administration for acute ischaemic stroke. Promising evidence from the clinical trials is emerging to suggest that patients with stroke can benefit from endovascular management strategies due to the restoration of cerebral blood flow. However, the recovery of cerebral blood flow, theoretically, cannot rescue the brains cells in the rim of infarct cores, which rapidly undergo death triggered by ischaemic cascades. Thus, the development of additive treatments that either provide acute brain protection or promote long-term functional recovery is of clinical significance to improve the beneficial effects of endovascular reperfusion strategies.

Hydrogen sulphide (H<sub>2</sub>S) is traditionally considered a toxic gas. However, the research of the past two decades has revealed that H<sub>2</sub>S is a gasotransmitter that displays profound and pleiotropic effects both physiologically and pathologically.<sup>2</sup> Particularly, large amount of evidence suggests that H<sub>2</sub>S, as a gasotransmitter, plays important functional roles in the

central nervous system.<sup>3,4</sup> The neuroprotective abilities of H<sub>2</sub>S have been investigated in the context of ischaemic stroke. Here, we briefly review the newest reports about the effects of H<sub>2</sub>S on the outcomes following cerebral ischaemia as well as underlying mechanisms. We also discuss the important issues that challenge the clinical translation of H<sub>2</sub>S-based therapies.

## PHYSIOLOGICAL FUNCTIONS OF H<sub>2</sub>S AND ITS ROLES IN ISCHAEMIC STROKE

### The role of endogenously produced H<sub>2</sub>S in ischaemic stroke

Few studies have been devoted to investigate the role of endogenously produced H<sub>2</sub>S, the level of which is markedly increased after middle cerebral artery occlusion (MCAO),<sup>5,6</sup> in the pathology of ischaemic stroke. Currently available publications suggest the endogenously produced H<sub>2</sub>S contributes to stroke pathology.

H<sub>2</sub>S is endogenously synthesised by three enzymes, namely cystathionine  $\gamma$ -lyase (CSE), cystathionine  $\beta$ -synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3MST) in conjunction with cysteine aminotransferase.<sup>7</sup> CSE is in abundance in the cardiovascular system and produces H<sub>2</sub>S from cysteine.<sup>8</sup> CSE expression is minor in the brain, and consistently, CSE inhibitors do not suppress the production of H<sub>2</sub>S in the rat brain.<sup>3,8</sup> CBS is considered the predominant H<sub>2</sub>S synthesising enzyme in the brain. CBS is mainly expressed by astrocytes, and could also be expressed by microglia and neurons in the brain.<sup>9</sup>  $\beta$ -replacement of cysteine with homocysteine to H<sub>2</sub>S and cystathionine, the most kinetically efficient reaction catalysed by CBS, contributes up to 95% of the net production of H<sub>2</sub>S by CBS in the brain.<sup>10</sup> It has been shown that cysteine administration increases the infarct damage dose-dependently, which is abolished by the CBS inhibitor aminooxyacetic acid (AOAA).<sup>5</sup> This indicates that endogenously produced H<sub>2</sub>S from cysteine is likely involved in ischaemic brain damage. Indeed, high plasma cysteine levels within 24 hours of



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stroke onset have been shown to be positively correlated with early stroke deterioration and long-term clinical outcomes assessed at 3 months.<sup>11</sup> Wong's group shows that H<sub>2</sub>S production via 3MST is reduced in astrocytes after stroke, further suggesting that CBS is responsible for the elevation of H<sub>2</sub>S level following cerebral ischaemia.<sup>12</sup> Moreover, by using a permanent MCAO, Wong's group showed that MCAO enhances both H<sub>2</sub>S levels and H<sub>2</sub>S synthesising activities in the ischaemic cortex. Besides, infarct damage is reduced by four inhibitors of H<sub>2</sub>S synthesis enzymes.<sup>5</sup>

However, a recent study found that AOAA confers protective effects at low dose, although at higher doses it does not protect or even worsen the ischaemic injury after MCAO.<sup>13</sup> Moreover, our group showed that CBS expression and H<sub>2</sub>S synthesising activity were significantly reduced in the microglia in the ischaemic brain.<sup>14</sup> Moreover, we showed that overexpressing CBS in microglia *in vitro* or supplementing the exogenous H<sub>2</sub>S donor *in vivo* suppressed microglia-mediated neuroinflammation and reduced infarct damage in the ischaemic models.<sup>14</sup> Collectively, these results suggest that endogenously produced H<sub>2</sub>S in microglia via CBS plays a protective role by inhibiting neuroinflammation following cerebral ischaemia. Thereby, more research is needed to shed light on the role of endogenous H<sub>2</sub>S in stroke pathology.

#### The effects of exogenous H<sub>2</sub>S donors on stroke outcomes following cerebral ischaemia

Conflicting evidence exists for the effects of H<sub>2</sub>S donors on stroke outcomes following cerebral ischaemia. The discrepancy may result from the use of different doses of inorganic H<sub>2</sub>S donors such as sodium hydrogen sulfide (NaHS) in the previous studies.

The first study about the effects of H<sub>2</sub>S on stroke outcomes was published in 2006. The study showed that NaHS at 180 mmol/kg, but not at 90 mmol/kg (intraperitoneal injection), exacerbated infarct damage following MCAO in rats.<sup>5</sup> Consistent with the publication, a paper published later also reported that NaHS at 180 mmol/kg remarkably exacerbated neuronal damage at 7 days of reperfusion in a rat global ischaemia-reperfusion model induced by permanent bilateral occlusion of vertebral artery and followed by 15 min of occlusion of common carotid artery, and no effects were detected at 90 mmol/kg.<sup>6</sup> The deleterious effects of H<sub>2</sub>S following cerebral ischaemia are likely to be mediated by two mechanisms. First, H<sub>2</sub>S is a potent inhibitor of cytochrome c oxidase (complex IV) comparable to cyanide.<sup>15</sup> The inhibition is reversible with a Ki of 10–30 μM for isolated mitochondria or cultured cells.<sup>16</sup> H<sub>2</sub>S donors, especially inorganic H<sub>2</sub>S donors that release excessive H<sub>2</sub>S instantaneously, are expected to impair mitochondrial functions adversely at concentrations used in the previous studies. It is likely that toxic inhibition on complex IV mainly contributes to the deleterious effects observed with inorganic H<sub>2</sub>S donors following ischaemic stroke. Moreover, H<sub>2</sub>S has been

shown to activate N-methyl-D-aspartate (NMDA) receptor function following ischaemic stroke.<sup>17</sup> Indeed, NMDA receptor antagonists inhibit H<sub>2</sub>S-induced cell death in neurons<sup>18</sup> and reduce infarction *in vivo*,<sup>5</sup> suggesting that H<sub>2</sub>S exacerbates infarct injuries by activating NMDA receptors.

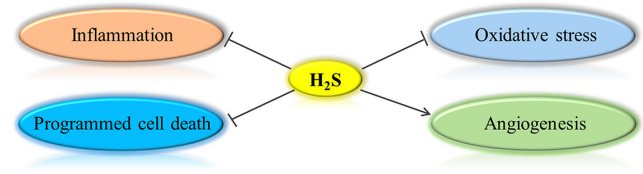
Current research suggests a dose-response pattern for the effects of H<sub>2</sub>S donors on stroke outcomes following cerebral ischaemia.<sup>19</sup> Strikingly, it is found that NaHS at 25 mmol/kg displayed remarkable neuroprotection against cerebral ischaemia-reperfusion injury in the same model.<sup>6</sup> The group also reports that NaHS at the same low dose significantly lowers mortality, improves neurological deficit and reduces infarct volume in rats following transient MCAO.<sup>20</sup> More recently, we also report that NaHS at 25 mmol/kg decreases infarct volumes and protects against the disruption of the blood-brain barrier in the well-established mouse transient MCAO model.<sup>21</sup> Moreover, we show that NaHS at low dose attenuates haemorrhagic transformation induced by tPA following cerebral ischaemia in the mouse transient MCAO model.<sup>22</sup> In addition, NaHS at 5 mg/kg significantly improves functional outcomes after 2 weeks in a rat MCAO model likely by augmenting angiogenesis in the peri-infarct area,<sup>23</sup> suggesting a potential value of H<sub>2</sub>S donors in regenerative recovery after stroke.

The slow-releasing organic H<sub>2</sub>S donors release H<sub>2</sub>S in a controlled manner. However, very few studies have investigated the effects of organic donors on stroke outcomes. The most widely used organic H<sub>2</sub>S donor is 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (ADT-OH), which requires cellular endogenous enzymes to release H<sub>2</sub>S.<sup>24</sup> We report that ADT-OH significantly inhibits microglia-mediated neuroinflammation upon lipopolysaccharide (LPS) stimulation.<sup>25</sup> Consistently, by using a transient mouse MCAO model, we show that 5-(4-methoxyphenyl)-3H-1,2-dithiole-3-thione (ADT), which is metabolised into ADT-OH *in vivo*, reduces infarct volumes and prevents disruption of the blood-brain barrier following MCAO via inhibiting the proinflammatory nuclear factor-κB (NF-κB) axis, matrix metalloproteinases 9 (MMP9) expression and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isoform 4-derived reactive oxygen species (ROS) production in the ischaemic brain.<sup>21</sup> Moreover, ADT is also able to inhibit haemorrhagic transformation induced by tPA following cerebral ischaemia in the mouse transient MCAO model.<sup>22</sup> In an innovative study by another group, the H<sub>2</sub>S donor moiety ADT-OH is combined with an NMDA antagonist (memantine) and the product is an H<sub>2</sub>S-releasing NMDA antagonist (S-memantine). Both ADT-OH and S-memantine protect against cell death in human neuroblastoma SH-SY5Y cells and primary neurons induced by oxygen and glucose deprivation. Importantly, S-memantine shows greater protective effects at the same concentration than ADT-OH does. In mice receiving bilateral carotid artery occlusion followed by reperfusion, at the dose of 25 mmol/kg, only S-memantine improves the survival rate and neurological deficits.<sup>26</sup>

Although inhalation of H<sub>2</sub>S does not find its use in clinical practice, inhalation of H<sub>2</sub>S gas has been shown to be protective in animal models following cerebral ischaemia. For instance, H<sub>2</sub>S inhalation at 40 ppm and 80 ppm decreases infarct volumes and brain oedema by suppressing the expression of aquaporin-4 (AQP-4) through activating protein kinase C in rats following MCAO.<sup>27</sup> Consistently, preconditioning with H<sub>2</sub>S inhalation decreases cerebral ischaemia/reperfusion injury and improves cognitive impairment in mice by the induction of HSP70 through the PI3K/Akt/Nrf2 pathway.<sup>28</sup> Inhalation of 80 ppm H<sub>2</sub>S leads to marked and reversible reduction of metabolic rate in mice,<sup>29</sup> indicating that H<sub>2</sub>S breathing may protect organ function when the nutrients and oxygen supply are compromised, such as after ischaemic stroke and cardiac arrest. Moreover, in a rabbit cardiac arrest model, inhalation of 80 ppm H<sub>2</sub>S reduces damage histologically and improves neurological functions during the acute phase.<sup>30</sup> More interestingly, inhalation of H<sub>2</sub>S gas has been shown to induce long-term hypothermia (2 days) and thus reduce infarct damage in aged rats after MCAO.<sup>31</sup>

### MECHANISMS UNDERLYING THE EFFECTS OF H<sub>2</sub>S IN ISCHAEMIC STROKE

Several mechanisms may contribute to the therapeutic effects of H<sub>2</sub>S. Following cerebral ischaemia, ischaemic insults trigger inflammation, oxidative stress, apoptotic cell death and endoplasmic reticulum stress, leading to ischaemic injuries. Our group shows that activation of adenosine 5'-monophosphate-activated protein kinase (AMPK) is a major mechanism underlying H<sub>2</sub>S inhibition on neuroinflammation.<sup>25</sup> Particularly, we show that both slow-releasing H<sub>2</sub>S donors and endogenously produced H<sub>2</sub>S protect against cerebral ischaemia by activating AMPK to inhibit microglia-mediated neuroinflammation.<sup>14</sup> NaHS at low doses reverses the ischaemia-induced elevation in malondialdehyde levels and decrease in Cu/Zu superoxide dismutase and glutathione (GSH) peroxidase activities in rats following transient MCAO,<sup>32</sup> suggesting that H<sub>2</sub>S may ameliorate cerebral ischaemic injuries by suppressing postischaemic oxidative stress. In addition, nuclear translocation of apoptosis-inducing factor (AIF) and poly(ADP-ribose) polymerase-1 (PARP-1) in the ischaemic brain is attenuated by NaHS in rats following transient MCAO,<sup>32</sup> indicating that NaHS likely reduces caspase-independent cell death by suppressing PARP-1/AIF signalling. Both NaHS at low dose and ADT protect blood-brain barrier integrity following mouse MCAO.<sup>21</sup> Moreover, NaHS promotes angiogenesis in the peri-infarct area after ischaemic stroke, possibly through augmenting the phosphorylation of AKT and extracellular signal-regulated kinase (ERK) and increasing the expression of angiopoietin-1 and vascular endothelial growth factor.<sup>23</sup> Collectively, current evidence suggests that H<sub>2</sub>S confers protection against cerebral ischaemia via the mechanisms of anti-inflammation, anti-oxidative



**Figure 1** Mechanisms underlying the effects of hydrogen sulfide (H<sub>2</sub>S) in ischaemic stroke.

stress, anti-programmed cell death and proangiogenesis (figure 1).

### THE CHALLENGES OF H<sub>2</sub>S-BASED THERAPIES FOR ISCHAEMIC STROKE

Several issues that have not been solved in the biology and effects of H<sub>2</sub>S following cerebral ischaemia hinder the clinical translation of H<sub>2</sub>S-based therapies from the bench. First, the signalling mechanisms underlying the therapeutic effects of H<sub>2</sub>S remain obscure. By definition, as a gasotransmitter, the effects of H<sub>2</sub>S should be specifically mediated by a limited number of cellular or molecular targets. Currently, the predominant H<sub>2</sub>S signalling mechanism is sulfhydrylation of target proteins. Since it is estimated that 10%–25% cellular proteins can be sulfhydrylated, the mechanism is unspecific. Moreover, we do not know whether the therapeutic mechanisms of H<sub>2</sub>S can be separated from its toxic mechanisms, such as inhibition on complex IV. Second, we know very little regarding the role of endogenous H<sub>2</sub>S in stroke pathology. Current studies mainly use the inhibitors of H<sub>2</sub>S synthases to investigate the role of endogenous H<sub>2</sub>S in stroke pathology. Since the unspecificity of these inhibitors, no clear conclusion has been reached based on these studies. We suggest that genetically engineered mice with deletion in H<sub>2</sub>S synthases, especially mice with cell-specific deletion of H<sub>2</sub>S synthases, will be the unique and valuable tools for the investigation of the role of endogenous H<sub>2</sub>S in stroke pathology. Third, current studies almost exclusively focus on the acute protection of H<sub>2</sub>S following cerebral ischaemia. Since most of the currently available stroke therapeutic targets display ‘biphasic’ effects,<sup>33</sup> it is important to examine the effects of H<sub>2</sub>S on long-term stroke outcomes.

### CONCLUSION MARKERS

Current evidence has suggested that H<sub>2</sub>S confers acute protection as well as promotes long-term functional recovery following cerebral ischaemia. Since inorganic H<sub>2</sub>S donors instantaneously release excessive H<sub>2</sub>S and high dose of inorganic H<sub>2</sub>S donors exacerbate brain injury after cerebral ischaemia, slow-releasing organic donors, especially those requiring endogenous enzymes to release H<sub>2</sub>S, open the opportunities to treat cerebral ischaemia with H<sub>2</sub>S-based therapies. Further investigations are



needed to elucidate the underlying signalling mechanisms mediating the therapeutic effects of H<sub>2</sub>S, and we believe that H<sub>2</sub>S-based therapies can be translated into clinical practice if we successfully address the issues.

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