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REVIEW ARTICLE

Protein S-sulfhydration by hydrogen sulfide in cardiovascular system

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Hydrogen sulfide (H_2S), independently of any specific transporters, has a number of biological effects on the cardiovascular system. However, until now, the detailed mechanism of H_2S was not clear. Recently, a novel post-translational modification induced by H_2S, named S-sulfhydration, has been proposed. S-sulfhydration is the chemical modification of specific cysteine residues of target proteins by H_2S. There are several methods for detecting S-sulfhydration, such as the modified biotin switch assay, maleimide assay with fluorescent thiol modifying regents, tag-switch method and mass spectrometry. H_2S induces S-sulfhydration on enzymes or receptors (such as p66Shc, phospholamban, protein tyrosine phosphatase 1B, mitogen-activated extracellular signal-regulated kinase 1 and ATP synthase subunit α), transcription factors (such as specific protein-1, kelch-like ECH-associating protein 1, NF-κB and interferon regulatory factor-1), and ion channels (such as voltage-activated Ca^{2+} channels, transient receptor potential channels and ATP-sensitive K^+ channels) in the cardiovascular system. Although significant progress has been achieved in delineating the role of protein S-sulfhydration by H_2S in the cardiovascular system, more proteins with detailed cysteine sites of S-sulfhydration as well as physiological function need to be investigated in further studies. This review mainly summarizes the role and possible mechanism of S-sulfhydration in the cardiovascular system. The S-sulfhydrated proteins may be potential novel targets for therapeutic intervention and drug design in the cardiovascular system, which may accelerate the development and application of H_2S-related drugs in the future.

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Abbreviation

ATP5A1, ATP synthase subunit α; CBS, cystathionine-β-synthase; CSE, cystathionine-γ-lyase; eNOS, endothelial NOS; ER, endoplasmic reticulum; IRF-1, interferon regulatory factor-1; K_{ATP}, ATP-sensitive K^+; Keap1, kelch-like ECH-associating protein 1; KLF5, krüppel-like factor 5; MEK1, mitogen-activated extracellular signal-regulated kinase 1; MMTS, methyl methanethiosulfonate; MSBT, methylsulfonybenzothiazole; NF-κB, nuclear factor κB; Nrf2, nuclear factor E2-related factor 2; PTEN, phosphatase and tensin homologue; PTP1B, protein tyrosine phosphatase 1B; SHR, spontaneously hypertensive rats; SP-1, specific protein-1; SUR2B, sulphonylurea 2B; TFAM, mitochondrial transcription factor A; TRP, transient receptor potential; VEGFR, VEGF receptor; WT, wild type

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Introduction

Hydrogen sulfide (H_2S) is a colourless, flammable, water-soluble gas with a characteristic smell of rotten eggs. H_2S was previously regarded as a toxic gas and environmental hazard. However, recent publications have revealed that H_2S is synthesized in mammalian tissues, and freely travels through cell membranes. H_2S acts independently of any specific transporters, and it has a number of biological effects on various systems (Meng *et al.,* 2015a; Cao and Bian, 2016; Feliers *et al.,* 2016; Ianaro *et al.,* 2016; Katsouda *et al.,* 2016; Cheng *et al.,* 2016b). Nowadays, H_2S is regarded as the third endogenous gasotransmitter followed by **NO** and CO (Hine *et al.,* 2015).

H_2S is produced, via the cysteine biosynthesis pathway, by three vital enzymes in mammalian species; these are **cystathionine-γ-lyase (CSE)**, **cystathionine-β-synthase (CBS)** and **3-mercaptopyruvate sulfurtransferase (MPST)**, and they have different distribution patterns in different tissues (Table 1). CSE is the main enzyme for H_2S production in the cardiovascular system (Wang, 2012; Wallace and Wang, 2015). Previous research found that H_2S has the potential to produce vasoconstriction or vasodilatation, angiogenesis, smooth muscle growth or apoptosis, cardioprotection and other effects (Mani *et al.,* 2013; Tsikas and Cooper, 2014; Ping *et al.,* 2015; Dunn *et al.,* 2016; Katsouda *et al.,* 2016; Marino *et al.,* 2016). Our studies have suggested that H_2S attenuates myocardial hypertrophy and fibrosis in spontaneously hypertensive rats (SHR) (Meng *et al.,* 2015a,c; Meng *et al.,* 2016), inhibits atherosclerotic plaque formation and inflammation in the aorta of apolipoprotein $E^{-/-}$ mice fed a high fat diet (Liu *et al.,* 2013), suppresses oxidative stress and apoptosis in myocardial ischaemia and reperfusion injury (Meng *et al.,* 2015b), augments mitochondrial function and the anti-oxidative capacity of endothelial cells (Xie *et al.,* 2016a), and activates **nuclear factor E2-related factor 2 (Nrf2)** to alleviate diabetes-accelerated atherosclerosis both *in vitro* and *in vivo* (Xie *et al.,* 2016b). H_2S also enhances the antioxidant activity, and regulates NO formation and kinase activity to maintain the homeostasis of the cardiovascular system (Liu *et al.,* 2015; Chen *et al.,* 2016; Shimizu *et al.,* 2016; Cheng *et al.,* 2016a). However, until now, the detailed mechanism of H_2S has not been clear. Recently, more and more researchers revealed that some of the above effects could be attributed to a novel post-translational modification induced by H_2S, named S-sulfhydration (Paul and Snyder, 2015a; Sen, 2017).

Table 1

Distribution of CBS, CSE and MPST

Names	Distribution
CBS	brain, astrocytes, liver
CSE	cardiovascular system, respiratory system
	liver, kidney, uterus, placenta, pancreatic islets
MPST	CNS
	aortic endothelium and smooth muscles

MPST, 3-mercaptopyruvate sulfurtransferase

S-sulfhydration is a chemical modification on specific cysteine residues of target proteins by H_2S. In the presence of H_2S, the free thiol groups of cysteine residues with a low pK_a become covalently converted into a persulfides group (Mustafa *et al.,* 2009; Paul and Snyder, 2015a). S-sulfhydration can be induced by H_2S on cysteine sulfenic acids (Cys-SOH) or cysteine disulfides (—S—S) (Figure 1 A-B), or by polysulfides on cysteine thiol (Cys-SH, Figure 1 C). It is important to note that H_2S also induces S-sulfhydration on cysteine thiols in oxidation conditions (Figure 1D,E). Similar to S-nitrosation, protein S-sulfhydration was reversed by the thioredoxin system (Paul and Snyder, 2015b; Wedmann *et al.,* 2016), which was closely correlated with cardiovascular diseases. This review will focus on the role of protein S-sulfhydration by H_2S in the cardiovascular system.

Detection of S-sulfhydration

Nowadays, it is very difficult to distinguish the persulfides group from the thiol group because of their similar reactivity. The biotin switch used previously for nitrosylation measurement has been modified to detect S-sulfhydration; this has been named as the 'modified biotin switch assay'. An alkylating agent S-methyl methanethiosulfonate (MMTS) was used to block thiol in proteins. The persulfides group was conjugated with N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP). The biotinylated protein was then immunoprecipitated and analysed by western blotting, which represents the level of protein S-sulfhydration (Mustafa *et al.,* 2009). However, the thiol and persulfides showed similar reactivity to MMTS, and the selectivity was not good. The basal S-sulfhydrated proteins account for as much as 25%, most of which might be a false positive (Pan and Carroll, 2013).

S-sulfhydration was also able to be measured with a maleimide assay with fluorescent thiol modifying regents. Fluorescent maleimide acts on both modified and unmodified sulfhydryl groups. DTT reduces only the modified cysteines, and the decreased fluorescent intensity representing S-sulfhydration is detected by SDS- PAGE (Sen *et al.,* 2012). Unfortunately, the propensity to determine both nitrosylation and sulfenic acids with the malemide assay weakened its credibility for detecting S-sulfhydration (Reisz *et al.,* 2013; Paul and Snyder, 2015b).

Zhang *et al.* proposed a novel measurement selective for S-sulfhydration, which was called 'tag-switch method' (Zhang *et al.,* 2014). Methylsulfonybenzothiazole (MSBT) was used to blocked thiols. Then a reagent containing nucleophile and biotin labelled only persulfides, while there was no binding with the blocked thiol groups. Finally, MSBT labelled persulfides representing S-sulfhydration were conjugated with streptavidin and visualized by western blots (Zhang *et al.,* 2014; Park *et al.,* 2015). However, the method without higher sensitivity posed a problem for S-sulfhydration measurement. To increase sensitivity, Wedmann *et al.* proposed an improved tag-switch method with new cyanoacetic acid derivatives such as fluorescent BODIPY moiety or the Cy3-dye (Wedmann *et al.*, 2016).

Mass spectrometry (MS) analysis was also used to filter and identify the protein S-sulfhydration. After protein

Figure 1

Reaction mechanisms for S-sulfhydration formation. S-sulfhydration can be induced by H_2S on cysteine sulfenic acids (Cys-SOH, A) or cysteine disulfides (−S-S, B), or by polysulfides on cysteine thiols (Cys-SH, C). H_2S induces S-sulfhydration on cysteine thiols in oxidation conditions (D-E).

samples were blocked with MSBT, only persulfide adducts reacted with CN-biotin to form biotin-labelled adducts. Then, these biotin-labelled proteins were broken into peptides for MS (Park *et al.,* 2015). By comparing the findings with the protein database, the names of the S-sulfhydrated proteins were identified and specific cysteine sites of protein were ascertained. However, it was very difficult to block the protein samples completely, and, therefore, it was easy to produce false positive results (Gao *et al.,* 2015; Park *et al.,* 2015).

Altogether, as yet, there is no ideal method to detect S-sulfhydration. More specific probes for the unique identification of S-sulfhydration are urgently required. The current methods combined with mass spectrometry might be beneficial for qualitative and quantitative detection of protein S-sulfhydration.

H_2S induced protein S-sulfhydration

Proteins with cysteine residues have the potential to be S-sulfhydrated. Mustafa *et al.* found that about 10–25% of proteins extracted from liver, including GAPDH, β-tubulin and actin, were S-sulfhydrated in physiological conditions, which suggests that protein S-sulfhydration might be a common form of post-translational modification (Mustafa *et al.,* 2009). LC-MS/MS analysis exhibited that GAPDH was S-sulfhydrated at Cys^{150}. Moreover, NaHS increased GAPDH activity as high as seven fold, which was absent after C150S mutation (Mustafa *et al.,* 2009). Mir *et al.* subsequently verified that GAPDH S-sulfhydration at Cys^{150} promoted its binding to the E3 ligase protein in brain (Mir *et al.,* 2014). However, one latest study failed to reproduce the conclusion on BL21 (DE3) *Escherichia coli* containing a pET15b vector expressing the wild-type (WT) His-tagged human GAPDH. They found that Cys^{156} or Cys^{247}, but not Cys^{152} (which refers to Cys^{150} in the liver of mice on GAPDH with the normal residue numbering system), was S-sulfhydrated by sulfide or polysulfide. And there was no increase in GAPDH activity after S-sulfhydration. In contrast, polysulfides decreased GAPDH activity to about half of the control (Jarosz *et al.,* 2015). Actually, not all cysteine-rich proteins are able to be S-sulfhydrated. The **VEGFR-2** contains several cysteine residues, and Cys^{1045} to Cys^{1024} serves as the molecular switch of vascular smooth muscle cell migration. However, there is no evidence that VEGFR-2 is able to be S-sulfhydrated by H_2S (Tao *et al.,* 2013). These discrepancies might be caused by the micro environment or the chemical structures of the proteins, and the different pathlogical states or specific characteristics of the cells or tissues. Generally, S-sulfhydration can alter the functions of a wide range of cellular proteins. The following is a summary of S-sulfhydrated proteins (Table 2) induced by H_2S in the cardiovascular system.

H_2S induced S-sulfhydration on enzymes or receptors in the cardiovascular system

NaHS induced S-sulfhydration in both cytosolic and membrane proteins of myocardium from isolated working frog hearts or langendorff-perfused rat hearts. Phospholamban, which is involved in myocardial relaxation through its modulation of intracellular calcium cycling, was identified to be S-sulfhydrated with the immunoprecipitation and modified biotin switch methods (Mazza *et al.,* 2013). This might be one of the selective post-translational modifications that maintains cardiac homeostasis.

Table 2

Categories and functions of protein S-sulfhydration in the cardiovascular system

Categories	S-sulfhydrated proteins	Functions in cardiovascular system
Enzymes/ receptors	ATP5A1	ATP synthase activity
	MEK1	Repair DNA damage
	PLN	Myocardial relaxation
	PPARγ	Adipogenesis
	PTP1B	Restore ER stress homeostasis
	p66Shc	Anti-oxidative stress
Transcription factors	IRF-1	Mitochondrial biogenesis
	Keap1	Anti-oxidative stress
	p65	Anti-apoptosis and anti-inflammation
	SP-1	Anti-myocardial hypertrophy
		Endothelial phenotypes regulation
Ion channels	$K_{ir}6.1$	Vasodilatation
	TRPV4	Vasodilatation

PLN, phospholamban

Protein tyrosine phosphatase 1B (PTP1B) plays a vital role in endoplasmic reticulum (ER) stress and is regarded as a potential target for therapeutic intervention in obesity-induced cardiomyopathy (Kandadi *et al.,* 2015) and septic shock-induced cardiovascular dysfunction (Coquerel *et al.,* 2014). Krishnan *et al.* found that H_2S induced PTP1B S-sulfhydration at Cys^{215} to inhibit its activity, which facilitated phosphorylation and activation of protein kinase-like ER kinase, and promoted restoration of ER homeostasis. All of these effects were unavailable in CSE-deleted HeLa cells (Krishnan *et al.,* 2011). This suggests that H_2S regulates ER stress via S-sulfhydration to inactivate PTP1B, which might be a novel mechanism for the protective effect of H_2S in the cardiovascular system.

Recently, H_2S was shown to directly S-sulfhydrate **PPARγ**. S-sulfhydration of PPAR γ at Cys^{139} increased nuclear PPARγ accumulation, enhanced DNA binding activity to promoter of the PPARγ response element, and promoted adipogenesis gene expression in adipocytes, which were blocked by DTT or Cys^{139} mutation of PPARγ (Cai *et al.,* 2016). As far as we know, PPAR is a dominant factor in blood lipid and glucose metabolism. S-Sulfhydration of PPARγ might be a novel target for diabetes, obesity, hyperlipidaemia and related complications of the cardiovascular system.

H_2S also increased **mitogen-activated extracellular signal-regulated kinase 1 (MEK1)** S-sulfhydration in both human endothelial cells and human fibroblasts, while there was lower S-sulfhydration of MEK1 in $CSE^{-/-}$ mice. S-sulfhydrated MEK1 facilitated **ERK1/2** phosphorylation, which subsequently transfers into the nucleus to activate PARP-1 and to repair DNA damage. Mutation of Cys^{341} on MEK1 inhibited ERK1/2 phosphorylation and PARP-1 activation, and failed to mediate DNA damage repair (Zhao *et al.,* 2014). Xie *et al.* found that both exogenous H_2S supplement and CBS overexpression increased p66Shc S-sulfhydration at Cys^{59}, which decreased the association of $PKC_{βII}$ with p66Shc and attenuated ROS production. However, H_2S failed to induce mitochondrial translocation of p66Shc, decrease ROS and protect H_2O_2-induced cell senescence if mutation of p66Shc at Cys^{59} (Xie *et al.,* 2014, 2016c). H_2S concentration-dependently increased S-sulfhydration of **ATP synthase subunit α (ATP5A1)** at Cys^{244} and Cys^{294} in HEK293 cells. Double mutation of C244S/C294S significantly attenuated ATP synthase activity. And there was lower S-sulfhydration and weaker activity of ATP5A1 in $CSE^{-/-}$ mice. This suggests that S-sulfhydration of ATP5A1 might be beneficial for the maintenance of ATP synthase homeostasis in mitochondrial energy disposal as well as antioxidant activity and redox signalling (Módis *et al.,* 2016). Most of the findings revealed that S-sulfhydration was the main post-translational modification induced by H_2S and played an important role in ROS production and redox signalling in the cardiovascular system.

Besides the cardiovascular system, several enzymes are also S-sulfhydrated by H_2S in the nervous system and endocrine system. NaHS augmented S-sulfhydration of hippocampal protein phosphatase type 2A, **PKA**, **PKC,** and **calcium/calmodulin-dependent protein kinase II (CAMKII)** to active postsynaptic signal pathways (Li *et al.,* 2016). H_2S also increased the S-sulfhydration of glucose-6-phosphatase and fructose-1,6-bisphosphatase to promote gluconeogenesis in primary hepatocytes (Untereiner *et al.,* 2016). S-sulfhydration and the activity of parkin, a neuroprotective ubiquitin E3 ligase, was up-regulated after H_2S administration, which was attenuated in the brains of patients suffering from Parkinson's disease (Vandiver *et al.,* 2014). However, whether these effects could be extrapolated to the cardiovascular system needs further exploration.

H_2S induced S-sulfhydration on transcription factors in the cardiovascular system

Specific protein-1 (SP-1) is an important transcription factor with multi-functions in the cardiovascular system (Yang *et al.,* 2013b). And there are a total of 11 cysteine residues on SP-1. Inhibiting H_2S production by **AOAA** (CBS inhibitor) or silencing CBS significantly reduced SP-1 S-sulfhydration at Cys^{68} and Cys^{755} in HUVECs, which enhanced proteasomal degradation of SP-1, followed by inhibited SP-1 binding with VEGFR-2 or neuropilin-1 promoter and impaired endothelial tube formation on Matrigel. All of these effects were dramatically restored by exogenous NaHS supplement. This suggests that H_2S regulates endothelial key phenotypes such as proliferation and migration via SP-1 S-sulfhydration (Saha *et al.,* 2016). Our latest study found that GYY4137, a H_2S slow releasing compound, increased S-sulfhydration on SP-1 in neonatal rat cardiomyocytes and in myocardium of SHR. There are four residues (Cys^{659}, Cys^{664}, Cys^{689} and Cys^{692}) in the domain of SP-1 for binding with krüppel-like factor 5

(KLF5, a key transcriptional factor involved in myocardial hypertrophy) promoter. And GYY4137 enhanced S-sulfhydration on SP-1 if there was an overexpression of WT SP-1 or SP-1 had mutations of C659A, C689A and C692A but not C664A in cardiomyocytes. Moreover, GYY4137 failed to attenuate KLF5 promoter activity and mRNA expression, reduce the binding between SP-1 and KLF5 promoter, decrease the mRNA expression of atrial natriuretic peptide and improve myocardial hypertrophy in angiotensin II-induced cardiomyocytes if the SP-1 mutation was C664A (Meng *et al.,* 2016). These findings suggest that S-sulfhydration at Cys^{664} is essential for the inhibitory ability of KLF5 transcription and protective effect against myocardial hypertrophy induced by H_2S.

Nrf2 is a vital transcription factor for protection against oxidative stress with **kelch-like ECH-associated protein 1 (Keap1)** as a negative receptor. Guo *et al.* found that NaHS increased Keap1 S-sulfhydration to promote dissociation of Keap1/Nrf2 and finally to increase transcription activity of Nrf2, which was involved in the protective effect against ischaemic reperfusion-induced oxidative stress and cell injury in human gastric epithelial cells (Guo *et al.,* 2014). Yang *et al.* verified that Keap1 was S-sulfhydrated in embryonic fibroblasts from WT mice but not CSE-knockout mice. NaHS S-sulfhydrated Keap1 at Cys^{151} to regulate the location, activity and target gene expression of Nrf2 in mouse embryonic fibroblasts. Tramtrack and Bric-á-Brac 2 dimerization domain (one functional domain on Keap1) deficiency completely abolished NaHS-induced Keap1 S-sulfhydration. The mutation of Cys^{151} in an intervening region, but not Cys^{288}, failed to enhance Keap1 S-sulfhydration, promote Nrf2 nuclear translocation or protect against cell senescence. This might be a novel mechanism for preventing from cell ageing by H_2S (Yang *et al.,* 2013a). Our latest study found that GYY4137 decreased aortic atherosclerotic plaque formation and ROS levels in aorta of streptozotocin-induced LDL receptor knockout out mice ($LDLr^{-/-}$) but not in $LDLr^{-/-}$ and $Nrf2^{-/-}$ double knockout mice. GYY4137 also attenuated foam cell formation and oxidative stress in peritoneal macrophages isolated from WT mice but not $Nrf2^{-/-}$ mice. This suggest that H_2S attenuates diabetes-accelerated atherosclerosis in an Nrf2-dependent manner. Further study showed that GYY4137 promoted the dissociation of Keap1 from Nrf2 in ox-LDL and high-glucose stimulated endothelial cells, which might be attributed to Keap1 S-sulfhydration at Cys^{151} and Cys^{273}. We also found that Keap1 mutation of C151A, but not C273A, abolished Keap1/Nrf2 dissociation, Nrf2 nuclear translocation and ROS inhibition induced by GYY4137 administration. It is proposed that protein S-sulfhydration by H_2S might be a novel therapeutic target to prevent diabetes-accelerated atherosclerosis (Xie *et al.,* 2016b). The latest research found that H_2S elevated Keap1 S-sulfhydration to reduce the association between Keap1 and Nrf2 in the kidneys of rats on a high-salt diet, followed by decreased collagen deposition and oxidative stress (Huang *et al.,* 2016). All of these results suggest that Keap1 is a key target of H_2S in several different cells or tissues. S-sulfhydration of Keap1 might be a potential target for attenuating oxidative stress and related cardiovascular diseases.

NF-κB is also a multi-functional transcription factor. Sen *et al.* found that TNF-α enhanced the binding activity between NF-κB and DNA, followed by increased p65 binding with the promoter of anti-apoptotic genes in macrophages. However, the anti-apoptosis effect was abolished in macrophages from $CSE^{-/-}$ mice, which was restored by CSE overexpression or H_2S supplement. Further experiments showed that H_2S S-sulfhydrated p65 at the highly conserved Cys^{38} residue and augmented its association with ribosomal protein S3 as a co-regulator of NF-κB to activate the promoter of anti-apoptotic genes. All these effects were absent after p65-C38S was transfected (Perkins, 2012; Sen *et al.,* 2012). However, Du *et al.* found that H_2S enhanced p65 S-sulfhydration in ox-LDL-induced macrophages, which was abolished by DTT or p65 mutation at Cys^{38}. Moreover, S-sulfhydration of p65 by H_2S helped to inhibit NF-κB activation and **monocyte chemoattractant protein 1 (MCP-1 also known as CCL2) g**eneration and suppressed ox-LDL-induced inflammation. The various effects of p65 S-sulfhydration can possibly be attributed to a different condition, which was anti-apoptosis in physiological but anti-inflammation in pathological conditions (Du *et al.,* 2014).

Li *et al.* found that a deficiency in CSE decreased mitochondrial DNA levels and mitochondrial transcription factor A (TFAM) expression in both smooth muscle cells and arteries, which resulted in mitochondrial function disorder. H_2S S-sulfhydrated transcription repressor interferon regulatory factor-1 (IRF-1), strengthened the binding between IRF-1 and DNA methyl transferase 3A promoter to inhibit TFAM promoter methylation. Finally, TFAM methylation was attenuated, while the expressions of TFAM and mitochondrial DNA were increased to restore the mitochondrial biogenesis (Li and Yang, 2015). Bioinformatics analysis found that only Cys^{53} is located in the DNA-binding domain of IRF-1.

H_2S induced S-sulfhydration on ion channels in cardiovascular system

Voltage-activated Ca^{2+} channels, one of the most important calcium channels in cardiovascular system, are regulated by H_2S (Fukami and Kawabata, 2015; Ping *et al.,* 2015; Zhang *et al.,* 2015). NaHS concentration-dependently inhibits L-type calcium currents in cardiomyocytes, which was abolished by DTT. And NaHS decreases the functional free sulfhydryl group in L-type calcium channel, which provides indirect evidence for S-sulfhydration of voltage-activated Ca^{2+} channels by H_2S (Zhang *et al.,* 2012). However, there was no direct evidence that voltage-activated Ca^{2+} channels were S-sulfhydrated by H_2S. Whether H_2S regulates related subunits or associated cysteine sites is also still unknown.

Transient receptor potential (TRP) channels, as putative pro-angiogenic Ca^{2+}-permeable channels, are also modulated by H_2S (Munaron *et al.,* 2013; Zhang *et al.,* 2015). Liu *et al.* found that H_2S S-sulfhydrated **TRPV6** at Cys^{172} and Cys^{329} sites in bone marrow mesenchymal stem cells. Overexpression of TRPV6 increased Ca^{2+} influx and activated the PKC/β-cateine signal pathway to promote osteogenic differentiation. Mutation of TRPV6 at both Cys^{172} and Cys^{329}, but not only one site alone, resulted in a suppressed PKC/β-cateine signal pathway and impaired osteogenic differentiation.

Moreover, there were also several cysteine residues at other Ca^{2+} TRP channels, such as **TRPV3** and **TRPM4**, which have the potential to be S-sulfhydrated by H_2S (Liu *et al.,* 2014b). A previous study confirmed that endothelial cells are responsible for endogenous H_2S production and H_2S-induced vasodilatation. Naik *et al.* found that H_2S-induced Ca^{2+} and K^+ influx to dilate vessels was blocked after **TRPV4** inhibition. Moreover, S-sulfhydration of TRPV4 was enhanced after Na_2S treatment in aortic endothelial cells (Naik *et al.,* 2016). This suggests that TRPV4 is activated after S-sulfhydration, which might be the key factor in vasodilatation.

K_{ir}6.1, a subunit of the ATP-sensitive K^+ (K_{ATP}) channels, was S-sulfhydrated after CSE overexpression, and this did not occur if CSE was absent or mutated. A further study confirmed that S-sulfhydrated K_{ir}6.1 at Cys^{43} attenuated ATP production but elicited more phosphatidylinositol 4,5-bisphosphate to bind with K_{ir}6.1, which promoted K_{ATP} channel activity and improved vasodilatation. Moreover, not only S-sulfhydration but also vasodilatation induced by H_2S was alleviated in K_{ir}6.1-Cys^{43} mutants (Mustafa *et al.,* 2011). This might be the key mechanism for H_2S to act as an endothelial derived relaxing factor. Kang *et al.* also found that H_2S increased S-sulfhydration on sulphonylurea 2B (SUR2B) at Cys24 and Cys1455, another subunit of K_{ATP} channels complex, to restore smooth muscle contraction (Kang *et al.,* 2015). Liu *et al.* found that H_2S decreased the membrane potential, inhibited the fast inactivation component of the voltage-dependent potassium channel current in gastric smooth muscle cells and promoted gastric motility, which was suppressed by KV4.3 knockdown. Meanwhile, KV4.3 was S-sulfhydrated by H_2S, which was attenuated by DTT (Liu *et al.,* 2014a). All these data suggested that different cysteine sites might be S-sulfhydrated in different cells, which have various effects responsible for physiological or pathological process.

Crosstalk with protein S-sulfhydration and other post-translational modifications

S-sulfhydration at cysteine residues usually alters the structure and function of a protein. It is possible that crosstalk occurs with several other post-translational modification to ultimately regulate a physiological or pathological process. But overall, there are relatively few studies on this type of crosstalk in cardiovascular research.

It was reported that **NO** elevates S-nitrosylation on **phosphatase and tensin homologue (PTEN)** at C83S to attenuate its activity followed by **Akt** activation. One study found that H_2S S-sulfhydrated PTEN at Cys^{71} and Cys^{124} in human neuroblastoma SH-SY5Y cells (Numajiri *et al.,* 2011). Furthermore, S-nitrosylation on PTEN increased if H_2S production was inhibited by knocking down CBS. Consistent with the reduced S-sulfhydration, Akt activity increased significantly (Ohno *et al.,* 2015). As far as we know, PTEN is also a key signal molecular in cardiomyocyte apoptosis (Ke *et al.,* 2016), ventricular remodelling (Yang *et al.,* 2016), angiogenesis (Serra *et al.,* 2015) and other cardiovascular diseases. It is thought that protein S-sulfhydration and S-nitrosylation of PTEN keep a dynamic balance, and compete with each other to maintain normal function of the protein. PTP1B at Cys^{215} also underwent S-nitrosylation and S-sulfhydration (Chen *et al.,* 2008; Krishnan *et al.,* 2011). S-sulfhydration of p65, one key submit of NF-κB, disrupted the S-nitrosylation of itself to suppress apoptosis in macrophages (Sen *et al.,* 2012). More recently it was verified that the effect of H_2S is determined by S-nitrosylation but not S-sulfhydration (Sun *et al.,* 2016). However, further studies need to be done on more proteins with simultaneous S-sulfhydration and S-nitrosylation to determine the possible physiological significance of each of these processes. Also, it would be beneficial to identify the potential mechanism for the common 'crosstalk' between H_2S and NO in the cardiovascular system.

Kang *et al.* reported that H_2S increases S-sulfhydration on SUR2B, one of the key subunits of the K_{ATP} channel complex, in mouse colonic smooth muscle cells. Furthermore the peroxynitrite donor SIN-1 enhances the tyrosine nitration of K_{ir}6.1 (another subunit of th eK_{ATP} channel complex). This SIN-1-induced up-regulation of tyrosine nitration on K_{ir}6.1 was restored by NaHS in Chinese hamster ovary cells transfected with K_{ir}6.1 and the SUB2B mutant at C263S but not C24S or C1455S. That is S-sulfhydration of SUB2B at C24S and C1455S inhibited the nitration of K_{ir}6.1. Moreover, NaHS also reduced the tyrosine nitration of **Ca_v1.2b channels** to improve Ca^{2+}-induced contractions in mouse ileum (Kang *et al.,* 2015). In accord with the previous study, NaHS reduced the activity of L-Ca^{2+} channels containing free sulfhydryl groups in cardiomyocytes (Zhang *et al.,* 2012). These results suggest that H_2S might directly S-sulfhydrate L-Ca^{2+} channels to regulate Ca^{2+} homeostasis in the heart. Moreover, H_2S S-sulfhydrates cysteine-rich proteins to induce several other post-translational modifications on different proteins. Both of the direct and indirect effects of S-sulfhydration play a vital role in maintaining the physiological function of proteins in the cardiovascular system.

A significant amount of endogenous H_2S anion is generated in rat cardiomyocytes and cardiac fibroblasts. The reduction in H_2S production caused by CSE or CBS deletion enhanced the protein S-guanylation induced by 8-nitro-cGMP. Moreover, exogenous NaHS treatment markedly enhanced S-sulfhydrated 8-nitro-cGMP, which attenuated 8-nitro-cGMP-induced H-Ras activation, but not the activation of the H-Ras C184S mutant, by H-Ras S-guanylation in rat cardiomyocytes and myocardium from a failing heart, due to myocardial infarction (Nishida *et al.,* 2012). As 8-nitro-cGMP is a key signalling molecule in cardiovascular system disorders (Akaike *et al.,* 2010), these findings suggest that 8-nitro-cGMP S-sulfhydration by H_2S not only inhibits S-guanylation but also antagonizes the oxidative stress-induced or electrophile-mediated cell injury, which might be a novel mechanism of cardioprotection.

At baseline conditions, in endothelial cells isolated from aortae of WT mice, 30% of total **endothelial NOS (eNOS)** is S-nitrosylated and 21% is S-sulfhydrated, and these values are elevated by NO or an H_2S donor respectively. Altogether about 5% of total eNOS is S-sulfhydrated in aortic tissue of WT mice, but S-sulfhydration is undetectable in CSE knock out mice. Moreover, H_2S abolished the S-nitrosylation of eNOS induced

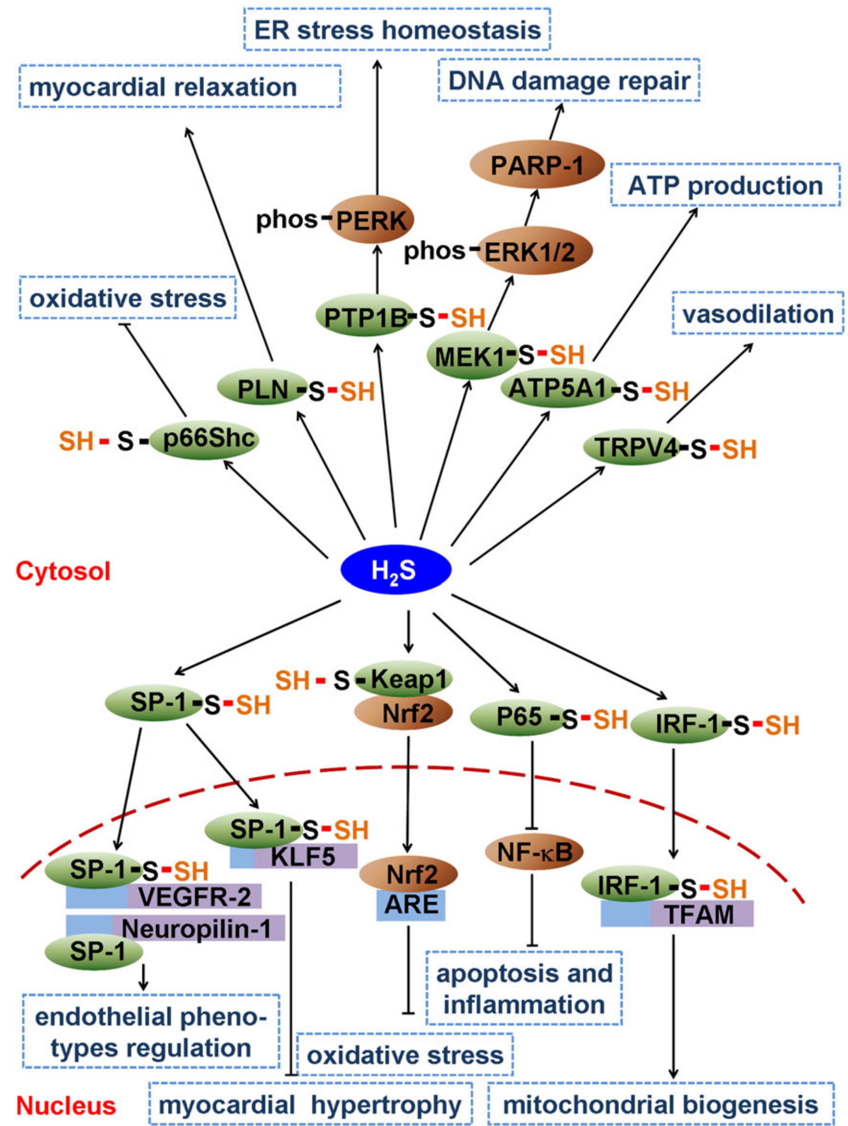

Figure 2

Schematic illustration of possible roles of S-sulfhydration by H_2S in the cardiovascular system. H_2S induces S-sulfhydration on p66Shc to inhibit oxidative stress. S-sulfhydration on phospholamban (PLN) promotes myocardial relaxation. S-sulfhydration on protein tyrosine phosphatase 1B (PTP1B) restores endoplasmic reticulum stress homeostatis. H_2S also S-sulfhydrates MEK1 to repair DNA damage. ATP5A1 and transient receptor potential V4 (TRPV4) S-sulfhydration improves ATP production and vasodilatation respectively. H_2S also S-sulfhydrates SP-1, Keap1, NF-κB and IRF-1 to regulate target gene transcription, which is vital for the regulation of endothelial phenotypes, myocardial hypertrophy, oxidative stress, mitochondrial biogenesis, apoptosis and inflammation.

by the NO donor **sodium nitroprussiate (SNP)**, but SNP had no effect on NaHS-induced S-sulfhydration of eNOS in endothelial cell lysates. NaHS increases S-sulfhydration, NO production and eNOS dimerization on WT eNOS and C689S-eNOS but not C443S-eNOS. NaHS also increases the phosphorylation of WT-eNOS or C443G-eNOS but not S1179A-eNOS. These results suggest that H_2S and NO compete for S-sulfhydration and S-nitrosylation at the cysteine residues to regulate the phosphorylation and activity of eNOS. H_2S, as a pivotal coordinator, maintains the dynamic homeostasis among several post-translational modifications of endothelial function (Altaany *et al.*, 2014).

In addition, S-sulfhydration of p66Shc at Cys^{59} promoted p66Shc phosphorylation at Ser^{36} due to an enhanced association with $PKC_{\beta II}$ and p66Shc (Xie *et al.*, 2014). S-sulfhydration is involved in the protective effect of the protein-O-GlcNAcylation in myocardial ischaemia reperfusion injury (Pagliaro *et al.*, 2011). H_2S also induces S-polythiolation, S-alkylation, S-arylation and other post-translational modifications (Rudolph and Freeman, 2009; Ida *et al.*, 2014). However, whether there is a crosstalk with S-sulfhydration and the possible effect in the cardiovascular system remains unknown. More knowledge of the crosstalk may speed

up our understanding of the role of protein S-sulfhydration in cardiovascular disease.

Concluding remarks and future perspectives

Over the past few years, significant progress has been achieved in delineating the role of protein S-sulfhydration by H_2S in the cardiovascular system (Figure 2). However, more scientific methods with enhanced sensitivity and specificity to detect S-sulfhydration are urgently needed. More proteins and detailed cysteine sites of S-sulfhydration need to be investigated in the cardiovascular system. However, not all of the proteins subjected to S-sulfhydration have an altered spatial configuration and activity. This might be determined by the location of the S-sulfhydrated cysteines. If S-sulfhydrated cysteines are located in the key domain, which is vital to maintain the structure and activity of that protein, protein S-sulfhydration will alter the protein function and signal transduction. In other words, there might be no significant difference after S-sulfhydration, which is known as 'ineffective S-sulfhydration'. Moreover, the significance of S-sulfhydration in the cardiovascular system, such as target gene transcription, enzymatic activity and ion channel permeability, are to be investigated in further studies. The exact nature of the crosstalk between S-sulfhydration and other post-translational modifications is not yet known and deserves to be better elucidated. In addition, the level of protein S-sulfhydration is controlled by the thioredoxin system, which suggests that some agents that alter thioredoxin activity or expression will be involved in regulating the intracellular levels of protein S-sulfhydration and H_2S-mediated biological and pharmacological effects (Wedmann *et al.*, 2016).

Protein S-sulfhydration, as a vital post-translational modification induced by H_2S, is a possible a molecular mechanism for the effects of H_2S. Clinically, the relevance of S-sulfhydration in cardiovascular diseases needs to be studied. More information about S-sulfhydration will help us to understand how S-sulfhydration at specific cysteines can have a beneficial effect in various cardiovascular diseases. Moreover, the S-sulfhydrated proteins may be potential novel targets for therapeutic intervention and drug design in the cardiovascular system, which may accelerate the development and application of H_2S-related drugs in the future.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b,c,d,e).

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Conflict of interest

The authors declare no conflicts of interest.

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