EFFECTS OF SULFIDE-CONTAINING COMPOUNDS ON DEVELOPMENT OF ATHEROSCLEROSIS IN HUMAN UNDOTHELIAL CELLS AND HYPERLIPIDEMIC RABBITS

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A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILISOPHY

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NATIONAL UNIVERSITY OF SINGAPORE
2013
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

WEN Yadan
25 July 2013
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As a chronic inflammatory disease of the arterial wall, atherosclerosis is a leading cause of death and morbidity worldwide. However, current treatments, statins, causing strong adverse drug reaction lead to unsatisfactory tolerance in patients experiencing coronary event. Hydrogen sulfide (H$_2$S), as the novel identified gaseous mediator in mammals, has emerged its protective effect on oxidative stress, inflammation, cardiovascular disease and neurodegenerative disease. In this study, the therapeutic potentials of H$_2$S and an analog of sulfide-containing garlic extraction, SPRC on atherosclerosis in vitro and in vivo were investigated.

In pilot study - experiment I, human umbilical vein endothelial cells (HUVECs), as the major cells evolved in the initial process of atherosclerosis, were protected by exogenous H$_2$S (NaHS) against hydrogen peroxide (H$_2$O$_2$) induced oxidative stress and mitochondrial dysfunction. H$_2$S showed no toxic to HUVECs at μM level. The results obtained from MTT, LDH releasing and Sulforhodomine B indicated that H$_2$S increased cell viability damaged by H$_2$O$_2$. For unveiling the mechanisms hidden behind, the mitochondria, redox status and program cell death were the three targets focused on. By observed by the staining of Hocheest, PI and Annexin V/PI, H$_2$S reduced apoptotic cells, which may be mediated by increased anti-apoptotic proteins (Bcl-2 and Bcl-X$_L$) and decreased pro-apoptotic proteins (cleaved caspase-3 and Bax). Mitochondrial function was reserved by H$_2$S through increasing ATP synthesis. H$_2$S also maintained the intact mitochondrial membrane by attenuating the dissipation of mitochondrial electrochemical potential ($\Delta \Psi_m$) and inhibiting cytochrome c releasing. The production of ROS detected by H$_2$DCFDA and DHE was inhibited by H$_2$S which elevated GSH, SOD, catalase, GST and GPx. The effects of H$_2$S can be reversed by inhibitor of CSE, PAG. The antioxidative and mitochondrial protective effects of H$_2$S may be through CSE/H$_2$S pathway.
In experiment II, the mitochondrial protective effect of \( \text{H}_2\text{S} \) was further demonstrated on New Zealand White (NZW) rabbit aortas. After the rabbits aortas were collected, mitochondria were isolated and accepted the injury from \( \text{H}_2\text{O}_2 \). \( \text{H}_2\text{O}_2 \) treatment resulted in oxidative stress to the aortic mitochondria, which showed a greater extent of ROS generation by the staining of \( \text{H}_2\text{DCFDA} \) and DHE. Under such circumstance, exogenous \( \text{H}_2\text{S} \) (NaHS) not only inhibited ROS generation, but also increased ATP synthesis. As the main location of producing ROS and ATP, mitochondrial respiration chain became the investigated target. Oxygen consumption by the respiration chain was suppressed by \( \text{H}_2\text{O}_2 \) and rescued by \( \text{H}_2\text{S} \). The activities of mitochondrial respiration chain complex I, II/III, IV and matrix enzyme \( \alpha\)-KGDHC was restored by \( \text{H}_2\text{S} \). \( \Delta\Psi_{\text{m}} \) and \( \Delta540 \) for testing mitochondrial swelling showed \( \text{H}_2\text{S} \) prevented the mitochondria rupture and maintained mitochondrial membrane. PAG showed the adverse effects of \( \text{H}_2\text{S} \).

In experiment III, \( \text{H}_2\text{S} \) showed the inhibition of atherogenesis on NZW rabbit hyperlipidemic model. The serum were collected to test the cholesterol level, LDL level and ox-LDL level, which significantly increased by high cholesterol feeding (HCD). Administration of \( \text{H}_2\text{S} \) leaded to a decrease of LDL level and ox-LDL level, which may be mediated by the activation of HO-1. Aortic lesions detected by H&E and carotid arterial lesions detected by high resolution ultrasonographic (HRUS) imaging, showed that the atherosclerotic lesions in arteries were inhibited by \( \text{H}_2\text{S} \) from the decreased intima-media thickness (IMT) and plaques sizes. The diminished plaques may be due to the suppression of free radicals, activation of antioxidants and inhibition of cell adhesive and inflammatory molecules. PAG showed the more severe atherosclerotic lesions. The cardiovascular protection of \( \text{H}_2\text{S} \) may be through CSE/\( \text{H}_2\text{S} \) pathway.

In experiment IV, S-Propargyl-cysteine (SPRC), a sulfide-containing molecule and the structural analog of a garlic extraction - S-allylcysteine (SAC), inhibited early
atherogenesis on NZW rabbit hyperlipidemic model. HCD treatment not only leaded to significantly increased body weight, serum cholesterol level and LDL level, but also formed the early atherosclerotic plaques. SPRC decreased LDL level and inhibited the plaques formation by the observation of aortas by H&E and carotids by HRUS. The mechanisms of anti-atherogenesis by SPRC may be through the regulation of redox status and suppression of inflammatory cell adhesion. The cardiovascular protective effect of SPRC was inhibited by PAG, showing greater atherosclerotic lesions. This anti-atherogenesis effect of SPRC may be through CSE/H₂S pathway.

In summary, H₂S and SPRC carry potential effects on atherosclerotic therapy, through the endothelial protection, modulation of mitochondrial function, antioxidant effects and anti-inflammatory cell adhesion.
List of Publication

Journal Papers


Conference Papers

1. Ya-Dan WEN, et al. SPRC, a novel water-soluble modulator of endogenous


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ATP = adenosine triphosphate
CAT = catalase
CBS = cystathionine β-synthase
CSE = cystathionine-γ-lyase
ELISA = enzyme-linked immunosorbent assay
GPx = glutathione peroxidase
GSH = glutathione
GST = glutathione S-transferase
H&E = hematoxylin & eosin
H₂S = hydrogen sulfide
H₂O₂ = hydrogen peroxide
HO-1 = heme oxygenase-1
HRUS = high resolution ultrasonography
HUVEC = human umbilical vein endothelial cell
ICAM-1 = intercellular adhesion molecule-1
IMT = intima-media thickness
LDL = low-density lipoprotein
MDA = malonaldehyde
NZW = New Zealand White
List of Abbreviation

NO = nitric oxide

ox-LDL = oxidized LDL

PAG = propargylglycine

PCR = Polymerase Chain Reaction

ROS = reactive oxygen species

RS = relative-sectional change

SAC = S-allylcysteine

SMCs = smooth muscle cells

SPRC = S-Propargyl-cysteine

SOD = superoxide dismutase

TC = total cholesterol

TEM = transmission electron microscope

Vp = peak flow velocity
Chapter 1

General Overview
1.1 Overview

Atherosclerosis is a chronic inflammatory disease occurring hand-in-hand with incipient accumulation of serum lipid in arterial blood vessels[1]. This high morbidity cardiovascular disease can silence for years, even though the atherosclerotic plaques formation in patient vasculature, called “stable plaques”. As the atherosclerotic conditions slowly grow and cumulate, the stable plaques become unstable and rupture to thrombus, which rapidly stop blood flow and result in death of tissues in the block areas[2]. The catastrophic ischemic symptoms can be myocardial infarction, stroke and claudication[3]. These complications can be lethal and disable, which are recognized as a leading cause to death in worldwide[3]. The patients carrying atherosclerotic unstable plaques and experienced the complications endure devastating impacts that may be chest pain, loss of vision, speech, paralysis and confusion, physical and mental disabilities. Therefore, atherosclerosis brings a substantial economical burden on individuals and society.

Although the mechanisms of atherogenesis are not fully understood, this vascular disease is highly related to the increased serum lipid, especially LDL[4]. Under this stimulation, the free radical species are dramatically generated, in turn, react with LDL to oxidized lipid molecules, which trigger a cascade of immune responses, like monocyte – endothelial cells adhesion, inflammation, fatty steak information and plaque core hardening[5]. There are also various anatomic, physiological and behavioral risk factors influencing atherosclerosis, including diabetes, dyslipoproteinemia, tobacco smoking, hypertension, vitamin B6 deficiency and raised serum C-reactive protein levels [6]. Therefore, reducing risk factors and developing therapies targeting the atherogenesis are the efficient solution for this cardiovascular disease.

Reducing risk factors can be done by doing regular exercise, eating healthily, maintaining an ideal body weight, avoiding excessive alcohol intake, doing physical
examination regally, preventing stress and giving up smoking. When these non-drug approaches become work little, the drugs turn to be main force. Current medications for atherosclerosis are usually the family “Statin”, which can lower serum cholesterol levels and stable plaques effectively. However, the Prove-It Trials found that intensive statin therapy for two years did not prevent 22.4% of patients from the coronary events occurred[7]. Moreover, liver dysfunction, rhabdomyolysis and elevated risk of cancer cause some patients to withdraw from statin treatments [8]. Therefore, it is high interest to investigate alternative therapies for atherosclerosis to extend patients treatments choices.

H₂S may have the potential to treat this ancient disease. H₂S, the novel gasotransmitter, is a hot research issue in recent years, due to its cardioprotective and anti-inflammatory characteristics [9]. The studies on myocardial infarction[10], ischemia/reperfusion[11, 12] and colitis [13] have been proven that H₂S regulates cellular adhesive molecular expression, expresses antioxidative abilities, suppresses inflammatory cytokines and antagonizes tissue program cell death. Atherosclerosis, an age-dependent and a multi-factorial disease with an important inflammatory component, is associated with oxidative stress and cell adhesion and program cell death[14], which can be triggered by H₂S, according to its novel features in previous peer studies. Additionally, some in vitro studies by using smooth muscle cells and in vivo studies focused on ICAM-1[15, 16] have already collected several inspiring results. Therefore, it is an interesting and encouraging attempt to link H₂S to atherosclerotic therapy that may provide a novel avenue to the treatments of this high prevalent disease.

Moreover, considering mitochondria is the main source of cellular energy plant[17], and mitochondria contribute to cardiac dysfunction and myocytes injury[18], this organelle functions were investigated for unveiling the protective effects of H₂S as one mechanism. There are several studies reported that H₂S can induce suspended
animation and create hypothermia by reducing metabolism in order to improve organ preservation [19]. Also, in wild nature, some bacteria and archaea produce and utilize H$_2$S as their energy supply for survival and proliferation [20]. Under these considerations, we hypothesize that H$_2$S may modulate the cellular energy supply through mitochondrial functions. Therefore, whether mitochondrial ultrastructure and function can be reserved by H$_2$S or not is an investigated direction we elucidate in this thesis.

In these studies, we found that administration of H$_2$S and the sulfide-containing chemical (SPRC) could attenuate the atherogenesis from cellular and animal levels that protect mitochondria, exhibited antioxidative abilities, suppression of lipid oxidation, inhibition of inflammatory cell adhesion. Therefore, our studies provided the new avenue for exploring novel therapeutic strategies for combating atherosclerosis and extended our understanding of the pathways of cardiovascular effects of H$_2$S.

This thesis focuses on the effects of H$_2$S and the sulfide-containing chemical (SPRC) on atherosclerosis and the mechanisms involved in protective effects on vasculature. Animal studies and cell studies were carried out for the general functional observations and specific mechanisms investigations in early stage of atherosclerotic process. Advanced stage of atherosclerosis and related complications are very complicated and involve many systemic issues. Therefore, investigations of advanced atherosclerosis are not central to this study and hence are beyond the scope of this thesis.
1.2 Objectives

The main objectives of this work are fourfold:

1. **Verify the possible therapeutic potential of exogenous H$_2$S on HUVECs against H$_2$O$_2$-induced mitochondrial dysfunction, oxidative stress and apoptosis.**

   In experiment I, the H$_2$S toxicity level and cell viability recovered by H$_2$S were tested. The underlying mechanisms of protective effects of H$_2$S were investigated in mitochondrial function (ATP production and $\Delta \Psi_m$), anti-oxidation (ROS production, MDA and antioxidative enzymes) and anti-apoptosis (apoptotic related proteins expressions and Akt pathway). These mechanisms of cardioprotective effects of H$_2$S were demonstrated through CSE/H$_2$S pathway.

2. **Elucidate the effects of exogenous H$_2$S on modulation of mitochondrial function in rabbit aortas mitochondria.**

   Since mitochondria are the primary source of determining the cellular oxidative stress, in experiment II, the reserved mitochondrial functions by H$_2$S were assessed in terms of mitochondrial respiration chain, ATP biosynthesis, ROS production and mitochondrial membrane permeability ($\Delta \Psi_m$ and mitochondrial swelling).

3. **Address the effects of exogenous H$_2$S on atherogenesis in New Zealand White rabbit hyperlipidemic model.**

   In experiment III, H$_2$S was target to identify the anti-atherogenesis in several parameters: cholesterol level, ox-LDL level, MDA level and HO-1 expressions to identify the effects of H$_2$S on lipid oxidation; aortic ultrastructure, thoracic aorta H&E and carotid imaging to identify the effects of H$_2$S on aortic plaque sizes; antioxidative enzymes activities and proteins and genes expressions to identify the effects of H$_2$S on oxidative stress; inflammatory cellular adhesive molecules expressions to identify the effects of H$_2$S on atherosclerotic inflammatory procedure. These mechanisms of cardioprotective effects of H$_2$S were also demonstrated through CSE/H$_2$S pathway.
4. Illustrate the effects of the sulfide-containing chemical, SPRC, on atherogenesis in New Zealand White rabbit hyperlipidemic model.

In experiment IV, a novel sulfide-containing chemical, SPRC, was target to identify the anti-atherogenesis in several parameters: serum lipid levels and MDA level to identify the effects of SPRC on lowering serum cholesterol; aortic ultrastructure, thoracic aorta H&E and carotid imaging to identify the effects of SPRC on aortic plaque sizes; antioxidative enzymes activities and proteins and genes expressions to identify the effects of SPRC on oxidative stress; inflammatory cellular adhesive molecules expressions to identify the effects of SPRC on atherosclerotic inflammatory procedure. These mechanisms of cardioprotective effects of H₂S were also demonstrated through CSE/H₂S pathway.
Chapter 2

Literature review
2.1 The Novel Gasotransmitter, Hydrogen Sulfide

2.1.1 Introduction

In an evolutionary perspective, the synthesis and catabolism of hydrogen sulfide (H$_2$S) by living organisms antedates the evolution of vertebrate. Bacteria and archaea produce and utilise the stinking gas as one of the essential sources for their survival and proliferation. For many decades, H$_2$S, the colorless gas with a strong odour of rotten gas, is recognized as a toxic gas and an environmental pollutant. The mechanism of its toxicity is a potent inhibition of mitochondrial cytochrome c oxidase, which is the important enzyme that is closely related with chemical energy in the form of adenosine triphosphate (ATP). Sulfide, together with cyanide, azide and carbon monoxide (CO), all can inhibit cytochrome c oxidase which leads to chemical asphyxiation of cells.

In the last two decades, the perception of H$_2$S has been changed from that of a noxious gas to a gasotransmitter with vast potential in pharmacotherapy. At the end of 1980s, endogenous H$_2$S is found in the brain [21]. Then, its enzymatic mechanism, physiological concentrations, specific cellular targets were described in the year of 1996 [22]. Subsequently, the physiological and pharmacological characters of H$_2$S were unveiled. Recently, H$_2$S, followed with NO and CO, is identified as the third gasotransmitter by Rui Wang [23]. The three gases share some common features. They are all colorless and poisonous gases. With the exception of gas pressure in atmosphere, they can dissolve in water at different solubility. All these small signaling molecules possess significant physiological importance, like anti-inflammation, anti-apoptosis, etc. The similarities and differences of the features of NO, CO and H$_2$S are summarized in Table 2-1.
<table>
<thead>
<tr>
<th></th>
<th>nitric oxide</th>
<th>carbon monoxide</th>
<th>hydrogen sulfide</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Formula</strong></td>
<td>NO</td>
<td>CO</td>
<td>H$_2$S</td>
</tr>
<tr>
<td><strong>Color and odor</strong></td>
<td>Colorless; a mild, sweet odor</td>
<td>Colorless; odorless</td>
<td>Colorless; smell like rotten egg</td>
</tr>
<tr>
<td><strong>Free radical</strong></td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Flammable</strong></td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Toxicity and dose</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Inhibition of</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>mitochondrial cytochrome c oxidase</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Resources</strong></td>
<td>L-arginine or nitrite</td>
<td>Protohaem IX</td>
<td>L-cysteine</td>
</tr>
<tr>
<td><strong>Intermediate Products</strong></td>
<td>L-NG hydroxyarginine, citrulline</td>
<td>Biliverdin IX-α</td>
<td>Cystathionine, L-cysteine, αketobutyrate, pyruvate</td>
</tr>
<tr>
<td><strong>Enzymes</strong></td>
<td>calmodulin-dependent nitric oxide synthase (NOS) (types 1, 2 and 3)</td>
<td>heme oxygenase (HO)( HO-1, HO-2 and HO-3)</td>
<td>Cystathionine β-synthase (CBS), Cystathionine γ-lyase (CSE), 3 mercaptopyruvate sulfide transferase (3-MST)</td>
</tr>
<tr>
<td><strong>Vascular effect</strong></td>
<td>vasodilation</td>
<td>vasodilation</td>
<td>vasodilation</td>
</tr>
<tr>
<td><strong>Inhibition inflammation</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Anti-apoptosis</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Haem effect</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
### Chapter 2

<table>
<thead>
<tr>
<th>Molecular targets</th>
<th>soluble guanylate cyclase (sGC)</th>
<th>soluble guanylate cyclase (sGC)</th>
<th>$K_{ATP}$ (ATP-gated potassium) channel</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Targeting outcome</strong></td>
<td>Stimulation of soluble guanylate cyclase and increase of intracellular cGMP concentration. But CO is a much weaker activator than NO.</td>
<td>Increase of cAMP, relaxation of smooth muscle</td>
<td></td>
</tr>
<tr>
<td><strong>Application on human</strong></td>
<td>pulmonary hypertension, lung transplantation, ARDS</td>
<td>not available</td>
<td>not available</td>
</tr>
</tbody>
</table>
2.1.2 Physical and biological characteristics

H₂S, a colorless and flammable gas with the characteristic foul odor of rotten eggs, is known for decades as a toxic gas and an environmental hazard. It is soluble in water (1 g in 242 ml at 20°C). In water or plasma, H₂S is a weak acid which hydrolyzes to hydrogen ion, hydrosulfide and sulfide ions as followings: \( H_2S \leftrightarrow H^+ + HS^- \leftrightarrow 2H^+ + S^{2-} \). The pKa at 37°C is 6.76. When H₂S is dissolved in physiological solution (pH7.4, 37°C), it yields approximately 18.5% H₂S and 81.5% hydrosulfide anion (HS⁻), as predicted by the Henderson–Hasselbach equation [24]. H₂S could be oxidized to sulfur oxide, sulfate, persulfide and sulfite. H₂S is permeable to plasma membranes as its solubility in lipophilic solvents is five-fold greater than in water. In other words, it is able to freely penetrate cells of all types.

The toxic effect of H₂S on living organisms has been recognized for nearly 300 years and until recently it was believed to be a poisonous environmental pollutant with minimal physiological significance. H₂S is more toxic than hydrogen cyanide and exposure to as little as 300 ppm in air for just 30 min is fatal to human. The level of odor detection of sulfide by the human nose is at a concentration of 0.02-0.1ppm, 400-fold lower than the toxic level. As a broad spectrum toxicant, H₂S affects many organ systems including lung, brain, kidney etc.

H₂S is often produced through the anaerobic bacterial breakdown of organic substrates in the absence of oxygen, such as in swamps and sewers (anaerobic digestion). It also results from inorganic reactions in volcanic gases, natural gas and some well waters. Digestion of algae, mushrooms, garlic and onions, are believed to release H₂S by chemical transformation and enzymatic reactions [25]. Structures of nature food releasing H₂S on digestion are shown in Fig. 2-1. Consuming mushrooms, garlic and onions, which contain chemicals and enzymes responsible for the transformation of the sulfur compounds, are responsible for H₂S production in
human gut [26]. Human body produces small amounts of H$_2$S and uses it as a signaling molecule. In different species and organs, the concentration of H$_2$S varies in different levels. In Wistar rats, the normal blood level of H$_2$S is 10 µM [27]; while in Sprague–Dawley rats, the plasma level of H$_2$S increase to 46 µM [28]; In human, 10–100 µM H$_2$S in blood was reported [29]. The tissue level of H$_2$S is known to be higher than its circulating level. The concentration of endogenous H$_2$S has been reported up to 50–160 µM in brains of rat, human and bovine [21, 30, 31]. Significant amounts of H$_2$S are generated from vascular tissues, and this production varies among different types of vascular tissues. For instance, the homogenates of thoracic aorta yielded more H$_2$S than that of portal vein of rats [28].
Fig. 2-1 Synthesis and catabolism of H₂S

AAT: aspartate aminotransferase
CDO: cysteine dioxygenase
CSE: cystathionine γ-lyase
H₂S: hydrogen sulfide
GNMT: glycine N-methyltransferase
GSH: glutathione
3-MST: 3-mercaptopyruvate sulfoxide transferase
MTHFR: methenyltetrahydrofolate reductase
SAH: S-adenosylhomocysteine
SO: sulfite oxidase
TSR: thiosulfate reductase
TSMT: thiol-S-methyltransferase

CBS: cystathionine β-synthase
CSD: sulfinate decarboxylase
HDH: hypotaurine dehydrogenase
GCS: γ-glutamyl cysteine synthase
GS: glutathione synthase
MAT: methionine adenosyltransferase
MS: methionine synthase
SAM: S-adenosylmethionine
THF: tetrahydrofolate
TSST: thiosulfate sulfurtransferase

S₀₆: elemental sulfur
2.1.3 Synthesis and catabolism of H$_2$S;

H$_2$S is endogenously formed by both enzymatic and non-enzymatic pathways [23]. The enzymatic procedure of synthesizing H$_2$S, in mammalian tissues, is involved in two pyridoxal 5’-phosphate-dependent enzymes: cystathionine $\gamma$-lyase (CSE) and cystathionine $\beta$-synthase (CBS) [32-34]. As shown in figure 1, H$_2$S is catalyzed from the desulfhydration of L-cysteine, a sulfur containing amino acid derived from alimentary sources, produced by the trans-sulfuration pathway of L-methionine to homocysteine, or liberated from other endogenous proteins [35, 36]. As the intermediate, CBS catalyzes homocysteine together with serine to yield cystathionine, which get converted to cysteine, $\alpha$-ketobutyrate and NH$_4^+$ by CSE. The two pyridoxal 5’-phosphate-dependent enzymes both or either catalyze the conversion of cysteine to H$_2$S, pyruvate, NH$_4^+$. CSE also could catalyze a $\beta$-disulfide elimination reaction that results in the production of thiocysteine, pyruvate and NH$_4^+$. Thiocysteine is associated with cysteine or other thiols to form H$_2$S [37].

The two enzymes are widespread in mammalian tissues and cells and also in many invertebrates and bacteria [38]. The activity of CSE is chiefly concentrated in liver, heart, vessels, kidney, brain, small intestine, stomach, uterus, placenta and pancreatic islets; whereas the amounts of CBS is mainly located in brain, liver, kidney and ileum, uterus, placenta and pancreatic islets [39]. The locations of H$_2$S-producing enzymes are seen in Table 2-2. In several species, the liver is the common organ containing the two enzymes in abundance. According to Zhao’s research, the intensity rank of biosynthesis of H$_2$S by origin of exogenous cysteine in different rat blood vessels was tail artery > aorta > mesenteric artery [40].
Table 2-2 Characteristics of H₂S-producing Enzymes

<table>
<thead>
<tr>
<th></th>
<th>Cystathionine γ-lyase (CSE)</th>
<th>Cystathionine β-synthase (CBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Localization</strong></td>
<td>liver, heart, vessels, kidney, brain, small intestine, stomach, uterus, placenta and pancreatic islets</td>
<td>brain, liver, kidney and ileum, uterus, placenta and pancreatic islets</td>
</tr>
<tr>
<td><strong>Activators</strong></td>
<td>Pyridoxal 5′-phosphate</td>
<td>Pyridoxal 5′-phosphate, S-adenosyl-L-methionine, Ca²⁺/calmodulin</td>
</tr>
<tr>
<td><strong>Inhibitors</strong></td>
<td>D,L-propargylglycine, β-cyano-L-alanine</td>
<td>Hydroxylamine, Amino-oxyacetate</td>
</tr>
<tr>
<td><strong>Functional roles</strong></td>
<td>H₂S production in liver and smooth muscle</td>
<td>H₂S production in the brain and nervous system</td>
</tr>
</tbody>
</table>

A third enzymatic reaction contributing to H₂S production has recently been identified in brain and vascular endothelium, i.e. 3-mercaptopyruvate sulfurtransferase (3-MST) in combination with aspartate aminotransferase (AAT) (also called cysteine aminotransferase) [41, 42], seen in Fig. 2-1. In mitochondria, L-cysteine and α-ketoglutarate as substrates, can be converted to 3-mercaptopuruvate by AAT; then the intermediate product is converted to H₂S by 3-MST [42]. In brain, 3-MST is found almost in neurons, while CBS in astrocytes [43]. It could speculate that the two enzymes of catalyzing H₂S play different roles in nervous system. In vascular tissues, 3-MST could be detected in both endothelial cells and vascular smooth muscle cells (SMCs), while AAT just occurs in endothelial cells. From another perspective, only vascular endothelial cells in vessel could utilize the two
enzymes to produce H\textsubscript{2}S, whereas vascular SMCs likely absorb 3-mercaptoppyruvate or other sources to generate H\textsubscript{2}S which exerts as a vasodilator.

The non-enzymatic route of yielding H\textsubscript{2}S is the conversion of elemental sulfur and transformation of oxidation of glucose. The non-enzymatic route is presented in vivo, involving phosphogluconate (<10%), glycolysis (>90%), glutathione (<5%) [23].

In the pathway of H\textsubscript{2}S production, there are some important amino acids: homocysteine and L-cysteine. Besides generation of H\textsubscript{2}S pathway, homocysteine is related to folate cycle and methionine cycle [44], the latter of which is participated in methionine, SAM and SAH, as previously stated. As the bridge of the two cycles, homocysteine could be remethylated to methionine by interacting with methyltetrahydrofolated (methyl-THF) and vitamin B\textsubscript{12} as cofactor under the synthesis of methionine synthase (MS). Methyl-THF is transformed from methylenetetrahydrofolate (methylene-THF) by methylenetetrahydrofolate reductase (MTHFR). Tetrahydrofolate (THF) is generated by remethylation and converted to methylene-THF, thus integrated the folate cycle. In another cycle, methionine, is transformed to S-adenosylmethionine (SAM) by methionine adenosyltransferase (MAT), then is converted to S-adenosylhomocysteine (SAH), which is subsequently hydrolyzed to homocysteine by glycine N-methyltransferase (GNMT).

Cysteine metabolism is engaged in three major routes. Apart from the conversion of H\textsubscript{2}S, one path is oxidation of –SH group by cysteine dioxygenase (CDO) to cysteine sulfinate, which is decarboxylated to hypotaurine by cysteine sulfinate decarboxylase (CSD), then further transformed to taurine by a non-enzymatic reaction or by hypotaurine dehydrogenase (HDH); or which is converted to sulfinyl pyruvate, subsequently to sulfite and further sulfate. Another path from cysteine is synthesis GSH by glutathione synthase (GS) from γ-Glutamyl cysteine, which is originated from cysteine and glutamate catalyzed by γ-Glutamyl cysteine synthase.
The concentration of H$_2$S is not only determined by the rate of formation but also by degradation of H$_2$S. Dissolved gaseous H$_2$S is in a pH-dependent equilibrium, with hydrosulfide anions (HS$^-$) and sulfide anions (S$^{2-}$), which can be catabolized to any sulfur-containing molecule. Sulfide, via non-enzymatic route, is catabolized to thiosulfate, which could be catalyzed to sulfite by thiosulfate reductase (TSR) in livers, brains or kidneys, or by thiosulfate sulfurtransferase (TSST) in livers, sequentially oxidized to sulfate via sulfite oxidase (SO) by a glutathione (GSH)-dependent reaction. The last product is excreted in urine [45]. H$_2$S could be breakdowned by rhodanese, methylated to CH$_3$SH, sequestrated by methemoglobin, interacted with superoxide or NO and scavenged by metallo- or disulfide-containing molecules such as oxidized glutathione [37],[38]. The major routes of degradation of H$_2$S through non-enzymatic oxidation of sulfide also yield elemental sulfur, polysulfides, dithionate, polythionates. The whole schematic version of synthesis and catabolism of H$_2$S is depicted in Fig. 2-1.

### 2.1.4 Donors and inhibitors of H$_2$S

#### 2.1.4.1 The donors of H$_2$S

#### 2.1.4.1.1 Sulfide-containing salts

Sodium hydrogen sulfide (NaHS) and disodium sulfide (Na$_2$S) are the common H$_2$S-releasing chemicals in research of hydrogen sulfide. These sodium salts purchased from pharmaceutical companies are usually aquo-compound, like NaHS·12H$_2$O, Na$_2$S·9H$_2$O, or anhydrous forms. The products of sodium hydrogen sulfide and disodium sulfide should be white. The pills with yellow color predicates the anhydrous forms have been converted to hygroscopic blocks and should not be
purchased. White sulfide products are likely to have greater purity, but may contain sodium salts of thiosulfate or higher oxidation state sulfur oxyanions [46]. Contamination by trace metal ions may also be important, as these catalyze oxidation processes. The sulfides should therefore be reserved in a vacuum dessicator to minimize oxidation.

The solution of NaHS, at physical pH and room temperature, hydrolyzes to sodium ion, hydrosulfide as followings: \( \text{NaHS} \leftrightarrow \text{Na}^+ + \text{HS}^- \). Solutions of HS\(^-\) are sensitive to oxygen, converting mainly to polysulfides, indicated by the appearance of yellow color. Hence, solutions of fresh prepared NaHS should be clear and put to use immediately. The purity of sulfides could be measured by determining the sulfide content either by titration with bromate, as described in standard analytical chemistry texts, or by UV spectroscopy in the case of sodium hydrogen sulfide, at pH 9, which has an absorption maximum at 230 nm with a molar absorptivity of 7200 L/mol/cm [47].

Considering the unstable chemical properties of NaHS and Na\(_2\)S, some researchers introduce another donor of H\(_2\)S, calcium sulfide (CaS), which is more steady [48]. CaS can be found as one of the effective components in a traditional herb, named “hepar sulfuris calcareum”, usually applied to homeopathic remedy. Oral administration of CaS will be decomposed to more H\(_2\)S in stomach acid environment. This review postulates CaS may carry out hypotension, arguing from its catabolism, relationship of calcium supplementation and blood pressure, dosage design and traditional application of homeopathic remedy on infection.

**2.1.4.1.2 H\(_2\)S releasing molecules:**

Thioacetamide is an organosulfur compound with the formula C\(_2\)H\(_3\)NS. This white crystalline solid is soluble in water and serves as a source of sulfide ions in the
synthesis of organic and inorganic compounds [49]. For lab safety, thioacetamide is carcinogen class 2B and has hepatotoxicity. Thioacetamide was widely used in classical qualitative inorganic analysis as an in situ source for sulfide ions.

Some research laboratories developed H₂S releasers. Lawesson’s reagent is a chemical compound used in organic synthesis as a thiation agent, is also a H₂S releaser. Lawesson's reagent is first synthesized in 1956 during a systematic study of the reactions of arenes with P₄S₁₀ [50]. After much time, it is first made popular by Sven-Olov Lawesson for introducing a thiation procedure as an example of a general synthetic method for the conversion of carbonyl to thiocarbonyl groups [50]. 2,4-bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane2,4-disulfide, Lawesson’s reagent, has a four-membered ring of alternating sulfur and phosphorus atoms. Normally in higher temperatures, the central phosphorus/sulfur four-membered ring can open to form two reactive dithiophosphine ylides (R-PS₂), which decompose to release H₂S. As its strong and unpleasant smell, it is best to prepare Lawesson's reagent within a fume hood and treat all glassware used with a decontamination solution before taking the glassware outside the fume hood.

Based on Lawesson’s compound, a series of compounds are synthesized. Professor Moore’s lab reports that morpholin-4-ium-4-methoxyphenyl (morpholino) phosphinodithioate (GYY4137) releases H₂S slowly both in vitro and in vivo. It has been proved that GYY4137 has vasodilator and antihypertensive activities and a useful H₂S-releasing chemical in the study of biological effects of H₂S [51]. In a later experiment, administration of GYY4137 to lipopolysaccharide(LPS) induced rats displays its anti-inflammatory effect by increasing plasma anti-inflammatory cytokine IL-10 and reducing plasma proinflammatory cytokines ((TNF-α, IL-1β, IL-6) and nitrite/nitrate, C-reactive protein, and L-selectin [52]. Structures of H₂S releasing molecules are shown in Fig. 2-2.
Considering pharmacological effects and adverse effects of H$_2$S, some pharmaceutical factories join in working on H$_2$S donors which is made up well-established parent compounds and H$_2$S-releasing moieties. CTG Pharma developed ACS series H$_2$S-releasing compounds to meet their interests on the aspects of hypertension, metabolic syndrome, thrombosis and arthritis (www.ctgpharma.com). Antibe Therapeutics synthesizes several ATB series H$_2$S-releasing derivatives for the treatments of inflammatory bowel disease, joint pain, and irritable bowel syndrome (www.antibe-therapeutics.com). The compound, IK-1001, from the company Ikaria, is an injectable form of Na$_2$S, which is pure, PH neutral and stable. IK-1001 has been used several basic studies and processed into clinical trials. One is a phase I safety trial for assessing pharmacokinetics of intravenous IK-1001(ClinicalTrials.gov ID: NCT00879645). Another is a phase II efficacy trial which administer IK-1001 in patients undergoing surgery for a coronary artery bypass graft (ClinicalTrials.gov ID: NCT00858936). The effects of some H$_2$S-releasing compounds are shown in Table 2-3.
Table 2-3 H$_2$S-releasing compounds used in basic scientific researches

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Constituents</th>
<th>Effects on Research fields</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAC</td>
<td>S-allylcysteine</td>
<td>Protection on cardiovascular and neural systems</td>
</tr>
<tr>
<td>SPRC</td>
<td>S-propargyl-cysteine</td>
<td>Anti-cancer, anti-inflammation, anti-hypoxic/ ischemia, impair cognition and Aβ-induced neuronal damage</td>
</tr>
<tr>
<td>GYY4137</td>
<td>morpholin-4-ium-4-methoxyphenyl (morpholino) phosphinodithioate</td>
<td>Antagonize endotoxic shock though anti-inflammatory effects</td>
</tr>
<tr>
<td>ACS-6</td>
<td>a H$_2$S-donating sildenafil</td>
<td>Inhibits superoxide formation and gp91$^{\text{phox}}$ expression in porcine PAECs</td>
</tr>
<tr>
<td>ACS-14</td>
<td>a H$_2$S-releasing aspirin</td>
<td>Regulate redox imbalance, such as GSH formation, HO-1 promoter activity, and isoprostane suppression</td>
</tr>
<tr>
<td>ACS-15</td>
<td>a H$_2$S-releasing derivative of diclofenac</td>
<td>Arthritis</td>
</tr>
<tr>
<td>ACS-67</td>
<td>a H$_2$S-releasing derivative of latanoprost acid</td>
<td>Glaucoma; retinal ischemia</td>
</tr>
<tr>
<td>ATB-284</td>
<td>a H$_2$S-releasing derivative</td>
<td>Irritable bowel Syndrome</td>
</tr>
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<tr>
<td><strong>trimebutine</strong></td>
<td></td>
<td>Gastrointestinal damage induced by NSAIDs</td>
</tr>
<tr>
<td><strong>ATB-337</strong></td>
<td>a H$_2$S-releasing derivative of diclofenac</td>
<td>Acute and chronic joint pain</td>
</tr>
<tr>
<td><strong>ATB-346</strong></td>
<td>a H$_2$S-releasing derivative of naproxen</td>
<td>Inflammatory bowel disease, antinociceptive and anti-inflammatory effects</td>
</tr>
<tr>
<td><strong>ATB-429</strong></td>
<td>a H$_2$S-releasing derivative of mesalamine</td>
<td>Suspended animation, multiple hypoxic/ischemic conditions, cardiac remodeling and congestive heart failure</td>
</tr>
<tr>
<td><strong>IK 1001</strong></td>
<td>calcium-crosslinked alginate polymer</td>
<td></td>
</tr>
</tbody>
</table>

### 2.1.4.1.3 Nature products containing sulfur

Digestion of algae, mushrooms, garlic and onions, are believed to form H$_2$S by chemical transformation and enzymatic reactions [25]. Structures of nature food releasing H$_2$S on digestion are shown in Fig. 2-3. Nearly all the allium families are sulfur rich containing. Several publications reports enumerated functional activities of garlic. It exhibits hypolipidemic, anti-microbial, anti-platelet and pro-circulatory effects [53-55]. It also demonstrates immune enhancement and provides anticancer, anti-mutagenic and anti-proliferative that are interesting in chemopreventive interventions. Additionally, aged garlic extract possesses hepatoprotective, neuroprotective, anti-oxidative activities [56]. The major sulfur-containing compounds in intact garlic are $\gamma$-glutamyl-S-allyl-L-cysteines and S-allyl-L-cysteine sulfoxides (alliin). Both are abundant as sulfur compounds, and alliin is the primary odorless, sulfur-containing amino acid, a precursor of allicin, methiin, (+)-S-(trans-1-propenyl)-L-cysteine sulfoxide, and cycloalliin [57].
S-allylcysteine (SAC), a major transformed product from \(\gamma\)-glutamyl-S-allyl-L-cysteine, is a sulfur amino acid detected in the blood that is verified as both biologically active and bioavailable \[58\], seen in Fig. 2-3. SAC has been enumerated in several research investigations mediating protective effects in neural system and cardiovascular system by the inhibition of cell damage in neuron, heart and endothelium. In neural system, it is reported that SAC may attenuate A\(\beta\)-induced apoptosis \[59\], destabilize Alzheimer’s A\(\beta\) fibrils in vitro \[60\]. SAC prohibits cerebral amyloid, cerebral inflammation, and tau phosphorylation in Alzheimer’s transgenic mouse model harboring Swedish double mutation \[61\]. In stroke-prone spontaneously hypertensive rats, intaking SAC diminishes incidence of stroke, impairs behavioral syndroms, abates mortality induced by stroke \[62\]. SAC inhibits free radical production, lipid peroxidation and neuronal damage in rat brain ischemia \[63\]. In cardiovascular system, SAC can help the acute myocardial infarction rats survived by significantly lowering mortality and reducing infarct size \[10\].

SPC and SPRC are structural analogues of SAC, differing only in the propargyl and allyl moiety respectively while containing the same cysteine structure as shown in Fig. 2-3. Wang et al., from our lab, reported that SPRC exhibited stronger cardioprotective effects than SAC in reducing mortality, increasing cell viability, reducing heart infarct size, lowering LDH and CK levels and activities, and having antioxidant properties \[64\]. These data suggest that the propargyl group of SPRC further increases the affinity and/or activity of SPRC towards the enzyme CSE as compared to SAC, where SPRC treatment is shown to have an increased CSE expression and activity to produce H\(2\)S for coping with ischemic damage. This observation suggests that the cardioprotective effects involving the CSE/H\(2\)S pathway were more effective using SPRC compared to SAC. Recently, our lab reported that SPRC showed neuroprotective effects of cognitive impairment and inhibition of neuronal
ultrastructure damage in Aβ-induced rats, affords a beneficial action on anti-inflammatory pathways [65]. SPRC has been demonstrated the anti-cancer effect on gastric cancer at high dose-50 mg/kg/d and 100mg/kg/d [66]. The effects of SAC and SPRC are shown in Table 2-3.

![Chemical structures of SAC, SPC, and SPRC](image)

**Fig. 2-3** The chemical structures of SAC, SPC and SPRC
2.1.4.2 The inhibitors and regulator of H\textsubscript{2}S

The production of H\textsubscript{2}S from cysteine by tissue/cell homogenate is decreased by the presence of inhibitors of H\textsubscript{2}S-producing enzymes, which are mainly attributed to CSE and CBS. CSE is also named cysteine desulphydrase [67]. The CBS locus is mapped to chromosome 21 (21q22.3) [68]. Several specific blockers for CSE and CBS are currently available. D,L-Propargylglycine (PAG) and b-cyano-L-alanine selectively inhibit CSE [28]. L-Cysteine metabolites, including ammonia, H\textsubscript{2}S, and pyruvate, cannot inhibit CSE activity [69]. CBS is inhibited by hydroxylamine (HA) and aminooxyacetate (AOAA) albeit these chemicals are not selective inhibitors of CBS [22]. The relationships between H\textsubscript{2}S-producing enzymes and their inhibitors are summarized in Table 2-2.

The currently known regulations of H\textsubscript{2}S-producing enzymes are glutamate and its receptors, S-adenosyl-methionine (SAM), hormones, other gasotransmitters- NO and CO, etc. In the brain, electrical stimulation and excitatory neurotransmitter, glutamate, rapidly increase CBS activity in Ca\textsuperscript{2+}/ calmodulin-dependent manner [70, 71]. Both \textalpha\textsubscript{-}amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptors and N-methyl-D-aspartate (NMDA) are involved in this effect. SAM is an intermediate product of methionine metabolism and a major donor of methyl groups. This allosteric regulator can activate CBS by approximately two-fold [22, 35]. Sex hormones seem to regulate brain H\textsubscript{2}S since CBS activity and H\textsubscript{2}S level are higher in male than in female mice and castration of male mice decreases H\textsubscript{2}S formation [35]. Sodium nitroprusside, a nitric oxide donor, increases the activity of brain CBS in vitro; however, this effect is NO-independent and results from chemical modification of the enzyme’s cysteine groups [72]. In contrast, NO itself may bind to and inactivate the CBS. Interestingly, CO is a much more potent CBS inhibitor than NO and it is suggested that CBS may be one of the molecular targets for CO in the brain [73, 74]. In homogenates of the rat aorta, NO donors acutely increase CSE-dependent H\textsubscript{2}S
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generation in a cGMP-dependent manner [40]. Moreover, prolonged incubation of cultured vascular smooth muscle cells in the presence of NO donors increases CSE mRNA and protein levels [28]. The physiological significance of NO in the regulation of H$_2$S production is also supported by the observation that circulating H$_2$S level as well as CSE gene expression and enzymatic activity in the cardiovascular system are reduced in rats chronically treated with NOS inhibitor. Thus, NO is probably a physiological regulator of H$_2$S production in the cardiovascular system.

2.1.5 H$_2$S measurements

2.1.5.1 Spectrophotometric method

The principle of spectrophotometric method of H$_2$S is depended on the formation of methylene blue. H$_2$S is chemiadsorbed by zinc acetate and transformed into stable zinc sulfide. The sulfide is recovered by extraction with water. In contact with an oxidizing agent such as ferric chloride in a strongly acid solution, it reacts with the N,N-dimethyl-p-phenylenediammonium (NNDPD) ion to yield methylene blue (C$_{16}$H$_{18}$N$_3$SCl). The equation is shown in Fig. 2-4:

\[
2 \text{NNDPD} + \text{H}_2\text{S} + 6\text{FeCl}_3 \rightarrow \text{C}_{16}\text{H}_{18}\text{N}_3\text{S}^- + 6\text{FeCl}_2 + \text{NH}_4\text{Cl} + 4\text{HCl}
\]

![Fig. 2-4 The equation of spectrophotometric method of H$_2$S](image)

The methylene blue method has been designed to different protocol. A common method is adding NNDPD and ferric chloride to the plasma or homogenized tissue,
then developing color and colorimetric estimating immediately. Owing to the volatile character of \( \text{H}_2\text{S} \), researchers modify the protocol, like using a filter paper to augment the contact surface and prolong the contact time \([75, 76]\). Based on published papers and previous experience, our lab revised the assay for \( \text{H}_2\text{S} \) by placing a sample in an airtight vessel with a central tube. The central tube contains a filter paper wick saturated with zinc acetate. The purpose of the filter paper wick is for trapping \( \text{H}_2\text{S} \) to zinc sulfide. The reactions are initiated by mixing of strong acid with the sample, which sulfide is driven out and adsorbed onto the wick. The driving time is usually 30-120 minutes which is modified based on lab condition and optimization in the sorts of samples. Reactions are stopped by injecting 0.5% trichloroacetic acid (TCA). After gas evolution and wick absorption, the sulfide in the central tube reacts with NNDPD in present of \( \text{Fe}^{2+} \) ion. The absorbance of the resulting solution at 670nm was measured with a micro plate reader.

This colorimetric method is not only widely used on the determination of \( \text{H}_2\text{S} \) on serum in animal experiment, but also the activity of CSE/CBS enzyme on tissues or cells. The concentrations of \( \text{H}_2\text{S} \) are reflected on the different shades of color of methylene blue and calculated by the plotting \( \text{H}_2\text{S} \) standard curve.

Two points need to be made. Firstly, most researchers assay \( \text{H}_2\text{S} \) using the spectrophotometric assay which involves acidifying zinc acetate-treated (to ‘trap’ free \( \text{H}_2\text{S} \)) biological samples in the presence of a dye and observing a colour change. This assay actually measures total sulfide and not the gas \( \text{H}_2\text{S} \). Secondly, \( \text{H}_2\text{S} \) is either broken down rapidly in the body by enzymes, sequestered by binding to haemoglobin, or can react chemically with a number of species abundant in tissues, including superoxide radical \([77]\), hydrogen peroxide \([75]\), peroxynitrite \([78]\) and/or hypochlorite \([79]\). All in all, making reasonably accurate measurements of such an evanescent and reactive gas in biological tissues is difficult. Indeed, the chemical nature of gases such as \( \text{H}_2\text{S} \), NO and CO might render it nonsensical even to try and
measure them in body fluids or tissues.

2.1.5.2 Sulfide ion-selective electrode

A sulfide ion-selective electrode (SISE) is immersed in an aqueous solution containing the ions to be measured, together with a separate, external reference electrode. The electrochemical circuit is completed by connecting the electrodes to a sensitive milli-volt meter using special low-noise cables and connectors. A potential difference is developed across the SISE membrane when the sulfide ions diffuse through from the high concentration side to the lower concentration side.

At equilibrium, the membrane potential is mainly dependent on the concentration of the target ion outside the membrane and is described by the Nernst equation. Briefly, the measured voltage is proportional to the Logarithm of the concentration, and the sensitivity of the electrode is expressed as the electrode slope - in millivolts per decade of concentration. Thus the electrodes can be calibrated by measuring the voltage in sulfide standard solution. Testing samples can then be determined by measuring the voltage and plotting the result on the calibration graph. The use of sulfide ion-selective electrode suffers from precipitation of metal sulfide, for example sliver sulfide (Ag₂S) from the filling solution on the electrodes.

Reproducibility is limited by factors such as temperature fluctuations, drift and noise. The electrode can be used at temperatures from 0 to 100 °C, and only used intermittently at temperatures above 80 °C. Interfering ions, like mercury, must be absent from all sulfide sample. In aqueous solution, H₂S is dissolved into HS⁻ and S²⁻. In acid solution, sulfide is chiefly in the form of H₂S, while in the intermediate pH range (up to approximately pH 12) almost all the sulfide is in the form HS⁻. Only in very basic does the sulfide exist primarily as free ion (S²⁻). The SISE from Thermo Scientific supplies sulfide anti-oxidant buffer could maintain a fixed level of H₂S.
Nevertheless, the alkaline condition of anti-oxidant buffer is regarded as an influencing factor to SISE measurements in plasma. Initially mixing samples to anti-oxidant buffer is reported to generate protein desulfuration and artificially increased sulfide values [80]. It is also observed that placing 5% bovine serum albumin into anti-oxidant buffer leads to a surging reading of total sulfide measured by SISE in first 20 minutes and following slow accumulation in 3 hours [81].

### 2.1.5.3 Other analyzing methods

Carbon nanotube (CNT) was introduced by Wu et al. for measuring low concentration and nano-quantity \( \text{H}_2\text{S} \) [82, 83]. One of the benefits of unfuctionalized CNT in analyzing \( \text{H}_2\text{S} \) is due to the special bond with \( \text{H}_2\text{S} \) but other proteins kept in serum. \( \text{H}_2\text{S} \) concentrations are reflected by the intensity of the fluorescence of the unfuctionalized CNT, due to the two values in a linear relationship. The lowest \( \text{H}_2\text{S} \) concentration can be tested is 20\( \mu \)M and smallest quantity of \( \text{H}_2\text{S} \) is 0.5\( \mu \)g. The series of experiments are trying to establish a new sensor to measure micro or nano quantity \( \text{H}_2\text{S} \), comprising unfuctionalized CNT as a transducer and LSM fluorescence as a signal acquisition modality.

Polarography is a voltammetric measurement which makes use of the dropping mercury electrode or the static mercury drop electrode. The value of diffusion current is depended on the speed of electroactive material (samples) diffusing to dropping mercury electrode. This principle contributes to the measurement of the concentration of analytes. Polarography is well known for the application of quantitative measurements of \( \text{O}_2 \) (polarographic oxygen sensor, POS) and NO (polarographic nitric oxide sensor, PNOS). By recent years of the appreciation of the third gasotransmitter, \( \text{H}_2\text{S} \), several analytical methods are utilized, including polarography. A novel polarographic hydrogen sulfide sensor (PHSS) has been
developed for the study of H$_2$S producing rates and consumption in mammalian tissues, with resolution of 10 nM [84]. The polarographic sulfide sensor is also applied to the investigation of kinetics of sulfide metabolism in organisms living in sulfide-rich environment [85]. PHSS permits direct and simultaneous measurement of H$_2$S gas in biological fluids without sample preparation. PHSS has provided an alternative method for sulfide measurement.

Gas chromatography is a recent method describes by Levitt et al. as a unique chemiluminescence-based technique to measure free and acid-labile H$_2$S in multiple tissues from mouse [86]. The tissues were first submerged in 50mM glycine-NaOH buffer (pH 9.3) and homogenized. The homogenate were then transferred to syringes, which were sealed and flushed with N$_2$. The homogenate in alkaline extraction turns to acidification to pH 5.8 by adding sodium hydrogen phosphate solution (pH 5.5). After vigorous mixture, the gas space was removed to gas chromatography to analyze free H$_2$S concentration. Next, adding 50% trichloroacetic acid to the syringe, the gas was collected to test the acid-labile H$_2$S concentration. The flow rate of N$_2$ was 25 ml/min. The concentration of H$_2$S was calculated by the plotting H$_2$S standard curve.

High-performance liquid chromatography (HPLC) is used to separate the sulfide mixture. Togawa et al. reported that using monobromobimane (MBB) with dithiothreitol (DTT) reacted with bound sulfide to produce sulfide dibimane, which is separated from MBB by HPLC and detected by its fluorescent probes [87]. Recently, MBB assay without DTT was used to measure available H$_2$S in rat blood [88] and mouse plasma [89]. The ranges or limits of H$_2$S measurements are in Fig. 2-5.
Fig. 2-5 The ranges or limits of H$_2$S measurements

### 2.1.6 H$_2$S in inflammation

Inflammation is an immune response to an injury or harmful stimuli, in order to self-protect body from avoiding pathogens assaults and initiating healing process. However, the adaptive immune system fails to counter invading agents, will turn to target host tissues, making deeply more serious damage. H$_2$S regulating inflammation and injury was initially contradictory, but in recent years more studies supported that H$_2$S inhibited the process of inflammation, except at high concentration [90]. This mediator possibly exerts its anti-inflammatory effects through reduction of leukocyte-endothelial cell adhesion [91], action on ATP-sensitive K$^+$ channels [92], scavenging of toxic free radicals [93], elevation of cyclic AMP and/or cyclic GMP [78, 79], inhibition of nuclear factor-κ B (NF-κB) and pro-inflammatory cytokines (e.g., COX-2 [94], iNOS [13] and interleukin (IL)-1β, IL-6 [95]).

Various diseases could be found inflammatory response, like atherosclerosis,
ischemia-reperfusion and colitis. Contributing to anti-inflammatory molecular mechanisms of this novel gasotransmitter, it is not surprising that H₂S may participate in the process of resolution of a variety of inflammatory diseases. In atherosclerosis, H₂S exerts its potent inhibitor of leukocyte adherence to vascular endothelium [15]. Meanwhile, the generation of reactive oxygen species (ROS), activation of NF-κB, increased expressions of cell adhesion cytokines and induction of apoptosis, which were all regarded as the key promoters of pathology, were all found suppressed by H₂S [15, 96]. These mechanisms of action described for H₂S may explain that H₂S can diminish the plaques in arteries and attenuate the atherosclerotic injury, suggesting the character of anti-inflammation of H₂S is benefit for the vascular protection.

Ischemia-reperfusion (I/R) is identified as an acute endogenous inflammatory response that characterizes release of toxic free radicals, leucocyte-endothelial cell adhesion and platelet-leucocyte aggregation [97]. In porcine myocardial I/R model, therapeutic sulfide improved myocardial function and diminished infarct size though decreased levels of inflammatory cytokines (IL-6, IL-8 and TNF-α), reduced left ventricular pressure and improved coronary microvascular reactivity, [11]. A similar tissue protection of H₂S also found in hepatic I/R injury by inhibition of inflammation (lipid peroxidation, IL-10, ICAM-1 and TNF-α) and apoptosis (caspase-3, Fas and Fas ligand) [98]. Another study suggested that the cardioprotective effects of H₂S may be mediated by opening the mitochondrial K₅TP channel and second window of protection caused by endotoxin [99].

Colitis is one form of gastrointestinal inflammation and ulceration. Administration of H₂S-generating agents or precursor for H₂S synthesis, L-cysteine, has been show to significantly accelerate ulcer healing [100, 101]. This ability of H₂S to enhance gastrointestinal resistance attracts investigators to exploit novel treatments of gastrointestinal injury and inflammation, like H₂S-releasing derivative of NSAIDs to
reduce the adverse drug reaction of NASIDs, retarding gastrointestinal ulcer healing [102]. Evidence of \( \text{H}_2\text{S} \) in resolution of colitis in rats or mice studies showed that administration of \( \text{H}_2\text{S} \) donor significantly inhibited the severity of colitis with marked reduction of granulocyte infiltration into colonic tissue. In inflamed colon, \( \text{H}_2\text{S} \) production was highly increase via CSE, CBS or other enzymatic pathways [103, 104]. Once \( \text{H}_2\text{S} \) synthesis was inhibited, the colitis tended to worsen the inflammation with thickening of the smooth muscle, perforation of bowel wall, even death [13].

### 2.1.7 \( \text{H}_2\text{S} \) in redox status

In a weak acid, \( \text{H}_2\text{S} \) dissociates in equilibrium with hydrosulfide anion (\( \text{HS}^- \)) and sulfide anion (\( \text{S}^{2-} \)). Under physiological conditions the amounts of \( \text{H}_2\text{S} \) and \( \text{HS}^- \) are early equal within the cell, whereas extracellular fluid and plasma exist approximately the ratio of 20% \( \text{H}_2\text{S} \), 80% \( \text{HS}^- \) and 0% \( \text{S}^{2-} \). \( \text{HS}^- \) is a potent one-electron reductant that eliminates free radicals by donating single electron. Hydrogen disulfide (\( \text{H}_2\text{S}_2 \)), also known as disulfane, is the production of oxidation of \( \text{HS}^- \) by two-electron oxidants, like hypochlorous acid [105] and hydrogen peroxide [106]. \( \text{H}_2\text{S}_2 \), a highly reactive oxidizing chemical, can generate \( \text{H}_2\text{S} \) by reacting with thiol [107] or disproportionation [105, 108].

\( \text{H}_2\text{S} \) is considered as an endogenous reducing agent which is produced in response to oxidative stress [109, 110]. Evidence showed that \( \text{H}_2\text{S} \) is a highly reactive molecule and may easily react with other compounds, especially with reactive oxygen and nitrogen species. \( \text{H}_2\text{S} \) reacts with at least four different ROS: superoxide radical anion [77], hydrogen peroxide [75], peroxynitrite [78] and hypochlorite [79]. All these compounds are highly reactive and their reactions with \( \text{H}_2\text{S} \) result in the protection of proteins and lipids against RNS/RNS-mediated damage [78, 79].

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Mitochondrial injury is an important source of reactive oxygen species (ROS), which is involved in a range of pathologies, such as ischemia/reperfusion, atherosclerosis and toxin exposure [111]. Under oxidative stress conditions, mitochondria will show unstable mitochondrial membrane potential ($\Delta \Psi m$), redox transitions, and negative changes in the mitochondrial permeability transition (MPT) pore and the inner membrane anion channel (IMAC) [112]. Our lab found that H$_2$S can reduce the H$_2$O$_2$-induced injury in HUVECs via increasing ATP production, saving mitochondrial ultrastructure, stabilizing mitochondrial membrane intact, decreasing ROS and MDA, and rising anti-oxidants, The same situation was also unveiled in H$_2$O$_2$-stimulated isolated rabbits aorta that H$_2$S ameliorated mitochondrial dysfunction through improving O$_2$ consumption and ATP production, protecting mitochondrial respiration chain complexes activities and matrix enzymes, decreasing mitochondrial membrane permeability and inhibiting mitochondrial ROS levels. These effects of H$_2$S indicated that the antioxidative ability of H$_2$S is through increasing antioxidants and prohibiting ROS levels, and also preserving mitochondrial function to reduce the production of toxic free radicals.

2.1.8 H$_2$S in cardiovascular system

2.1.8.1 Hypertension

Before identified as the third gasotransmitter, together with NO and CO, H$_2$S has been speculated to regulate an array of physiological processes in regulating cardiovascular functions, distinctive from its toxicological effect. A great number of studies have been carried on investigation of the modulating of blood pressure by exogenous and endogenous H$_2$S. Early at the end of last century, it is first reported that H$_2$S relaxes the contracted smooth muscles (SM) induced by 1 $\mu$m norepinephrinein in rat thoracic aorta and portal vein [113]. The relaxations in these tested aortas and veins present a NaHS dose dependent manner, but the potency of
relaxation by exogenous H₂S in the thoracic aorta is less than the portal vein, even by 10⁻³ M NaHS, which are around 25% and 90%, respectively. The data also showed that the relaxation effects of H₂S and NO can be enhanced by each other. 30 μM NaHS can augment the loosening effect of NO by up to 13 fold. Thus, endogenous cysteine and glutathione do not have synergistic effect with NO. Subsequently, the vasorelaxant effect of H₂S is found in vivo of SD rats, ex vivo of aortic rings and in vitro at rat aortic smooth muscle cells [34]. This is a literature that first demonstrated the underlying mechanism of vasorelaxation which is a consequence of opening K⁺ATP channels. The endogenous production of H₂S is generated by CSE which is identified in vascular SMCs, not in endothelium. Nonetheless, this paper does not mention the third enzymatic pathway of synthesizing H₂S by the enzymes MPST and AAT. Therefore, it does not mean endothelium cannot produce H₂S. Additionally, this paper also refers to the association of and NO on elevated H₂S production from vascular tissues. Above experiments are implemented on normal animals, while more functions of H₂S are indicated in spontaneous hypertensive rats (SHRs) and CSE gene knockout mice (SMCs-KO) [28, 76, 114, 115].

Different from NO and CO, the relaxed effect of vascular tissues by H₂S is not through the activation of cGMP pathway. 1H-[1,2,4] oxadiazolo [4,3,-a]quinoxalin-1-one (ODQ), a specific inhibitor of soluble guanylyl cyclase, could abolish the vasorelaxation induced by NO, but not by H₂S [116, 117]. One possible mechanism involved in the hypotensive effect of H₂S is K⁺ATP channels is opened by H₂S that increasing K⁺ currents result in hyperpolarizing membrane of smooth muscle cells in vascular tissue. The vasodilation effect of H₂S is inhibited significantly by either using a calcium-free bath solution or with the normal bath solution but in the presence of nifedipine, a voltage-gated Ca²⁺ channel inhibitor, on aortic rings [28]. This indicates that the vascular effects of H₂S are also likely mediated by the attenuation of intracellular inward Ca²⁺ currents. H₂S not only
influence blood pressure, but also exerts cardioprotective effect by relieving vascular structural remodeling observed during hypertension, including and suppression of VSMC proliferation via the activation of cardiac extracellular-signal-regulated kinase (ERK) and/ or Akt pathway [118] and attenuation of collagen accumulation through reduction of collagen type I level, [3H] thymidine and [3H] proline incorporation and [3H] hydroxyproline secretion in the SHRs [114] and through nitrogen-activated protein kinase (MAPK) pathway [16]. In CSE-KO mice, other mechanisms were found that due to the feature of CSE activated by calcium-calmodulin, the H\textsubscript{2}S formation may be involved in vascular activation to reduce blood pressure [115].

2.1.8.2 Atherosclerosis

Atherosclerosis is a chronic and slowly progressive cardiovascular disease that affects arterial blood vessels by thickening and hardening as consequences of the high plasma cholesterol concentrations, especially cholesterol in low density lipoprotein [119]. Cholesterol deposition, lipid oxidization, cell adhesion, vascular inflammation, foam cell accumulation, smooth muscle cell migration and plaque calcification are involved in different stages of the pathological process [120]. The cumulative plaques consequentially narrow the arterial lumen and restrict blood supply. Severe atherosclerotic lesions are the high risk factors of ischemic diseases such as stroke and heart attack [121].

Recent years, H\textsubscript{2}S draws attentions from researchers by its cardiovascular protective effects, while there are not many studies on its effects on the progress of atherosclerosis. Fortunately, increasing evidence has indicated that H\textsubscript{2}S plays a potentially significant role in number of biological processes and potential cardiovascular protections, which suggest that H\textsubscript{2}S may contribute to the inhibition of pathogenesis of atherosclerosis. First, H\textsubscript{2}S shows inhibitory effects on the
development of atherogenesis, such as oxidative stress, modified oxidation of LDL, cell adhesion and calcification. In vascular smooth muscle cells (SMCs), low levels of NaHS (30 or 50 μM), a donor of H₂S, decrease toxic reactive oxygen species, including H₂O₂, ONOO⁻, and O₂⁻ [122]. At the same time, NaHS also enhances the functions of antioxidative enzymes. In addition, H₂S inhibits atherogenic modification of LDL (such as oxidized LDL, shorted as oxLDL) induced HOCl in vitro. As a potent atherogenic agent, oxLDL particle is a important product of atherogenic oxidation, that stimulate endothelial cells to express various adhesion molecules for consequent inflammatory reactions and formation of foam cells. Therefore, inhibition of oxLDL by potential treatments of H₂S implies that H₂S may interfere atherosclerotic progress [123]. Furthermore, H₂S attenuates atherosclerotic lesions by reducing cell adhesion molecules, such as ICAM-1, involving the NF-κB pathway in vivo and in vitro [15]. Adhesion molecules are the significant causes to promote bindings between monocytes and T-lymphocytes to endothelial cells, which will lead to sequential inflammation and advanced process. Reduced expressions of adhesion molecules prohibit monocytes migration and later inflammation, which may also benefit in ameliorate atherosclerotic lesions. Lastly, calcification, presented in the advanced process of atherosclerosis, is a potent factor of plaque stability. There was a study found that the link between H₂S with plaque calcification [124]. In calcified arteries, H₂S level, CSE activity and CSE mRNA were down-regulated, while after administration of H₂S a dose response was shown in decreasing vascular calcium content, Ca²⁺ accumulation, ALP activity, and aortic OPN mRNA. These changes speculated the effect on atherogenesis of H₂S might be induced by suppressing vessel calcification.

Second, H₂S possesses vascular protective capacities from inhibition of proliferation of vascular cells, such as intima and SMCs, and angiosteosis. It has been demonstrated that H₂S suppresses neointima hyperplasia on rat carotid after balloon injury [125]. In another balloon-injured arteries experiment, NaHS (30 μmol/kg
bodyweight) enhances methacholine induced vasorelaxation and significantly ameliorates neointimal lesion formation. Additionally, evidences are also pointing to the fact that H₂S relieves apoptosis and proliferation of SMCs [126]. SMCs migrate from the medial layer into the sub-endothelial space where they may proliferate, ingest modified lipoproteins, secrete extracellular matrix proteins and contribute to lesion development. The suppression of proliferation of SMCs by H₂S can restrict atherosclerotic damages. Moreover, H₂S prevents the process of angiosteosis [16, 127, 128]. Angiosteosis, ossification or calcification of a vessel, is an advanced change in the pathology of atherosclerosis. Its development leads to the narrowing of the caliber of an artery, stimulates thrombosis, or even worse, generates the abruption of unstable plaques. Vascular calcifications induced by vitamin D3 and nicotine in rats are ameliorated by exogenous H₂S. The responses after administration of H₂S show the decreased calcium concentration in vessels, reduced expressions of angiosteosis accompanied acidic phosphatase and osteopontin.

Third, H₂S alleviates the vascular damage induced by an established risk factor, for instance homocysteine. Homocysteine is an amino acid, biosynthesized from methionine and converted into cysteine and sulfur. Augmented levels of homocysteine in plasma, termed hyperhomocysteinemia, are considered as a high risk factor of atherogenesis. Early plaque development in apolipoprotein E deficient mice, a knock out genetic model of atherosclerosis by 8 weeks high cholesterol diet intake, could be enhanced by dietary supplementation with methionine or homocysteine [129]. A research shows that low concentrations of NaHS (30 or 50 μM), a H₂S donor, potentiates cell viability of rat aortic SMCs by abating cytotoxicity and reactive oxygen species stimulated by hyperhomocysteinemia [130].

Although atherosclerosis is chronic, systemic disease with multi-factor involved in its initiation and progression, previous studies have shown that the specific characteristics and functions of H₂S may contribute to the inhibition of atherogenesis.
The multiaspect recognitions of cardiovascular protective effects of H\(_2\)S provide a new avenue of antagonism towards the complicated cardiovascular disease.

### 2.1.8.3 Myocardial injury

Plenty of work have documented that the CSE/H\(_2\)S pathway participates in the regulation of cardioprotective effects [122]. Administration of exogenous H\(_2\)S reduces “infarct-like” myocardial necrosis induced by isoproterenol in the rat [75, 131, 132]. This protection is accompanied with the reduced concentrations of H\(_2\)S in myocardium and plasma, decreased CSE protein activity and up-regulated CSE gene expression in myocardium [75]. NaHS attenuates the myocardial ischemic injury by evidences of reduced mortality and shrinked infarct size in vivo of rat and recovered SMCs viability induced by hypoxia [75]. Further study discovers that 14\(\mu\)mol/kg/d NaHS improves ECG and blood pressure, diminishes infarct size, as well as the greater survivin expression [132].

Oxidative stress injury is an important mechanism of myocardial injury. Direct or indirect antioxidative effects will lead to cardioprotection from myocardial ischemia. The data in above literature reveal that NaHS may antagonize MDA production in vitro of myocytes by oxygen-free radicals or directly react with hydrogen peroxide and superoxide anions [133]. Another experiment also proves that H\(_2\)S provided profound protection against ischemic injury by significant decreases in infarct size, circulating troponin I levels, and oxidative stress [75]. The protections by Na\(_2\)S in early and late preconditioning are all though stimulating the increased antioxidants, which could be itemized to the elevated Nrf2 in early stage and increased expressions of heme oxygenase-1 and thioredoxin 1 in late preconditioning. The antioxidant effect of H\(_2\)S is also embodied in the preservation of mitochondrial functions and ultrastructure by Na\(_2\)S after myocardial ischemia-reperfusion (MI-R) injury [134]. These observations have been recently confirmed by cysteine analogues,
SAC, SPC and SPRC [135, 136]. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), as well as glutathione redox status are preserved by cysteine analogues. The mitochondrial ultrastructure of cysteine analogues treatments appeared more normal than MI vehicle group. These evidences demonstrate the CSE/H₂S pathway is involved in reducing the deleterious effects of oxidative stress.

Furthermore, recent discoveries indicate the observed protection of H₂S is related to regulate leukocyte adhesion and leukocyte-mediated inflammation, increase anti-inflammatory cytokines and reduce several pro-inflammatory cytokines [136]. The anti-inflammatory effect of H₂S is reflected in amplification of heat shock protein (HSP) 70, HSP 90 and cyclooxygenase-2 [11], and reduction of MPO activity [134], nuclear factor-κ B (NF-κB), interleukin (IL)-6, IL-8, and tumor necrosis factor-alpha (TNF-α) [134]. The cardioprotection of H₂S is associated with inhibition of cardiomyocyte apoptosis after myocardial injury. H₂S amplifies anti-apoptosis proteins (Bcl-2, Bcl-xL) and inactivates pro-apoptogen (Bad) [11]. It is also suggested that H₂S ameliorates cardiomyocyte apoptosis after MI-R injury in vitro and in vivo, significant abatement of caspase-3 activity and declining of the number of TUNEL positive nuclei, respectively [134].

Finally, multiple studies have elucidated that a protective effect of K_{ATP} channel activators in myocardial MI-R injury [135]. By virtue of the relaxant effect of H₂S as an opener of K_{ATP} channels, it is easy to hypothesize that H₂S protects myocardial cells against ischemic injury. In the isolated Langendorff-perfused rat hearts, administrations of NaHS result in a dose-dependent limitation of infarct size induced by left coronary artery ligation and reperfusion, while this protective effect is abolished by K_{ATP} channel blockers [137]. There is a report that H₂S preconditioning presents cardioprotective effects against ischemia though signaling pathways of K_{ATP}/PKC/ERK1/2 and PI3K/Akt [12].
2.2 Pathophysiology of atherosclerosis

2.2.1 The structure of vessel wall and functions

There are three layers in healthy arteries and veins:

1. Tunica intima, a single layer of squamous endothelial cells stucked with their matrix and connective tissue;
2. Tunica media, the thickest layer in arteries, which is rich in vascular smooth muscle cells (SMCs), elastic fiber and polysaccharide substances and controls vascular tone;
3. Tunica adventitia, the thickest layer in veins, which constitutes by connective tissue and nerves [1].

Endothelial cells comprise the lumen of blood vessels. Importantly, the vascular endothelium maintains vascular tone, balances the vasodilation and vasoconstriction, and regulates growth, thrombosis, inflammation and oxidation [138]. Endothelium is a bridge of blood flow and vascular wall that can inhibit platelets activation and aggregation, suppress monocyte adhesion and migration, and regulate vascular smooth muscle contraction and proliferation [1]. Endothelium is also a biggest endocrine organ in human body that nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) are released as vasodilators, and vasoconstrictors endothelin-1, angiotensin II (AngII) and thromboxane A2 as vasoconstrictors [139]. SMCs relax or contract to control the volume of blood flow and blood pressure. Three type receptors, $\alpha_1$, $\alpha_2$, $\beta_2$, are the adrenoceptors in sympathetic nervous system to innervate vascular smooth muscle. Activation of $\alpha_1$ receptors leads to vasoconstriction, while agonists of $\alpha_2$ and $\beta_2$ receptors cause vasodilation and hypotension in clinic practice [140]. The main cells involved in the progression of atherosclerosis are endothelial cells and SMCs in vessel, and platelets and monocytes from blood.
2.2.2 Modification of oxidized lipoproteins

Atherosclerotic plaques consist of subendothelial foam cells which are identified as macrophages derived from monocytes circulating in the blood and scavenged lipid supplied by blood lipoproteins and modified by free radicals [141]. The oxidative modification of lipoprotein, especially low density lipoprotein (LDL) is a principal inducer for the progression of atherosclerosis [142]. Oxidized LDL has been demonstrated its proatherogenic potentials that stimulate the cell adhesion between endothelial cells and monocytes, stimulate endothelial cells to generate inflammatory cytokines, chemokines and adhesion molecules, and induce monocyte/macrophages to attach scavenger receptors and matrix metalloproteinase, eventually leading to the formation of foam cells, the hallmark of arterial fatty steak [143, 144].

Oxidized modification of LDL is assumed to change in two major stages [4, 145-147]. In the first stage of LDL oxidation, the LDL lipids are oxidized in the absence or little changes in apolipoprotein B 100, which called minimally oxidized LDL[145]. Such modified LDL still retains its affinity to the LDL receptors, has a little negative charge, but activates anti-apoptotic signaling, and stimulates more inflammatory chemokines and cytokines expressions[148, 149]. The changes to induction of inflammatory molecules may lead to large more variety of enhanced cytokines and process the oxidized modification of LDL. During second stage of LDL oxidation, the LDL lipids are further oxidized, including the modification of LDL lipoprotein[145, 147]. The advanced oxidized LDL gradually loses the recognition of LDL receptor and shifts to the attachment of scavenger receptors, also called oxidized LDL receptors. This change results in the accumulation of foam cells lesion development. The LDL diversity degrees of oxidation provide different biological effects to arterial walls. Mildly oxidized LDL could induced endothelial cell expressing tissue factors, while highly oxidized LDL is cytotoxic and pro-apoptotic, and stimulates the proliferation
of smooth muscle cells[149].

The mechanisms of oxidized modification of LDL may be through the transition metal ions and oxidation by free radicals[147]. Without tissue or cells circumstances, high concentrations of free metal ions can oxidize LDL, which reactions were established and used for monitoring the extent of oxidized modification of human LDL[150]. In vitro, most of cells exist in the arterial intima can induce oxidized modification of LDL thorough the catalysis by lipoxygenase, myeloperoxidase and/or metal ions, like microconcentration of iron or copper[147]. The oxidized modification of LDL mediated by macrophage forms enhanced cholesteryl ester-core aldehydes of LDL[151]. This type of cell-mediated oxidation of LDL can be blocked by metal chelators[4]. There is also found that the metal ions are increased in the advanced human atherosclerotic lesions[152]. The study using electron paramagnetic resonance (EPR) spectroscopy and inductively coupled plasma mass spectrometry (ICPMS) showed a relationship between elevated iron levels and cholesterol accumulation in the human carotid samples[152]. Others studies indicated that thiols may play a vital role in oxidation of LDL[153, 154]. Incubated with iron at acidic environment, LDL is oxidized to hydroperoxides and aldehydes and enhanced by increased levels of cysteine through transferring Fe$^{3+}$ to Fe$^{2+}$ and α-tocopherol radicals of oxidized LDL to α-tocopherol[155].

2.2.3 Monocyte-endothelial adhesion

Early phase of atherosclerosis involves the interaction of monocytes with the vascular endothelium [156]. Some cell-surface proteins control these interactions, including integrins, selectins, and members of the immunoglobulin gene superfamily [157]. First, the accumulated ox-LDL particles activate endothelial cells to express adhesive molecules for recruiting blood cells [158]. After recruitment and contact, the next step is tethering and rolling of blood cells to the surfaces of activated endothelial
monlayers, which is mediated by selectins family. Then, the further attachment is
firmed by the interaction of integrins with immunoglobulin superfamily, especially
intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 [159]. Furthermore, integrins and platelet endothelial cellular adhesion molecule 1 (PECAM-1) help
leucocytes transformation and transmigration to subendothelial layers for advanced
inflammatory reactions [160].

2.2.3.1 Selectins

Selectins include three members and named according to their main expression site:
L-selectin in leukocytes, E-selectin specifically in endothelial cells, and P-selectin
mainly in platelets but also in endothelial cells [161]. The three molecules have their
own specific pattern of expression. L-selectin is present on some T cells, B cells and
NK cells depending on the activated state. E-selectin cannot be tested in resting
endothelial cells but is transcriptionally induced by inflammatory cytokines.
P-selectin is just stored in resting cells of Weibel-Palade bodies of endothelial cells
and α-granules of platelets and moves to cell surfaces after activation [161, 162].
After inflammatory stimuli, selectin family molecules are internalized, lysosomal
targeted or shed/proteolytic cleaved to their activated states and rapidly recruited to
the cell surface [162, 163]. Selectins and their ligands (mainly P-selectin glycoprotein
ligand, PSGL-1) participate in the early stage of leukocyte recruitment: rolling and
tethering of leukocytes on the vascular wall [159, 164]. Besides that, selectins are also
involved in the recruitment of bonds between platelets and leukocytes, or between
platelets, or between leukocytes [162]. P-selectin/PSGL-1 binding not only creates
weak bonds between activated endothelium and leukocytes, also activates more
leukocytes, mobilizes integrins, stimulates advanced inflammatory reactions and
thrombosis [165]. Leukocytes traffic and homing is controlled by L-selectin [166,
167].

44
2.2.3.2 Integrins

Integrins belong to the group of heterodimeric transmembrane glycoproteins consisted of an α chain and a β chain with a non-covalent bond [168, 169]. Integrins are usually non-adhesive and present low affinity for ligands in resting cells, while develop higher affinity to ligands when activation by modifying their conformation [170, 171]. Integrins are expressed in various cells with different functions. In white blood cells can be found the class of β-2 integrins, which activated conformation interact with ICAM subfamily and lead to leucocytes transformation and migration at the surfaces of endothelial cells with firm arrests [170, 172, 173]. Integrins α4/β1 distributed in endothelial cells and binded with VCAM-1 can also firm adhesion in arresting step [170, 172]. The large class of β1 integrins is expressed in SMCs and help SMCs attaching to extracellular matrix [174, 175]. The β3 family integrins are distributed in platelets that control the fibrin formation, cell survival, migration and proliferation [174, 176, 177]. Until now, there is no soluble form of either integrin been found [169, 178].

2.2.3.3 Immunoglobulin family

The adhesion molecules in immunoglobulin (Ig) superfamily are transmembrane glycoprotein receptors containing various extracellular Ig domains and participate in the antigen recognition. VCAM-1 is upregulated by TNF-α and IL-1, expressed on early dysfunctional endothelial surfaces [179, 180]. The attachment assisted by VCAM-1 is exclusively to monocytes and lymphocytes [181]. The ligands of VCAM-1 include integrins α4/β1 and α4/β7 in monocytes and lymphocytes to firm the adhesion. ICAM-1 is activated and expressed in endothelial cells by IFN-γ, TNF-α, IL-1β, and bacterial endotoxin [182]. The ligands of ICAM-1 is comprised of integrins αL/β2 and αM/β2 located on the surfaces of leukocytes, monocytes and lymphocytes [183]. Owing to interactions between leukocytes and endothelial cells
reflecting the inflammatory-immune response, the upregulation of ICAM-1 is also a characteristic feature of atherosclerosis [184]. Lastly, PECAM-1 is adhesive molecule in the Ig family distributed on platelets, leukocytes and endothelial cells [185]. PECAM-1 is particularly located at the junctions between endothelial cells where influence the homophilic bindings among adjacent endothelial cells [186]. Therefore, PECAM-1 is involved in endothelial integrity, leukocyte extravasation which helps leukocyte transmigration to media layer [160].

2.2.4  Endothelial dysfunction in atherosclerosis

Endothelial dysfunction is a systemic disorder and an important viable in the pathogenesis of atherosclerosis and its complications. Enhanced NO inactivation is a hallmark of endothelial dysfunction in vascular disease [139]. Several studies have investigated that impairment of endothelium-dependent vasodilation is found in coronary, renal vasculature in patients with cardiovascular disease [187, 188]. In addition to the increase of endothelium-derived contracting factors, endothelial dysfunction also includes a distinct state of endothelial activation, which is represented by a pro-coagulatory, proliferative, and pro-inflammatory milieu that can be found at all stages of atherogenesis [189].

ROS is generated at sites where the cell layers has been damaged or under metabolic stress. Endothelial dysfunction is also strong relationship with production of ROS, including superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH), hypochlorous acid (HOCl), NO, and peroxynitrite (ONOO\(^-\)) [138]. The decreased bioavailability of NO, a feature of endothelial dysfunction, can be induced by the reaction with the increased generated ROS that form ONOO\(^-\) which binds to guanylate cyclase, leads to lipid peroxidation, protein oxidation, and DNA fragmentation [1]. The vicious interaction between oxidative stress and endothelia dysfunction promotes the development of atherosclerosis.
Endothelial dysfunction can be treated as the “ultimate risk of the risk factors” presenting a specific atherogenic vascular milieu [190]. Many risk factors, including hyperlipidemia, hypertension, diabetes and smoking, are associated to atherosclerosis, complications and cardiovascular events. These risk factors found to be related to endothelial dysfunction may be due to the overproduction of ROS and oxidative stress [191]. In turn, the increased oxidative stress will lead to the reduction of vascular NO bioavailability. Therefore, the ROS generation may serve as a major mechanism involved in atherogenesis and enhance the risk of endothelial dysfunction on the progression of atherosclerosis. Moreover, some studies also found that the variable endothelial susceptibility of individual patients to different cardiovascular risk factors, prevented or promoted the endothelial dysfunction [192-194], indicating the genetic predisposition is a factor that may vary the impact of risk factor observed in cardiovascular and metabolic diseases on endothelial dysfunction.

2.2.5 Pathogenesis of atherosclerosis

Atherosclerosis is no longer considered as a disorder rather than an inflammatory insult due to its bridge role of linking hyperlipidemia with atherosclerosis [195]. Amounting evidences suggest that high plasma cholesterol levels result in atherosclerotic lesion formation [196]. The other risk factors, like diabetes, hypertension, male gender and smoking, appear to accelerate a disease driven by atherogenic lipoproteins, the first of which being LDL [5]. Inflammation in atherogenesis is likely initiated when endothelial cell over-expresses various adhesion molecules in response to turbulent flow in the setting of an unfavorable serum lipid profile, which leads to the attachment of leucocytes to endothelial cells [195]. It has been demonstrated vascular cell adhesion molecules-1 (VCAM-1), an early and necessary step in the pathogenesis of atherosclerosis, induces the binding of monocytes and T-lymphocytes to endothelium [161]. Subsequently, leukocytes
express monocyte chemoattractant protein-1 (MCP-1) to recruit more leukocytes and magnify the inflammatory cascade \[197\]. After the attachment to endothelial cells, monocytes migrate to subendothelial layer and differentiate to macrophages. Macrophages produce scavenger receptors which bind ox-LDL, with no feedback by intracellular cholesterol levels \[120\]. Continued scavenging lipid droplets, macrophages are full of cholesterol esters in their cytoplasm and gradually change to foam cells, which accumulate to fatty steaks. Fatty steak is the earliest observable pathological change in the arterial walls \[14\]. Subsequently, foam cells release inflammatory cytokines and ROS, which promote the inflammatory process \[198\]. In response to cytokines and growth factors, atherosclerotic plaques may accumulate vascular smooth muscle cells (VSMCs) migration and proliferation, which contribute to the narrowing of blood lumen. VSMCs also secrete extracellular matrix proteins, such as collagen and elastin, which may progress to the formation of fibrous cap \[199\]. At the advanced stage, the death of macrophages leads to the necrotic lipid core and finally the plaque ruptures, which yield blood components contact with plaque lipids and tissue factors, generating the thrombus and related clinical complications \[200, 201\].

2.2.6 Mitochondria and vascular disease

2.2.6.1 Endothelial mitochondria

Mitochondria are estimated to account for 5% of endothelial cell volume\[202\]. Mitochondrion is not only an organelle generating ATP, also plays a vital role in regulating the homeostatic triangle of ROS, NO, and Ca$^{2+}$ \[202\]. Whether mitochondrion is the major source of ATP production is a debating issue. Recently, some observations showed that the data of mitochondria respiratory flux in primary endothelial cells of bovine aortas suggested mitochondria respiration was greatly coupled to ATP production\[203\]. There were evidences showed that under oxidative
stress, the reliance of endothelial cells upon oxidative phosphorylation increased, indicating the mitochondrial reserve capacity was involved in the protective effect to endothelial cells response to oxidative stimulation [203, 204]. Therefore, the more important role of endothelial mitochondria is the generation of ROS and NO, and regulation of intracellular Ca\(^{2+}\) signaling.

Fig. 2-6 Oxidative phosphorylation, superoxide production and scavenging pathways in mitochondria [205].

Mitochondria ETC located at mitochondria inner membrane, consists of complex I, II, III, IV and V, seen Fig 2-6 [205]. Electrons (e\(^{-}\)) from nicotine adenine dinucleotide (NADH) and FADH\(_{2}\) pass thorough complex I and II, respectively, then via ubiquinol (coenzyme Q) transfer to complex III. Through cytochrome c, electrons are transferred from complex III to IV, reducing O\(_{2}\) to H\(_{2}\)O. In the meantime, the protons (H\(^{+}\)) are pumped from the mitochondrial matrix to intermembrane space by complex I, III and IV and reenter to the mitochondrial matrix through complex V by proton-motive force (Δp) to produce ATP [205]. Among these protein complexes, complex I leaks electrons to produce to matrix and
complex III produces both matrix and intermembrane space [205]. Superoxide is dismutated to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) by Mn-SOD (SOD-2) in the mitochondrial matrix and Cu,Zn-SOD (SOD-1) in the mitochondrial intermembrane space. Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) is degraded to H\textsubscript{2}O through glutathione peroxidase (GPx) by using glutathione (GSH) as the hydrogen donor [206].

### 2.2.6.2 Mitochondria and ROS

Mitochondria are the major source of ROS within most mammalian cells, generating superoxide then dismutated to hydrogen peroxide [111, 207]. In tranquil status, mitochondria produce ROS as the natural byproduct of the normal metabolism of oxygen; while in the cardiovascular diseases, ROS increase dramatically and overwhelm antioxidant defenses, resulting in damage to cell structures, known as oxidative stress [208].

There are several potential sites of ROS generation in mitochondria. The majority of superoxide produced from mitochondria is on the complexes of the electron-transport chain (ETC) [209]. Others sources of ROS comprise Krebs cycle enzymes participating in redox reactions, like monoamine oxidase A and B [210, 211], a-ketoglutarate dehydrogenase[212], tricarboxylic acid cycle enzymes aconitase [213], cytochrome bs reductase and glycerol-3-phosphate dehydrogenase from the glycerol phosphate shuttle [214]. The production of ROS by these proteins relies on the proteins to be abundantly charged with electrons [215]. Therefore, when available electrons are relatively small, the ROS generation is reduced.

From the way of transferring electrons on ETC, the major sites of mitochondrial ROS are complexes I and III. In physiologic conditions prone to ATP generation, complexes I accept electrons donated from NADH, then through iron-sulfur centers, pass electrons to ubiquinone-biding sites[216]. Through ubiquinol and cytochrome c,
complex I pass electrons to complex III, which release O$_2^-$ to mitochondrial matrix and intermembranous space\textsuperscript{[217]}. Under oxidative stress, the high ratios of NADH/NAD$^+$, low electron flow, low oxygen consumption, and low ATP production need, electrons can be transferred from the fully reduced flavin mononucleotide (FMN) sites on complexes I and Q cycle in complex III to oxygen to yield superoxide\textsuperscript{[212]}. ROS generation could also be enhanced together with the release of cytochrome c during apoptosis as absence of cytochrome c results in the block of electron flow to complex III and a rise in reverse flow of electron to complex I with a subsequent rise in ROS generation at this site\textsuperscript{[218]}.

ROS, the natural byproduct in the metabolism, can also damage to cellular macromolecules, such as nucleic acids, phospholipids and proteins\textsuperscript{[213]}. Oxidative stress inflicts to DNA leading to the modification of the bases (purine and pyrimidine), the backbone (deoxyribose), and single and double strand-breaks\textsuperscript{[219]}. These modifications of DNA structures are mutagenic and have close relationship with cancer, aging and neurodegenerative disease\textsuperscript{[220]}. Polyunsaturated fatty acid (PUFA) located in phospholipids is highly sensitive to ROS. One potent inducers of lipid peroxidation is hydroxyl radical generated by the Fenton reaction\textsuperscript{[221]}. Another powerful catalyst of lipid peroxidation is Fe$^{2+}$, which has been demonstrated to increase the mitochondrial PUFA oxidation and the lysosomal fragility\textsuperscript{[222]}. Proteins assaulted by ROS have been demonstrated several types of damage, such as protein-protein cross-linking, oxidation of sulfhydryl groups, reaction with aldehydes, alteration of tertiary structure of proteins and protein fragmentation\textsuperscript{[223]}. The outcome of protein oxidation is the loss of proteins normal functions, like channel forming properties, enzymatic activities, etc. and the increasing of the tendency to proteolytic degradation\textsuperscript{[224]}. In addition to being the primary source of intracellular ROS in aerobic cells, mitochondria are also the targets of oxidative stress. Mitochondrial DNA (mtDNA)
is a target of oxygen radicals, because mtDNA is more sensitive to free radicals than nuclear DNA due to the close proximity to ETC where ROS is generated. Additionally, mtDNA is deficiency in protective histone and has limited base excision repair mechanisms[225]. A mechanism of superoxide toxicity leading to oxidative stress is oxidation and inactivation of iron-sulfur (Fe-S) proteins, like aconitases, and association of leaking iron[226-228]. The consequences are the simultaneous release of Fe²⁺ and H₂O₂ and generation of potent hydroxyl radicals, which amply the oxidative damage[229]. Other deleterious effects of ROS generation in mitochondria are suppression of mitochondrial metabolism [207] and opening of Ca²⁺-dependent mitochondrial permeability transition (MPT) [230, 231]. These effects will change mitochondrial functions and lead to apoptosis which contribute to vascular diseases.

2.2.6.3 Mitochondria and apoptosis

Apoptosis is a form of cell death that is an evolutionarily conserved and genetically regulated procedure which plays a crucial role in morphogenesis, embryonic development, and for the maintaining homeostasis in mature cells[232]. Apoptosis is characterized by plasma membrane blebbing, cell shrinkage, condensation of chromatin, nuclear fragmentation, and chromosomal DNA fragmentation [233].

All mammalian cells posses contain two major apoptotic signaling pathways, known as intrinsic pathway and extrinsic pathway. The extrinsic pathway initiates apoptosis through the gathering of plasma membrane death receptors, tumor necrosis factor receptor (TNFR) family, which transmit the apoptotic signaling through binding with their death ligands. Fas/FasL, the TNFR/ligand binding, is reported to involve in apoptosis of endothelial cells and smooth muscle cells [234-236].

Another important apoptotic protein is caspase family, a group of proteases with low
intrinsic activity and activation by proteolytic maturation or interaction with an allosteric activator [237]. Fas-associated death domain protein (FADD), Fas, FasL and procaspase-8 or -10 combine to a death-inducing signaling complex (DISC), which switch off the positions of death effector domain (DED) with initiator caspase (procaspase-8) [238]. Therefore, the initiator caspases activate caspase-3 that generates the execution of apoptotic program by cleavage of downstream signaling [239]. Moreover, caspase-8 first cleaves Bid, a protein leading to apoptosis in Bcl-2 family[240], which, results in translocation, oligomerization and insertion of other apoptotic Bcl-2 proteins, like Bax or Bak, into the mitochondrial outer membrane (OMM) [241]. The permeabilization of OMM leads to the release of cytochrome c, which forms a cytosolic apoptosome complex that activate caspase-9, then in turn cleaves and activates caspase-3 and downstream caspase cascade[242].

![Fig.2- 7 Role of the mitochondria in apoptosis and necrosis [243].](image)

The intrinsic pathway includes mitochondria swelling, causing the mitochondrial
outer membrane rupture and various pro-apoptotic proteins from the mitochondrial intermembrane space to the cytoplasm, including cytochrome c, apoptosis inducing factor (AIF), Smac/Diablo, HtrA/Omi, and DNaseG \[244\], seen in Fig. 2-7. This pathway can be regulated by Bcl-2 family proteins to control cytochrome c release, Smac to activate mitochondrial apoptosis \[245\], inhibitor of apoptosis proteins (IAPs) to prevent caspase \[246\] and HtrA/Omi to suppress IAPs\[247, 248\]. Meanwhile, the swollen or ruptured mitochondria can generate and release high toxic ROS, which promotes the progress of apoptosis and cell death. These factors are all important for the mitochondrial regulation of apoptosis.

An important protein family governing mitochondrial outer membrane permeabilization in apoptotic program is the Bcl-2 family, including pro- and anti-apoptotic proteins\[249\]. The group of anti-apoptotic proteins contains Bcl-2 and Bcl-XL, which inhibit permeabilization of OMM and release of pro-apoptotic proteins from the mitochondrial intermembrane space\[250\]. The other group of pro-apoptotic proteins includes Bax, Bak, BAD and Bok, which are activated by their oligomerization and insertion into OMM, leading to release of pro-apoptotic proteins from mitochondria, then cell death \[251\].

In the intrinsic pathway, the generated ROS in mitochondrial ETC can be withstood by mitochondrial antioxidant defense systems. Within the mitochondrial matrix, \(\text{O}_2^\cdot^-\) is converted to \(\text{H}_2\text{O}_2\) by Mn-SOD (SOD-2). In the mitochondrial intermembrane space, \(\text{O}_2^\cdot^-\) is catalyzed by Cu,Zn-SOD (SOD-1). Then, \(\text{H}_2\text{O}_2\) can be readily metabolized to \(\text{H}_2\text{O}\) by GPx-1 and peroxiredoxin (Prx III) which oxidize reduced GSH to oxidized glutathione (GSSG). GSSG can be converted back to GSH by glutathione reductase \[206\]. The formation, effects and inactivation of ROS in mitochondria are shown in Fig. 2-8. In addition to GSH, multiple GSH-linked antioxidant enzymes are involved in mitochondrial antioxidant defense, like GPx-1, GPx-4 and glutaredoxin (Grx) \[252\], seen in Fig. 2-8. Small fractions of GPx-1
present at mitochondrial matrix to show its biological effect. GPx-4 catalyzes lipid hydroperoxides and is considered as the primary enzymatic defense mechanism against oxidative stress to cellular membranes. Glutaredoxins catalyze glutathione-dependent dithiol reaction mechanisms \[253\], supply intramolecular disulfide bond to two cysteines\[254\], and carry electrons in the glutathione-dependent synthesis of deoxyribonucleotides \[255\].

![Diagram of ROS in mitochondria](image)

**Fig.2-8** Formation, effects and inactivation of ROS in mitochondria \[206\].

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione disulfide</td>
</tr>
<tr>
<td>Gpx</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>Grx</td>
<td>glutaredoxin</td>
</tr>
<tr>
<td>IDHm</td>
<td>mitochondrial isocitrate dehydrogenase</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>Prx</td>
<td>peroxiredoxin</td>
</tr>
<tr>
<td>Trx</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>thioredoxin reductase</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TH</td>
<td>transhydrogenase</td>
</tr>
</tbody>
</table>

Besides glutathione antioxidative system, there are several mitochondrial enzymatic antioxidants, such as peroxiredoxin (Prx), thioredoxin (Trx) and thioredoxin reductase (TrxR), seen in **Fig.2-8**. Thioredoxins show higher reduction of protein
disulfides than Grx and interact with Prx, which in turn utilize Trx to detoxify H$_2$O$_2$ [256, 257]. Nonenzymatic antioxidants include coenzyme Q10, cytochrome c, α-tocopherol, and glutathione.

In structurally and functionally intact mitochondria, the abundant antioxidant defense network balances with physiological ROS generation, therefore there is little net ROS generation for cellular apoptosis. Nevertheless, the affliction of mitochondria causes largely increase of ROS generation outweighing the capability of mitochondrial antioxidant defense systems. Once this happens, a vicious cycle will ensue that more generation of toxic free radicals and loss of antioxidants capacities [258].
Chapter 3
Methods and Materials
3.1 Drug preparation

3.1.1 Materials

Cysteine, D, L-Propargylglycine (PAG), DHE and LDH assay kit were purchased from Sigma, USA. Sodium hydrosulfide (NaHS) was purchased from Aldrich, USA. H$_2$DCFDA and Annexin V/PI kit were purchased from Invitrogen, USA. MDA levels kit, JC-1 assay kit were purchased from Beyotime, China. DPPP, kits for antioxidant enzyme assays, SOD, catalase, GPx and GST assay were purchased from Cayman Chemicals, USA.

3.2 Synthesis of SPRC

SPRC (also named as ZYZ-802) was synthesized from the reaction of L-cysteine with propargyl bromide and purified by recrystallization from an ethanol-water mixture (96.1%). The final product was verified with $^1$H nuclear magnetic resonance spectroscopy.

3.2 Animals and cells

3.2.1 Animals

NZW rabbits were provided by the Centre for Animal Resource (CARE) of National University of Singapore (NUS). The experimental protocol for animal study was approved by NUS Institutional Animal Care and Use Committee (IACUC). New Zealand White rabbits weighing around 2.5 kg were caged individually in the Animal Holding Unit (24 ± 1°C and 55 ± 5% humidity) with dark-light cycles of 12 hours. Water and feeds were available to the animal ad libitum with standard rabbit chow.
(with or without 1% cholesterol). Rabbits were randomly divided into different experimental groups of 6 animals each. The rabbits in the normal control (sham) group were fed a chow diet for 8 weeks. The other rabbits fed 1% cholesterol diet were treated with either saline (0.5 ml/kg/day, vehicle group) with or without treatments of NaHS (1, 10 and 30 μmol/kg/d) or SPRC (10 μmol/kg/d) or PAG (10 mg/kg/d) administered daily through subcutaneous injection for 8 weeks. These diets were high-cholesterol diet (HCD) SF00-221 (modified guinea pig and rabbit + 1% cholesterol) and purchased from Glen Forrest Stockfeeders, Western Australia. Rabbit health and weight were monitored throughout the study.

3.2.2 Cell culture

HUVECs (Lonza, Singapore) were grown in EGM-2 (Lonza, Singapore) containing vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), hydrocortisone, heparin, gentamicin sulfate amphotericin, 1‰ ascorbic acid and 2% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO₂. HUVECs were passaged every three days. The 4th to 8th passages of HUVECs were used for this study.

3.3 Hyperlipidemic rabbit model

New Zealand white (NZW) male rabbits weighing on average 2.5 kg were obtained from the Laboratory Animal Centre (Sembawang, Singapore). The NZW rabbits were caged individually in the Animal Holding Unit (24 ± 1°C and 55 ± 5% humidity) with dark-light cycles of 12 hours. Water and feeds were available to the animal ad libitum with high-cholesterol diet (HCD) SF00-221 (modified guinea pig and rabbit + 1% cholesterol) for eight weeks. The food intake and animal body weights were monitored, and all rabbits gained weight during the experimental period of 8 weeks.
3.4 Experimental protocols

3.4.1 Experimental protocol I

3.4.1.1 Objectives

Hydrogen sulfide (H$_2$S) has been shown to have cytoprotective effects in models of hypertension, ischemia/reperfusion and Alzheimer’s disease. However, little is known about its effects or mechanisms of action in atherosclerosis. Therefore, in this part studies we have examined the protective effects of H$_2$S in endothelial cells against H$_2$O$_2$ induced cellular stress.

Our major focus is to determine whether H$_2$S,

i) executes protective effects through CSE/ H$_2$S pathway,

ii) preserves mitochondrial functions and ultrastructure,

iii) promotes the induction of cytoprotective antioxidant enzymes that can detoxify toxic free radicals.

The current findings provide further evidences for a functional role of H$_2$S in endothelial cells in relation to the prevention of atherosclerosis.

3.4.1.2 Experimental design

The experimental design is illustrated in Fig. 3-1. For all experiments, HUVECs were grown to confluence in 96-well plates, 35 mm$^2$ dishes or 100 mm$^2$ dishes. Cells were pre-incubated with NaHS (10, 30, 100, 300, 500 or only 300 µM) or PAG (10 mM) for 6 hours before exposure to H$_2$O$_2$ (600 µM). Following exposure to H$_2$O$_2$, cells were harvested for further analysis. Normal control cells are in “control group”
without H₂O₂ stimulation; cells accepted H₂O₂ (600 μM) only are in “vehicle +H₂O₂ group”; treatment groups are included NaHS (10, 30, 100, 300, 500μM) groups or only NaHS group (300 μM) and PAG group (10 mM), seen in Table 3-1. First, we evaluated the cytotoxicity of H₂S by MTT assay. Then, the cellular protective effects of exogenous H₂S were tested by MTT assay, LDH assay and apoptosis – Annexin V/PI staining. In order to investigate the cardioprotective effects by H₂S though the CSE/ H₂S pathways, we measured the H₂S concentrations in the medium, CSE activity, and CSE protein and gene expressions in cells. For the mitochondrial studies, mitochondrial membrane potential-JC-1 staining, ΔΨm assay, ROS production and Transmission Electron Microscopy (TEM) were used to detect the mitochondrial healthy status in HUVECs. More specific measurements were needed for detect mitochondrial dysfunction in HUVECs. Therefore, cells were isolated mitochondrial suspension for subsequent experiments. ATP biosynthesis assay and cytochrome c release measurement were done to evaluate the effects of H₂S in isolated mitochondria. Moreover, the effects of antioxidants activities and antioxidants enzymes protein expressions were evaluated. Furthermore, the protective mechanism of H₂S was also investigated by detecting apoptotic protein expressions.
Table 3-1 Grouping for studies of effects of H₂S on HUVECs

<table>
<thead>
<tr>
<th>Group #-1. Cytotoxicity study</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control – no drug</td>
</tr>
<tr>
<td>2</td>
<td>NaHS (10 µM) + H₂O₂</td>
</tr>
<tr>
<td>3</td>
<td>NaHS (30 µM) + H₂O₂</td>
</tr>
<tr>
<td>4</td>
<td>NaHS (100 µM) + H₂O₂</td>
</tr>
<tr>
<td>5</td>
<td>NaHS (300 µM) + H₂O₂</td>
</tr>
<tr>
<td>6</td>
<td>NaHS (500 µM) + H₂O₂</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group #-2. Cellular protection study</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control – no drug</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle + H₂O₂ (600µM)</td>
</tr>
<tr>
<td>3</td>
<td>NaHS (30 µM) + H₂O₂</td>
</tr>
<tr>
<td>4</td>
<td>NaHS (100 µM) + H₂O₂</td>
</tr>
<tr>
<td>5</td>
<td>NaHS (300 µM) + H₂O₂</td>
</tr>
<tr>
<td>6</td>
<td>NaHS (500 µM) + H₂O₂</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group #-3. Mechanisms study</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control – no drug</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle + H₂O₂ (600µM)</td>
</tr>
<tr>
<td>3</td>
<td>NaHS (300 µM) + H₂O₂</td>
</tr>
<tr>
<td>4</td>
<td>PAG (10 mM) + H₂O₂</td>
</tr>
</tbody>
</table>
Fig. 3-1 A flow chart represents the general outline of the experiment I.
3.4.2 Experimental protocol II

3.4.2.1 Objectives

Hydrogen sulfide (H\textsubscript{2}S) has been shown to have cardioprotective effects in models of hypertension, ischemia/reperfusion and Alzheimer’s disease. However, few evidence is provided to show its effects or mechanisms of action mitochondrion, especially in vascular system. Therefore, in this part studies we have examined the protective effects of H\textsubscript{2}S on isolated rabbit aortas mitochondria against H\textsubscript{2}O\textsubscript{2} induced mitochondrial dysfunction.

Our major focus is to determine whether H\textsubscript{2}S,

(i) prevents oxidative stress (ROS production),

(ii) protects mitochondrial functions (mitochondrial respiration-oxygen consumption, ATP biosynthesis, and mitochondrial respiratory chain and matrix enzyme activity);

(iii) preserves mitochondrial membrane intact (mitochondrial membrane potential and cytochrome c release)

The current findings provide further evidences for a functional role of H\textsubscript{2}S in isolated rabbit aortas mitochondria in relation to the prevention of vascular mitochondrial dysfunction.

3.4.2.2 Experimental design

The experimental design is illustrated in **Fig. 3-2.** New Zealand White rabbits were anaesthetized with 2 ml/kg ketamin/xylazine (i.p) and killed by 1ml phenobarbitone in order to collect the aorta samples. Aortas were fasted collected, immediately peeled the surrounding fats and muscles, and then processed for isolation mitochondria.
Before the experiments were to be carried out, quality of aortic mitochondrial preparations was assessed by JC-1 assay to ensure the quality of mitochondria suitable for bioenergetics studies. Isolated mitochondria suspension was divided into groups as listed in Table 3-2 for subsequent experiments. ROS production, mitochondrial respiration, ATP biosynthesis, mitochondrial respiratory chain and matrix enzyme activity, mitochondrial membrane potential and cytochrome c release were used to detect the effects of H₂S on isolated aortic mitochondria.

Table 3-2 Grouping for studies of effects of H₂S on isolated mitochondria

<table>
<thead>
<tr>
<th>Group #</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control – no drug</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle + H₂O₂ (1mM)</td>
</tr>
<tr>
<td>3</td>
<td>NaHS (10 µM) + H₂O₂</td>
</tr>
<tr>
<td>4</td>
<td>NaHS (100 µM) + H₂O₂</td>
</tr>
<tr>
<td>5</td>
<td>NaHS (300 µM) + H₂O₂</td>
</tr>
<tr>
<td>6</td>
<td>PAG (10 mM) + H₂O₂</td>
</tr>
</tbody>
</table>

Fig. 3-2 A flow chart represents the general outline of the experiment II.
3.4.3 Experimental protocol III

3.4.3.1 Objectives

Due to the limited knowledge of effects of H$_2$S on atherosclerosis, in this part study we have examined the vascular protective effects of H$_2$S in the high cholesterol-fed rabbit model.

Our major focus is to determine whether, in the hyperlipidemic rabbits, H$_2$S
(i) executes its cardioprotective effects via the CSE/ H$_2$S pathway;
(ii) inhibits the development of atherosclerotic plaques;
(iii) inhibits atherogenic oxidized LDL;
(iv) reduces toxic free radicals and improves activities of antioxidant enzymes, thereby improving the redox status;
(v) suppresses inflammatory cell adhesion molecules in the atherogenesis.

The results of this study will provide a better understanding of the biological properties of H$_2$S vis-à-vis atherosclerosis, and unveil a potential therapeutic avenue for this challenging and ubiquitous disorder.

3.4.3.2 Experimental design

The experimental design is illustrated in Fig. 3-3. Thirty-six New Zealand White male rabbits weighing around 2.5 kg were caged individually in the Animal Holding Unit (24 ± 1°C and 55 ± 5% humidity) with dark-light cycles of 12 hours. Water and feeds were available to the animal ad libitum with standard rabbit chow (with or without 1% cholesterol). Rabbits were randomly divided into 6 experimental groups of 6 animals each. The rabbits in the normal control (sham) group were fed a chow diet for 8 weeks.
The other rabbits fed 1% cholesterol diet were treated with either saline (0.5 ml/kg/day, vehicle group), NaHS (1, 10 and 30 μmol/kg/d) or PAG (10 mg/kg/d) administered daily through subcutaneous injection for 8 weeks, seen in Table 3-3. Rabbit health and weight were monitored throughout the study. First, in order to investigate the effects of H₂S though CSE/H₂S pathway, we detected the H₂S levels in plasma, aortic CSE activities, and aortic CSE gene expressions. Then, the lipid files were tested for veiling the cholesterol-lowering effects of H₂S. H & E staining and HRUS were used to detect the plaques in thoracic and carotid aortas. TEM were shown the vascular ultrastructure of thoracic aorta in different treatment groups. The mechanisms of protective effects of H₂S were investigated on oxidative modification of LDL and antioxidative effects.
### Table 3-3 Grouping for studies of effects of H\(_2\)S on hyperlipidemic NZW rabbits

<table>
<thead>
<tr>
<th>Group #</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sham – no drug</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle + high cholesterol diet (HCD)</td>
</tr>
<tr>
<td>3</td>
<td>NaHS (1 (\mu \text{mol/kg/d})) + HCD</td>
</tr>
<tr>
<td>4</td>
<td>NaHS (10 (\mu \text{mol/kg/d})) + HCD</td>
</tr>
<tr>
<td>5</td>
<td>NaHS (30 (\mu \text{mol/kg/d})) + HCD</td>
</tr>
<tr>
<td>6</td>
<td>PAG (10 mg/kg/d) + HCD</td>
</tr>
</tbody>
</table>

![Flow chart](chart.png)

**Fig. 3-3** A flow chart represents the general outline of the experiment III
3.4.4 Experimental protocol IV

3.4.4.1 Objectives

SPRC, a structural analog of garlic extract - S-allylcysteine (SAC), plays cardioprotective roles in hypertension and ischemia/reperfusion. But no evidence was shown its effects on atherosclerosis. Therefore, in this part study we have examined the vascular protective effects of SPRC, in the high cholesterol-fed rabbit model.

Our major focus is to determine whether, in the hyperlipidemic rabbits, SPRC (i) executes its cardioprotective effects via the CSE/ H$_2$S pathway; (ii) inhibits the development of atherosclerotic plaques; (iii) inhibits toxic free radicals and enhances activities of antioxidant enzymes, thereby improving the redox status. (iv) suppresses the inflammatory cytokines and cell adhesive cytokines to play its anti-inflammatory effects.

3.4.4.2 Experimental design

The experimental design is illustrated in Fig. 3-4. Thirty New Zealand White male rabbits weighing around 2.5 kg were caged individually in the Animal Holding Unit (24 ± 1°C and 55 ± 5% humidity) with dark-light cycles of 12 hours. Water and feeds were available to the animal ad libitum with standard rabbit chow (with or without 1% cholesterol). Rabbits were randomly divided into 5 experimental groups of 6 animals each. The rabbits in the normal control (sham) group were fed a chow diet for 8 weeks. The other rabbits fed 1% cholesterol diet were treated with either saline (0.5 ml/kg/day, vehicle group), SPRC (1, 10 μmol/kg/d) or PAG (10 mg/kg/d) administered daily through subcutaneous injection for 8 weeks, seen in Table 3-4.
Chapter 3

Rabbit health and weight were monitored throughout the study. First, in order to investigate the effects of SPRC through CSE/H₂S pathway, we detected the H₂S levels in plasma, aortic CSE activities, and aortic CSE gene expressions. Then, the lipid files were detected for analyzing the cholesterol-lowering effects of H₂S. H & E staining and HRUS were used to detect the plaques in thoracic and carotid aortas. TEM were shown the vascular ultrastructure of thoracic aorta in different treatment groups. The mechanisms of protective effects of H₂S were investigated on antioxidative and anti-inflammatory effects.

Table 3-4 Grouping for studies of effects of SPRC on hyperlipidemic NZW rabbits

<table>
<thead>
<tr>
<th>Group #</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sham – no drug</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle – high cholesterol diet (HCD)</td>
</tr>
<tr>
<td>3</td>
<td>SPRC (1 μmol/kg/d) + HCD</td>
</tr>
<tr>
<td>4</td>
<td>SPRC (10 μmol/kg/d) + HCD</td>
</tr>
<tr>
<td>5</td>
<td>PAG (10 mg/kg/d) + HCD</td>
</tr>
</tbody>
</table>

Fig.3-4 A flow chart represents the general outline of the experiment IV.
3.5 Experimental techniques

3.5.1 Cytotoxicity assays

The cell viability was determined by the colorimetric MTT assay. Briefly, HUVECs were seeded on 96-well plates in culture medium and maintained in regular growth medium for one day. Cells were pre-treated with different concentrations of NaHS (30, 100, 300 µM) and PAG (10 mM) for 6 hours then exposed to 600 µM H₂O₂ for 4 hours. Following H₂O₂ treatment, 10 µL of MTT (final concentration 0.5 mg/mL) was added to each well and cultures were incubated for 4 h at 37 °C. The medium was then removed and the cells were washed twice with phosphate-buffered saline (PBS). The metabolized MTT was solubilized with dimethylsulfoxide and the absorbance of the solubilized blue formazin dye was read at 530 nm, with 690 nm as reference. The reduction in optical density produced by NaHS treatment was considered to represent the decrease in cell viability. The cells incubated with control medium were considered to be 100% viable. Cell viability% = absorbance of each injured group/absorbance of normal group × 100. The effective concentration of H₂O₂ and NaHS chosen for further experiments was based on these MTT results.

Cell death was determined by measuring LDH activity. At the end of incubation, the supernatant was collected, and the amount of LDH released from cells was determined using LDH assay kit according to the manufacturer’s instructions. The absorbance was measured on a microplate reader at 490 nm. The data in each treatment group is expressed as a percentage of control.

Sulforhodamine B assay is an alternative method to measure total biomass (the number of cells) by staining cellular proteins with the dye, Sulforhodamine B. The result indicates the degree of cytotoxicity caused by the desired treatments. First, HUVECs were cultured in a 96-well plate. After drug treatments, add 25 µl of 50%
TCA in 100 µl medium in each well to fix the cells at 4 °C for 1 hour. The excess TCA was rinsed with distilled water. The plate was air dried, then added 50 µl of 0.4% Sulforhodamine B solution (in 1% acetic acid) to cover the culture surface area for 30 minutes at room temperature. At the end of staining, the plate was washed with fresh 1% acetic acid for 3 times. The incorporated dye was the solubilized in 100 µl Sulforhodamine B solubilization solution (10 mM Tris base solution) for 5 minutes at room temperature. The intense color was measured at 565 nm wavelength and background absorbance was subtracted at 690 nm wavelength. The data in each treatment group is expressed as a percentage of control.

3.5.2 Fluorescent staining of nuclei

HUVECs nuclei were stained with chromatin dye (Hoechst 33258). Briefly, cells were fixed with 3.7% paraformaldehyde for 10 min at room temperature, washed twice with phosphate buffered solution (PBS), and incubated with 10 µM Hoechst 33258 in PBS at room temperature for 30 min. After three washes, cells were observed under a fluorescence microscope (Olympus DP72).

3.5.3 Cell apoptosis assay

HUVECs were cultured in 35 mm² disks. After treatments, cells were collected by trypsinization and centrifugation at 1500rpm for 5 minutes, followed by washing cell pellet twice with cold PBS and resuspending cell pellet in 1* Annexin-binding buffer. Then, cells were added 5 µL Annexin V and 1µL 100 µg/ml PI working solution in 100 µL cell suspension, and incubated at room temperature for 15 minutes in the dark. After the incubation, 400 µL 1* Annexin-binding buffer was added. After mixing gently, the fluorescence intensity was detected with a flow cytometry (CyAn ADP, Beckman Coulter, USA) at emission at 530 nm and 575 nm and excitation at 488 nm. The percentage of cells stained by Annexin V/PI which indicates early apoptosis was
3.5.4 Measurement of H$_2$S concentrations

500 μl medium or plasma was mixed with 250 μl of zinc acetate (1% w/v) in duplicates. Subsequently, NNDPD (20 μM; 133 μl) in 7.2 mol/L HCl was added, followed by FeCl$_3$ (30 μM; 133 μl) in 1.2 mol/L HCl. Thereafter, TCA (10% w/v; 250 μl) was used to precipitate any protein. This final solution was then centrifuged at 24000 g for 5 min at 4°C. The optical absorbance of the resulting solution was measured at 670 nm using a 96-well microplate reader (Tecan Systems Inc., Switzerland). H$_2$S concentration for each sample was calculated against a calibration curve made using NaHS standard (3.125 μM-250 μM). Results are expressed as μM.

3.5.5 Measurement of CSE activity

Briefly, HUVECs were harvested by a cell lysis buffer and aortae were digested by a tissue lysis buffer, then centrifuged at 24000 g for 5 min at 4°C. The supernatant was used for this assay. The reaction mixture contained 20 μl of 10 mM L-cysteine, 20 μl of 2 mM pyridoxal-5-phosphate, 30 μl of saline and 430 μl cell lysis supernatant. The catalytic reaction was initiated by transferring the reaction mixture contained in microtubes from ice to a 37°C water bath for 30 min. Then 250 μl of 1% zinc acetate was added to the tubes using a syringe to trap any evolved H$_2$S. 250 μl of 10% trichloroacetic acid (TCA) was added next to quench the enzymatic reaction. Finally, 133 μl N,N-dimethyl-pphenylenediamine sulphate (NNDPD) in 7.2 M HCl and 133 μl of FeCl$_3$ in 1.2 M HCl were added. The absorbance of the final reaction mixture was measured at 670 nm using a 96-well microplate reader (Tecan Systems Inc., Switzerland). All samples were assayed in duplicates. H$_2$S concentration for each sample was calculated against a calibration curve made using NaHS standard (3.125 μM-250 μM). Results are expressed as μmol/h/g protein. Protein content was
determined using a BCA assay kit (BIO-RAD).

3.5.6 Preparation of HUVECs Mitochondria

Treated and untreated HUVECs were harvested by centrifugation at 1000g for 3 min at room temperature. Mitochondrial and cytosol extractions were carried out using a Mitochondrial Isolation Kit (Pierce Chemical) according to manufacturer’s instructions.

3.5.7 Preparation of intact rabbit aorta mitochondria

Briefly, New Zealand White rabbits weighed 2.8 – 3 kg were anaesthetized and aortas were collected. The aorta was immediately excised and transferred into ice-cold isolation buffer containing 20mM Tris-HCl, 250mM sucrose, 40mM KCl, 2mM EGTA, and 1mg/ml bovine serum albumin, pH7.2. All the isolation procedures were carried out at 4°C. The cortex was homogenized using hand-held glass dounce tissue grinder with 15 tight upward and downward strokes, in isolation buffer. The crude homogenate was centrifuged at 2000 × g for 3 minutes to remove cell debris and nuclei. The supernatant, containing the mitochondrial fraction was further centrifuged at 12000 × g for 11 minutes. Subsequently, the mitochondrial pellet was suspended in 3.5ml of 15% (v/v) Percoll. This suspension was overlain on two preformed percoll layers consisting of 3.5ml 40% and 3.5ml 23% (v/v) Percoll. The percoll gradient was established by centrifugation at 30700 × g for 5 minutes. The distinct creamy layer in between 15% and 23% percoll was collected and resuspended in 500μl isolation buffer. The suspension was again subjected to centrifugation at 12000 × g for 11 minutes. Mitochondrial pellet was washed by resuspending the pellet with isolation buffer (without EGTA) and centrifugation at 12000 × g for 11 minutes. The resulting mitochondrial pellet was suspended in 200μl of isolation buffer to give the protein concentration of 25-35mg/ml as measured by nanodrop (Thermo Scientific).
The mitochondrial suspension was kept on ice before the subsequent experiments.

3.5.8 ATP Synthesis Recording

1 mg/ml of mitochondria were collected for ATP synthesis analysis. The rate of ATP production was measured using a bioluminescence assay kit (Beyotime, China). Briefly, isolated mitochondria were immediately incubated with 2.5 mM ADP, 1 mM pyruvic acid and 1 mM malic acid. The ATP synthesis was kinetically recorded every 30s for 2 minutes. Then, lyciferin substrate and luciferase enzyme were added and bioluminescence was measured by Luminometer (Varioskan Flash Multimode Reader, Thermo). Standardization was performed with known quantities of standard ATP provided in the kit and measured in the same conditions. The rate of ATP synthesis was calculated using a linear regression. Results were expressed in µmol ATP/min/g of mitochondrial protein.

3.5.9 Mitochondrial respiration measurement

Mitochondrial respiration was measured polarographically by monitoring the rate of oxygen consumption in an airtight chamber, equipped with a magnetic stirring device using a Clark type oxygen electrode (Hansatech, UK). Mitochondrial isolation was done for rabbit aortas from each treatment group. Subsequently, 1 mg of mitochondria was added into the 2ml respiratory buffer consisting of 0.5mM EGTA, 3 mM MgCl₂-6H₂O, 60mM K-lactobionate, 20 mM Taurine, 10 mM KH₂PO₄, 20mM HEPES, 110mM Sucrose and 1mg/ml bovine serum albumin, pH7.1 for oxygen consumption measurement. After the addition of mitochondria, the suspension was stirred with magnetic stirrer to ensure the equal distribution of oxygen and contents within the solution. Once the system had reached the equilibrium, state 2 respiration was initiated by the addition of 10mM succinate. Subsequently, 125µM of ADP was added into the chamber for state 3 respiration, followed by state 4
respiration by adding 0.5μg/ml of oligomycin. Oligomycin is a F₀F₁ ATP synthase inhibitor. During the oxygen consumption measurement, oligomycin will return the mitochondrial respiration to the basal rates. Respiratory control ratio (RCR) was then calculated as ratio of mean of state 3 slope over state 4 respiration slopes.

### 3.5.10 Mitochondrial respiratory chain and matrix enzyme activity assays

After incubation with the indicated compounds, mitochondrial suspensions were subjected to three freeze–thaw cycles to disrupt membranes and expose enzymes. All enzyme assays were performed spectrophotometrically at 30°C using a thermostatically regulated UV/visible spectrophotometer (U-2550 spectrophotometer; Shimadzu Corp., Japan). Mitochondrial respiratory chain complex activities were measured by the following methods: complex I (NADH–ubiquinone oxidoreductase) \[259\], complex II/III (succinate–cytochrome c oxidoreductase) \[260\], and complex IV (cytochrome c oxidase) \[261\].

The matrix enzyme citrate synthase was determined using the previously described method \[262\]. α-Ketoglutarate dehydrogenase complex (α-KGDHC) activity was measured as described previously \[263\]. Pyruvate dehydrogenase complex (PDHC) activity was measured using “method 3” of Elnageh and Gaitonde \[264\].

### 3.5.11 Mitochondrial membrane potential - JC-1 staining

Loss of mitochondrial membrane potential (ΔΨₘ) was assessed by fluorescence microscopy using the dye 5,5’,6,6’-tetrachloro-1,1’,3,3’- tetracylbenzimidazole-carbocyanide iodine (JC-1 assay kit, Beyotime, China). After each treatment, HUVECs were stained with JC-1 for 20 min at 37 °C. Cells on 8-chamber slides were
scanned with a fluorescence microscope (Olympus DP72). Fluorescence was analyzed with a Texas red-FITC filter cube. Red emission of the dye represented a potential-dependent aggregation in the mitochondria, reflecting $\Delta \Psi_m$. Green fluorescence represented the monomeric form of JC-1, appearing in the cytosol after mitochondrial membrane depolarization. Cells treated with 10 µM CCCP were used as positive control. CCCP is a protonophore which can cause dissipation of $\Delta \Psi_m$.

### 3.5.12 $\Delta \Psi_m$ measurement

JC-1 was used to measure $\Delta \Psi_m$ of HUVECs and aortic mitochondria. Total cells or mitochondria were collected into 2 ml tubes and incubated with JC-1 for 20 min at 37 °C. The fluorescence intensity of cells was detected with a flow cytometry (BD FACS Aria I cell sorter, Becton Dickinson Company). The fluorescence intensity of aorta mitochondria was detected with a fluorescent microplate reader (Molecular Devices, Gemini XS, USA). The wavelengths of excitation and emission were 514 nm and 529 nm for detection of monomeric form of JC-1. 585 nm and 590 nm were used to detect aggregation of JC-1. The ratio of aggregated JC-1 and monomeric JC-1 represented $\Delta \Psi_m$ of HUVECs or aortic mitochondria.

### 3.5.13 Measurement of ROS

The fluorescent probe, H$_2$DCFDA, was used to measure the intracellular generation of ROS by H$_2$O$_2$. Briefly, confluent HUVECs in 96-well plates were pretreated with 300 µM NaHS or 10 mM PAG for 6 hours and then stimulated with 600 µM H$_2$O$_2$ for 4 h. The reactions were stopped by removing medium, and washing with PBS followed by staining with 10 µM H$_2$DCFDA for 20 min at 37 °C. DHE was also used to detect ROS production. After drug treatments, cells were incubated in 5 µM DHE for 30 min at 37 °C. The fluorescence intensities of H$_2$DCFDA and DHE were kinetically measured at an excitation and emission wavelength of 485 nm and 530 nm for
H$_2$DCFDA, and 520nm and 610nm for DHE, respectively, using a fluorescent microplate reader (Molecular Devices, Gemini XS, USA). The same methods were used to test the ROS production of aorta mitochondria.

### 3.5.14 Lipid peroxidation assays

For *in vitro* study, HUVECs were cultured in 35 mm$^2$ disks. A cell lysis buffer (RIPA buffer) was used to collect cell samples. For *in vivo* study, a tissue lysis buffer (RIPA buffer) was used to lyse liver samples. The tissue samples were then homogenized by Polytron homogenizer (Janke and Kunkel, Germany) followed by centrifugation at 3000rpm/minute for 15 min, supernatant was collected for malondialdehyde (MDA) measurement. MDA levels were determined by measuring the thiobarbituric acid-reactive substances using a commercial kit (Beyotime, China) according to the manufacturer's introduction. The kit is based on the MDA in the samples that forms 1:2 adduct with thiobarbituric acid (TBA). The MDA-TBA adduct formed from the reaction of MDA in samples with TBA can be measured spectrophotometrically at 532nm. MDA values are expressed as nmol/mg protein.

Lipid peroxidation was further estimated using a fluorescent probe, DPPP. After treatments, cells were incubated with 100 μM DPPP for 60 min in the dark. The fluorescent intensities of DPPP fluorescence were analyzed with a fluorescent microplate reader (Molecular Devices, Gemini XS, USA) at an excitation of 351 nm and an emission of 380 nm.

### 3.5.15 Cytochrome c Release Assay

Cells were harvested by centrifugation at 1000g for 3 min at room temperature. Mitochondrial and cytosol extractions were separated using the Mitochondrial Isolation Kit (Pierce Chemical) according to manufacturer’s instructions. The
presence of cytochrome c was detected from mitochondrial and cytosol extractions by immunoblot analysis using anti-cytochrome c antibody (1:1000, Cell Signaling).

3.5.16 Transmission Electron Microscopy (TEM)

For \textit{in vitro} study, HUVECs were harvested then prefixed in 2.5\% glutaraldehyde solution overnight. Postfixation was in cold 1\% aqueous osmium tetroxide for 1 h. After rinsing with PBS, the samples were dehydrated in a graded ethanol series of 25 to 100\% and then embedded in fresh resin and polymerized at 60 °C for 24 h. Ultra-thin sections were sliced with glass knives on a LKB-V ultramicrotome (Leica), stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope CM120 Bio TWIN (Philips).

For \textit{in vivo} study, thoracic aortae sections were fixed using 2.5\% glutaraldehyde in 0.01 M phosphate buffer at a pH 7.38. Subsequently, vessel sections were fixed in 2\% OsO$_4$. Then after a process of dehydration, infiltration, embedment, and polymerization, the vessel tissues were cut into 50 nm sections with a vibratome and prepared for examination under TEM (CM120, Philips, Holland), following standard procedures. Ultrastructure of smooth muscle cell was observed.

3.5.17 Antioxidant enzyme activities assay

For \textit{in vitro} study, HUVECs were cultured in 100 mm$^2$ disks. Cells were harvested through centrifuging (1,500 g for 10 minutes at 4 °C). Then, cells were homogenized in cold buffer and centrifuged at 10,000 g for 15 min at 4 °C. The supernatants were used for all the assays. For \textit{in vivo} study, livers were homogenized in cold buffer and centrifuged at 10,000 g for 15 min at 4 °C. The supernatants were used for all the assays. The antioxidants assays were performed using commercially available kits.
3.5.18 High resolution ultrasonographic (HRUS) imaging

At the end of week 8, HRUS imaging was performed as described before. Rabbits under light anesthesia with intravenous sodium pentobarbital (30mg/kg) were examined by using a high resolution ultrasound system (Vevo 770, Visualsonics, Canada) equipped with a 30MHz mechanical transducer. The transverse section was first viewed on the B-mode to visualize the left common carotid arterial site, and after a clockwise 90° rotation, the longitudinal view of the artery at the site 0.5-1 mm proximal to the artery bifurcation was employed to depict the plaque length and thickness. After the transverse and longitudinal axis imagings of carotids were obtained, the maximal intima-media thickness (IMT) of the plaque, aortic end-diastole diameter (Dd) and end-systolic diameter (Ds) were measured from the imagings. Blood flow velocity was recorded by pulsed Doppler technique. The maximal systolic velocity of blood flow in the lumen of artery proximal to lesion is recorded to be carotid peak flow velocity (Vp) \[265\]. Relative-sectional change (RS) of the left common carotid arteries was calculated as: \[ RS = (Dd^2 - Ds^2)/Dd^2 \]. All the measurements were repeated twice at the same site.

3.5.19 H&E staining

Sections from the thoracic aortae were fixed in 10% buffered formalin (Sigma, USA) for 1 week. 4 μm-thick tissue sections were stained with H&E for normal histological assessment, with the cytoplasm stained pink, and the nuclei deep purple. Hematoxylin, Eosin and Orange G stains were obtained from Merck, Germany while Light Green SF Yellowish and Pararosaniline hydrochloride were obtained from Sigma, USA.

3.5.20 Measurement of serum lipid levels

After overnight fasting, blood was drawn from the marginal ear vein at baseline and
the end of 8 weeks. Blood samples were put on ice and centrifuged (3000 rpm, 15 min, 4 °C) to obtain serum. Serum TC and LDL levels were measured with an automatic analyzer (Au5600 OLYMPUS).

3.5.21 Oxidized LDL (ox-LDL) in serum

The level of ox-LDL in serum was measured using commercially available kits for rabbits (R&D systems), following the manufacturer’s instructions. Final results were expressed as μg/L.

3.5.22 Inflammatory cytokines in serum

The level of soluble vascular adhesion molecular-1 (sVCAM-1), soluble intercellular adhesion molecular-1 (sICAM-1), MCP-1, IL-6 and TNF-α in the serums were measured using commercially available kits for rabbits (R&D systems), following the manufacturer’s instructions. Final results were expressed as nanograms cytokine per milliliter serum.

3.5.23 Immunoblotting

Cultured HUVECs were harvested by scraping and centrifugation. The pellets of cells or aortas were washed twice with ice-cold PBS, and re-suspended in RIPA buffer. Soluble proteins were collected by centrifugation at 15,000 g for 15 min. Protein lysates were subjected to 10-12% SDS-PAGE and transferred onto a PVDF membrane (Millipore Corporation). After blocking with 5% skim milk, the membranes were incubated with the respective primary antibodies (SOD-1 1:800, SOD-2 1:400, catalase 1:500, GPx 1: 500, GST 1:500, cytochrome c 1:1000, CTH 1: 1000, Santa Cruz; CBS 1:800 Abcam) in PBS 0.1% Tween-20 overnight at 4 °C. The
membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated IgG antibodies at a 1:10,000 dilution for 1 h at room temperature (Santa Cruz). Immunoreactive proteins were then visualized enhanced chemoluminescence (Pierce). The signals were quantified by densitometry using a Kodak Image Station 4000R (Kodak). β-actin served as the loading control. Protein content was determined using a BCA assay kit (BIO-RAD).

### 3.5.24 Real-time Polymerase Chain Reaction (R-T PCR)

Total RNA from HUVECs or aorta was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Real-time PCR was performed in triplicate on a Corbett RG6000 5plex with HRM sequence detector, using 100ng RNA, 0.1 μl PCR master Mix (QuantiTect, QIAGEN), 5 μl SYBR (QuantiTect, QIAGEN), and 5 μM each of forward and reverse primers, in a final volume of 10 μl. Samples were incubated at 50 °C for 30 min, then at 95 °C for 15 min; denaturation was performed for 45 cycles at 94 °C for 15 s, and followed by annealing and extension at 62 °C for 30s and 72 °C for 30s. Amplifications were normalized by β-actin. The amount of the target gene, normalized to β-actin, is given as $2^{-\Delta\Delta CT}$. Results were analyzed using the Rotor-Gene series software (1.7). Expressions of CSE and CBS SOD, CAT, GST, GPx, CSE and HO-1 mRNA were determined.

The primers used for real-time PCR in experiment I are shown in Table 3-5 and The primers used for real-time PCR in experiment III and IV are shown in Table 3-6.
Table 3- 5 The primers used for real-time PCR in experiment I.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSE</td>
<td>5'-ccatctctattgatttgctctct-3'</td>
<td>5'-cactgacgcttaccaaactc-3'</td>
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<tr>
<td>CBS</td>
<td>5'-tcaagagcaacgatgagggg-3'</td>
<td>5'-atgtcgctccgactgagtc-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-gagagggaaatgtagtgac-3'</td>
<td>5'-ctgcttaagttgacgtag-3'</td>
</tr>
</tbody>
</table>

Table 3- 6 The primers used for real-time PCR in experiment III and IV.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
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<td>5'-gtctctctccaatatgtcaac-3'</td>
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<tr>
<td>SOD-1</td>
<td>5'-gacctggggaaagggtggaatg-3'</td>
<td>5'-caaccagcagctactagtagag-3'</td>
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<tr>
<td>GPx</td>
<td>5'-tctacgtctctcagcgcaccg-3'</td>
<td>5'-tccagagcagcgcaccaattcaat-3</td>
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<tr>
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<td>5'-aagggaaatggegaaggt-3'</td>
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<tr>
<td>VCAM-1</td>
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<td>5'-tatgagccagctctctct-3'</td>
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<td>5'-tgccgtttggtggtctgtg-3'</td>
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<tr>
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<td>5'-gcacagaaacaggtgaatg-3'</td>
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<tr>
<td>β-actin</td>
<td>5'-agtgccgtgagacatcc-3'</td>
<td>5'-tggcttaacagtcgctag-3'</td>
</tr>
</tbody>
</table>
3.5.25 Statistical analysis

Statistical analyses were performed using SPSS 13.0 for Windows software (SPSS Inc., USA), with normality of data tested by Komogorov-Smirnov tests. Since the sample sizes of the groups were unequal, comparisons of normally distributed variables between control, SIVD and MIX were performed by one-way analyses of variance (ANOVA) followed by post-hoc Tamhane’s T2 tests. Pearson’s product moment was used in correlations of neurochemical variables with dementia severity (defined as mean CAMCOG and MMSE scores up to one year pre-death). Appropriate non-parametric tests were used for ordinal or non-normally distributed variables. Results were considered statistically significant if p< 0.05. All values are represented as means ± SD.
Chapter 4

Results
4.1 Results of Experiment I: Hydrogen sulfide protects HUVECs against hydrogen peroxide induced mitochondrial dysfunction and oxidative stress

4.1.1 NaHS is non-toxic to HUVECs

Sodium hydrosulfide (NaHS) at concentrations of 10, 30, 100, 300 and 500 μM with incubation of 12, 24, 48 hours was found to be non-toxic to endothelial cells, as determined by the MTT viability assay (Fig. 4.1-1, n=9).
Fig. 4.1-1 The cell viability of HUVECs subjected to different concentrations of NaHS. (A)-(C) MTT assay. (A) HUVECs were treated with 10-500 μM NaHS for 12 hours. (B) HUVECs were treated with 10-500 μM NaHS for 24 hours. (C) HUVECs were treated with 10-500 μM NaHS for 48 hours. Cell viability in each treatment group is expressed as a percentage of control. The data shown are mean ± SEM (n=9).

4.1.2 Protective effects of exogenous H₂S on H₂O₂ induced cell death

The dose response of H₂O₂ treatments on HUVECs cell viability is shown in Fig. 4.1-2 (n=9). After incubating with H₂O₂ for 4 hours, cell viabilities were significantly decreased in 0.6-1.5 mM H₂O₂ treatment groups (P< 0.01). The less toxic concentration, 0.6 mM of H₂O₂ was chosen for later studies.
Fig. 4.1-2. The Cell viability of HUVECs subjected to different concentrations of H₂O₂. HUVECs were treated with 0.2-1.5 mM H₂O₂ for 4 hours. Cell viability in each treatment group is expressed as a percentage of control. The data shown are mean ± SEM (n=9). ** p<0.01 vs control.

Another MTT assay (Fig. 4.1-3 A) showed the cellular protective effect of NaHS on HUVECs damaged by H₂O₂. The cell viability fell to 57.71 ± 2.96% when exposed to H₂O₂ (600 µM) for 4 h (P< 0.01, vs. control). A dose response was observed in cell viabilities 62.38 ± 2.12%, 78.74 ± 4.23%, 89.26 ± 3.45% and 82.41 ± 1.78% for 30, 100, 300 and 500 µM NaHS, respectively. The differences between the H₂O₂ group and NaHS (100, 300 and 500 µM) groups were statistically significant (P< 0.01). The MTT results were further supported by lactate dehydrogenase (LDH) release assay (Fig. 4.1-3 B). Compared with control, vehicle + H₂O₂ induced 252.23 ± 1.79% LDH release, while NaHS pretreatment significantly decreased LDH release to 180.63 ± 3.13% and PAG increased to 297.26 ± 5.28% Pretreatment with different concentrations of NaHS could reverse H₂O₂-induced cell death dramatically, showing the ability of H₂S to reduce H₂O₂ cytotoxicity. Sulforhodomine B assay showed the similar results of LDH data (Fig. 4.1-3 C). The higher absorbance of Sulforhodomine B solubilization solution means higher total biomass (equal to cell viability). Vehicle
+ H₂O₂ showed lower total biomass than control group, while NaHS had the denser total biomass and PAG showed much lower total biomass than Vehicle + H₂O₂ (P< 0.01).

Fig. 4.1- 3. Cell viability and death assay of HUVECs subjected to different concentrations of NaHS with or without H₂O₂. (A) MTT assay showed cell viability of HUVECs pretreated with vehicle or 30-500 μM NaHS for 6 hours, followed by exposure to 600 μM H₂O₂ for another 4 hours. (B) LDH release assay showed cell death of HUVECs pretreated with vehicle or 300 μM NaHS and 10mM PAG for 6 hours, followed by exposure to 600 μM H₂O₂ for another 4 hours. (C) Sulforhodamine B assay showed total biomass (the number of cells) of HUVECs pretreated with vehicle or 300 μM NaHS and 10mM PAG for 6 hours, followed by exposure to 600 μM H₂O₂ for another 4 hours. Cell viability or LDH release in each treatment group is expressed as a percentage of control. The data shown are mean ± SEM (n=9). ** p<0.01 vs control. ## p<0.01 vs vehicle + H₂O₂.
Similar results were obtained using Hoechst staining (Fig. 4.1-4), in which apoptotic nuclei were brighter. There were fewer apoptotic cells in the NaHS pre-treated groups than in the H_2O_2 group, while much more apoptotic cells in the PAG pre-treated groups were observed than in H_2O_2 group.

**Fig. 4.1- 4 The cell viability of HUVECs by Hoechst staining.** HUVECs were pretreated with vehicle, 300 μM NaHS or 10mM PAG for 6 hours, followed by exposure to 600 μM H_2O_2 for another 4 hours. Cells were observed under ×200 microscopy. Scale bar is shown at 100μm.
The cell viability was also evaluated by propidium iodide (PI) staining, which is a DNA dye to detect the nucleus and other DNA containing organelles. Under microscopy, we found the \( \text{H}_2\text{O}_2 \) group had more PI stained cells than that of control, while NaHS pretreated cells showed less bright and PAG increased the PI staining light, seen in Fig. 4.1-5.

![Fig. 4.1-5 The cell viability of HUVECs by PI staining. HUVECs were pretreated with vehicle, 300 \( \mu \text{M} \) NaHS or 10mM PAG for 6 hours, followed by exposure to 600 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for another 4 hours. Cells were observed under \( \times200 \) microscopy. Scale bar is shown at 50 \( \mu \text{m} \).](image-url)
The protective effects of H₂S on cells in the stage of early apoptosis induced by H₂O₂ were showed in Fig. 4.1-6. The percentage of cells stained by Annexin V/PI which indicated early apoptosis, was 11.5 ± 0.53% in control, and significantly increased to 21.33 ± 0.89% in vehicle + H₂O₂, decreased to 13.59 ± 1.77% in NaHS and highly rocketed to 27.81 ± 3% in PAG (P< 0.01). H₂S can protect endothelial cells against H₂O₂-induced apoptosis.

Fig. 4.1- 6 The percentage of early apoptotic cells stained by Annexin V/PI by flow cytometry. HUVECs were pretreated with vehicle or 300 μM NaHS and 10mM PAG for 6 hours, followed by exposure to 600 μM H₂O₂ for another 4 hours. The data shown are mean ± SEM (n=3). ** p<0.01 vs control. ## p<0.01 vs vehicle + H₂O₂.
4.1.3 CSE protein and gene expression, CSE activity and H$_2$S concentration after H$_2$O$_2$-induced injury

H$_2$O$_2$ treatment was found to decrease of H$_2$S concentration in the medium, Fig. 4.1-7 (n=6) ($P$< 0.05, vs. control). In contrast, the H$_2$S donor - NaHS elevated H$_2$S concentrations in the medium ($P$< 0.05, vs. vehicle + H$_2$O$_2$). These effects were significantly reduced by PAG treatment ($P$< 0.05, vs. control).

![Graph showing H$_2$S concentration (μM) in medium for each treatment group.](image)

Fig. 4.1- 7 The H$_2$S concentration (μM) in medium for each treatment group. The data shown are mean ± SEM (n=6). * $p$<0.05 vs control. # $p$<0.05 vs vehicle + H$_2$O$_2$ group.

Similarly, cellular CSE activities were analyzed in the cell lysates from all treatment groups, as shown in Fig. 4.1-8 (n=6). CSE activity in the control group was 28.73 ± 0.69 (μmol/h/g) with this been decreased to 15.02 ± 0.91 (μmol/h/g) in the H$_2$O$_2$ group ($P$< 0.05, vs. control). NaHS was found to preserve CSE activity in cells when exposed to H$_2$O$_2$ (21.07 ± 0.52 μmol/h/g; $P$< 0.05, vs. vehicle + H$_2$O$_2$), while PAG reduced CSE activity levels to 13.02± 0.97 (μmol/h/g) ($P$< 0.01, vs. control).
Fig. 4.1-8 CSE activities (μmol/h/g) in HUVECs lysate of each group. The data shown are mean ± SEM (n=6). * p<0.05, ** p<0.01 vs control. # p<0.05 vs vehicle + H₂O₂ group.

Using western blot and PCR analysis, we also determined the relative protein and mRNA levels of CSE in HUVECs (Fig 4.1-9). In the H₂O₂ treatment groups CSE protein and mRNA levels were reduced while in the NaHS pretreatment groups CSE protein and mRNA levels were increased and in the PAG pretreatment groups CSE protein levels were decreased and CSE mRNA levels were increased (P< 0.05). Interestingly, CBS protein and mRNA levels, an additional H₂S synthesizing enzyme, remained unchanged in all treatments groups as shown in Fig. 4.1-9. Taken together, these results indicate that the cardioprotective effects by H₂S might be though the CSE/ H₂S pathways.
Fig. 4.1- 9 Effects of NaHS on H2S synthesizing enzyme protein and gene expressions. (A) CSE protein expressions levels as determined using western blot analysis. (B) CSE mRNA expression levels as determined by real-time PCR. (C) CBS protein expressions levels and (D) The CBS mRNA expression tested by real-time PCR. The values in (A)-(D) were normalized against the control values. The data shown are mean ± SEM (n=6). * p<0.05, ** p<0.01 vs control. # p<0.05 vs vehicle + H2O2 group.

4.1.4 Effects of exogenous H2S on mitochondrial ATP synthesis

In aerobic eukaryote cells the major site of adenosine triphosphate (ATP) production occurs in mitochondria. As shown in Fig. 4.1-10 (n=6) cellular ATP levels responded
to H₂O₂, the H₂S donor (NaHS) and inhibitor (PAG). The ATP content of the control cells was 144.66 ± 21.13 (µmol/min/g). After incubation with H₂O₂, the rate of ATP production by mitochondria was greatly decreased to 54.16 ± 2.79 (µmol/min/g) ($P < 0.01$, vs. control). Meanwhile, ATP production in the pretreated NaHS group significantly increased to 102.87 ± 22.34 (µmol/min/g) ($P < 0.01$, vs. vehicle + H₂O₂). A significantly decreased to 25.47 ± 8.90 (µmol/min/g) in the pretreated PAG group was also noted ($P < 0.01$, vs. control). These results indicated that H₂S could attenuate H₂O₂ induced inhibition of ATP synthesis.

**Fig. 4.1- 10 Effect of NaHS on ATP synthesis.** After pretreatment with vehicle, 300 µM NaHS or 10mM PAG for 6 hours and followed by 600 µM H₂O₂ for another 4 hours, HUVECs were harvested to collect mitochondria. The rate of ATP synthesis was expressed by µmol ATP/min/g of mitochondrial protein.
4.1.5 Effects of exogenous H$_2$S on mitochondrial membrane permeability

JC-1 aggregates in healthy mitochondria and has a red fluorescence (Fig. 4.1-11 A). Exposure of HUVECs to H$_2$O$_2$ resulted in an increase in green fluorescence, indicating a loss in mitochondrial membrane potential ($\Delta \Psi_m$) (Fig. 4.1-11 B). Pretreatment of NaHS reduced the effects of H$_2$O$_2$ on mitochondrial membrane potential, indicating a protective effect of NaHS (Fig. 4.1-11 C). Pretreatment with PAG also resulted in the dissipation of mitochondrial membrane potential (Fig. 4.1-11 D). Carbonyl cyanide m-chlorophenylhydrazone (CCCP), the positive control of the JC-1 staining in mitochondria, promoted mitochondrial inner membrane permeable leading to the dissipation of the proton gradient across the inner mitochondrial membrane (Fig. 4.1-11 E). The ratio of red and green fluorescence was also used to demonstrate the toxicity of H$_2$O$_2$ treatment to mitochondria and the protective effect of NaHS (Fig. 4.1-11 F). Moreover, the ratio of aggregated JC-1 and monomeric JC-1 were measured by flow cytometry. In control cells, JC-1 aggregated in mitochondria with the strong PI intensity and light FITC intensity, presenting to the ratio of PI/FITC. In contrast, H$_2$O$_2$-treated cells was observed a lower ratio ($P<0.05$, vs. control), because the monomeric form of JC-1 appeared in the cytosol indicating the dissipation of $\Delta \Psi_m$. Cells pre-treated with NaHS attenuated the dissipation of $\Delta \Psi_m$ ($P<0.05$, vs. vehicle + H$_2$O$_2$), while PAG further increased the loss of membrane potential ($P<0.05$, vs. control).
Fig. 4.1-11 Effects of NaHS on mitochondrial membrane potential ($\Delta \Psi_m$). (A)-(E) JC-1 staining. Red fluorescence represents the mitochondrial aggregate form of JC-1, indicating intact $\Delta \Psi_m$. Green fluorescence represents the monomeric form of JC-1, indicating dissipation of $\Delta \Psi_m$. (E) CCCP was the positive control. Cells were observed under ×200 microscopy. Scale bar is shown at 100μm. (F) Ratio of red to green fluorescence, indicating ratio of JC-1 polymer/monomer. The data shown are mean ± SEM (n=6). * $p<0.05$, vs control. # $p<0.05$, vs vehicle + H$_2$O$_2$ group.
One possible mechanism by which oxidative stress may trigger cellular toxicity in HUVECs is the induction of the mitochondrial apoptotic pathway that is triggered through the release of cytochrome c into the cytosol. To verify this possibility, the protective effect of NaHS on H₂O₂-induced toxicity was measured by determining the release of cytochrome c from mitochondria using Western-Blot analysis (Fig. 4.1-12) (n=6). In the control group, the presence of relatively low levels of cytochrome c was released from the mitochondria to cytosol. After incubation with H₂O₂, the cytochrome c levels were significantly increased in the cytosol and decreased in mitochondria (P< 0.01, vs. control). Pretreatment with NaHS inhibited the release of cytochrome c (P< 0.01, vs. vehicle + H₂O₂), while pretreatment of PAG potentiated the release of cytochrome c to the cytosol (P< 0.01, vs. control).

**Fig. 4.1- 12 Effects of NaHS on release of cytochrome c from mitochondria.** After treatments as previous description, HUVECs were harvested to collect mitochondria and cytosol. The protein expression was tested by western blot. The bar chart showed the ratio of cytochrome c in cytosol to that in mitochondria, indicating the intensity of release of cytochrome c. The data shown are mean ± SEM (n=6). **p<0.01 vs control. ##p<0.01 vs vehicle + H₂O₂ group.
4.1.6 Endothelial cell ultrastructure observation

Fig. 4.1-13 shows ultrastructural changes in HUVECs exposed to H₂O₂. In control cells, the ultrastructure was normal with intact nuclei and healthy looking mitochondria, endoplasmic reticulum and lysosomes (Fig. 4.1-13 A, E). In contrast, H₂O₂-treated HUVECs displayed condensed chromatin, an irregular nuclear outline, dilated or fragmented endoplasmic reticulum, swollen, ruptured or engulfed mitochondria, and darkened and clumping lysosomes. In addition the cytoplasm had significant vacuolization and protrusions (Fig. 4.1-13 B, F). In the NaHS pretreated HUVECs, the cellular ultrastructure appeared similar to that of the control endothelial cells although some mitochondria had expanded cristas, and darkened lysosomes, vacuoles and engulfed organelles (Fig. 4.1-13 C, G). In the PAG pretreated cells a more severe ultrastructural change was observed including nuclear chromatin condensation, multiple cytoplasmic protrusions or blebs, and ruptured or fragmented organelles (Fig. 4.1-13 D, H). Essentially, the transmission electron microscopic observations indicated that exogenous H₂S could preserve cellular ultrastructural changes induced by H₂O₂ damage.
Fig. 4.1-13 Ultrastructural changes in HUVECs induced by H2O2 using transmission electron microscopy. (A)-(D) showed HUVECs with legible nucleus. Scale bar is shown at 1 μm. (E)-(H) showed mitochondria. Scale bar is shown at 0.2 μm. (A) and (E) cell and mitochondria in the control group; (B) and (F) cell and mitochondria in vehicle + H2O2 group; (C) and (G) cell and mitochondria in NaHS + H2O2 group; (D) and (H) cell and mitochondria in PAG + H2O2 group.

† normal mitochondria; ▲ abnormal mitochondria; △ apoptotic blebbing; ▲ bubbles; ‡ condensed lysosomes.
4.1.7 Effects of exogenous H\textsubscript{2}S on MDA formation and ROS production

MDA is a product of lipid peroxidation. The data for the levels of cellular MDA are shown in Fig. 4.1-14 A (n=6). MDA levels were low in the normal control cells. Treatment with H\textsubscript{2}O\textsubscript{2} significantly increased cellular MDA levels indicating the elevation of oxidative stress ($P<0.01$, vs. control). Pretreatment with NaHS reduced the formation of MDA induced by H\textsubscript{2}O\textsubscript{2} ($P<0.01$, vs. vehicle + H\textsubscript{2}O\textsubscript{2}), while pretreated PAG reversed the inhibition caused by NaHS ($P<0.01$, vs. control). The study of DPPP staining was in line with MDA result, which showed the increase in fluorescent intensity of DPPP induced by H\textsubscript{2}O\textsubscript{2} was reduced by NaHS and enhanced by PAG ($P<0.01$) (Fig. 4.1-14 B).

![Fig. 4.1-14 Effects of NaHS on lipid peroxidation. HUVECs were pretreated with vehicle, 300 \textmu M NaHS or 10mM PAG for 6 hours, then 600 \textmu M H\textsubscript{2}O\textsubscript{2} for 4 hours. (A) MDA changes in HUVECs mediated by H\textsubscript{2}O\textsubscript{2}. The data are expressed at nmol/mg. (B) Fluorescent intensity of DPPP in HUVECs mediated by H\textsubscript{2}O\textsubscript{2}. The data shown are mean ± SEM (n=6). ** $p<0.01$ vs control. ## $p<0.01$ vs vehicle + H\textsubscript{2}O\textsubscript{2} group.](image-url)
Redox status was observed in H$_2$DCFDA study (**Fig. 4.1-15** A) (n=6). Administration of H$_2$O$_2$ induced an increase in the fluorescence intensity of H$_2$DCFDA, as compared to the control group (P< 0.05). Pre-incubation with NaHS inhibited the levels of ROS induced by H$_2$O$_2$ (P< 0.05). However, pretreatment with PAG intensified the fluorescence intensity of H$_2$DCFDA induced by H$_2$O$_2$ (P< 0.05). DHE is another dye for ROS detection, especially superoxide. Similar results were found that cells stimulated by H$_2$O$_2$ expressed higher fluorescent intensity of DHE than that of control, which were suppressed by NaHS and strengthened by PAG (P< 0.05) (n=6) (**Fig. 4.1-15** B). Our results suggest that exogenous H$_2$S can suppress the productions of toxic free radicals.

**Fig. 4.1-15 Effects of NaHS on ROS production.** (A) ROS production was stained by 10 μM H$_2$DCFDA for 20 min, whose oxidation product (DCF) fluorescence indicated ROS formation. (B) ROS production was stained by 5 μM DHE for 30 min, which fluorescence indicated ROS formation. The absorbance values in (D)-(F) of HUVECs were normalized against the values for normal controls and expressed as a percentage of control. The data shown are mean ± SEM (n=6). * p<0.05, vs control. # p<0.05, vs vehicle + H$_2$O$_2$ group.
4.1.8 Effects of exogenous H₂S on antioxidants activities and antioxidants enzyme protein expressions

The activities of tissue antioxidant enzymes are shown in Table 4.1-1. When cells were stimulated with H₂O₂ a significant decreases in the activities of superoxide dismutase (SOD), catalase, glutathione Peroxidase (GPx) and glutathione S-transferase (GST) were observed ($P<0.05$, vs. control, n=6). However, pretreatment with NaHS significantly elevated the activities of SOD, catalase, GST and GPx ($P<0.05$, vs. vehicle + H₂O₂), while PAG strongly reduced the activities of these antioxidative enzymes ($P<0.01$, vs. control). In our study, exogenous H₂S was found to increase the activities of antioxidant enzymes as compared with H₂O₂-stimulated group.

Table 4.1-1 Antioxidant enzyme activities in each study groups

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<tr>
<th></th>
<th>SOD (U/g)</th>
<th>Catalase (nmol/min/g)</th>
<th>GPx (nmol/min/g)</th>
<th>GST (nmol/min/g)</th>
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<tr>
<td>Control</td>
<td>4.32±0.84</td>
<td>12.39±1.75</td>
<td>68.85±5.63</td>
<td>45.66±4.8</td>
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<tr>
<td>H₂O₂</td>
<td>1.24±0.14**</td>
<td>7.35±1.08**</td>
<td>48.11±6.21**</td>
<td>27.41±4.0**</td>
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<td>NaHS</td>
<td>3.98±0.53##</td>
<td>9.42±1.38#</td>
<td>55.39±13.67#</td>
<td>32.48±3.35##</td>
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<tr>
<td>PAG</td>
<td>0.82±0.11**</td>
<td>6.09±1.34**</td>
<td>42.85±6.23**</td>
<td>19.41±1.62**</td>
</tr>
</tbody>
</table>

The data shown are mean ± SEM (n=6). ** $p<0.01$ vs control. # $p<0.05$, ## $p<0.01$ vs vehicle + H₂O₂ group.

To support these findings, we examined the protein expression levels of SOD, catalase, GPx and GST in HUVECs, as shown in Fig. 4.1-16 (n=6). Protein expression levels were all decreased in the H₂O₂ and PAG groups as compared to the control group ($P<0.05$). In the NaHS group higher protein expressions levels of
antioxidant enzymes was observed as compared to the H₂O₂ group (P< 0.05). These results are in line with the corresponding enzymes activities in the above experiments. Combined these data indicates that H₂S can enhance the antioxidative systems in cells under H₂O₂-stimulated stress and thus preserve cellular redox balance. These data correlating with the preservation of mitochondrial integrity, reduced oxidative stress levels and the maintenance of cell viability by H₂S.

Fig. 4.1- 16 Effects of NaHS on protein expressions of antioxidant enzymes. (A) Western-blot analysis showing the intensities of Catalase, SOD-1, SOD-2, GST and GPx in each group, (B)-(F) bar charts indicating the different intensities of antioxidant proteins between groups. Values were normalized against the control values. The data shown are mean ± SEM (n=6). * p<0.05, ** p<0.01 vs control. # p<0.05, ## p<0.01 vs vehicle + H₂O₂ group.

4.1.9 Protective effects of exogenous H₂S against H₂O₂-induced injury though the inhibition of program cell death pathway and elevation of Akt pathway

Expression levels of apoptosis-related proteins were shown in Fig. 4.1-17 (n=6). Bcl-2 and Bcl-X₁ are known to promote cell survival as well as to suppress cell death by various apoptotic stimuli, while Bax is a pro-apoptotic protein. Caspase-3, a key protein in apoptosis, is activated by cleaved. In our study, H₂O₂-induced cytotoxicity
accompanying with decreased the expression of Bcl-2 and Bcl-XL, increased the Bax and cleaved caspase-3 expressions \((P<0.05, \text{ vs. control})\). Pretreatment with NaHS prevented the decrease in the expression of Bcl-2 protein and Bcl-XL protein whereas it suppressed the increase of Bax expression and cleaved caspase-3 \((P<0.05, \text{ vs. vehicle } + \text{H}_2\text{O}_2).\) The PAG showed the reverse effects of NaHS in Bcl-2, Bcl-XL, Bax and cleaved caspase-3 protein expressions \((P<0.05, \text{ vs. control})\).

It is also determined the relative expression levels of the serine/threonine kinase Akt, shown in **Fig. 4.1-17** \((n=6)\). NaHS increased the level of phosphor-Akt/total Akt as compared with \text{H}_2\text{O}_2 \((P<0.05, \text{ vs. vehicle } + \text{H}_2\text{O}_2).\) However, PAG inhibited the increase in the level of phosphor-Akt/ total Akt induced by NaHS \((P<0.05, \text{ vs. control}).\) These results strongly suggest that suppressing mitochondrial program cell death by \text{H}_2\text{S} may be responsible for the cellular and mitochondrial protection against the oxidative stress.

![Fig. 4.1-17](image)

**Fig. 4.1- 17 Effects of NaHS on protein expressions of proapoptotic and antiapoptotic proteins.** (A) Western blot analysis showed intensities of Bcl-2, Bcl-XL, Bax, Cleaved Caspase 3, p-Akt and Akt in each group. (B)-(F) the bar charts showed the different intensities of apoptosis-related proteins between groups. The values were normalized against the control values. The data shown are mean ± SEM \((n=6)\). * \(p<0.05, ** p<0.01\) vs control. # \(p<0.05, ## p<0.01\) vs vehicle + \text{H}_2\text{O}_2 group.
4.2 Results of experiment II: Hydrogen sulfide protects isolated rabbit aorta mitochondria against hydrogen peroxide

4.2.1 Effects of H$_2$S on mitochondrial ROS production in isolated rabbits aorta

ROS is generated from mitochondrial respiration chain. Dramatic and overwhelm ROS production will lead to oxidative stress. In our study, redox status was first observed by a ROS dye, H$_2$DCFDA (Fig. 4.2-1 A) (n=6). Administration of H$_2$O$_2$ 1mM for 30min induced an increase in the fluorescence intensity of H$_2$DCFDA, as compared to the control group ($P<0.01$). Pre-incubation with NaHS (10, 100 and 300 μM) for 1 hour inhibited the levels of ROS induced by H$_2$O$_2$ ($P<0.05$, vs. control). However, pretreatment with PAG (10 mM) for 1 hour intensified the fluorescence intensity of H$_2$DCFDA induced by H$_2$O$_2$ ($p<0.01$, vs. vehicle + H$_2$O$_2$). Redox status was further evaluated by another ROS dye DHE, which can detect free radicals especially superoxide. Similar results were found that cells stimulated by H$_2$O$_2$ expressed higher fluorescent intensity of DHE than that of control ($P<0.05$, vs. control). The fluorescent intensity of DHE were suppressed by NaHS ($P<0.05$, vs. vehicle + H$_2$O$_2$) and strengthened by PAG pretreatment ($P<0.05$, vs. control) (n=6) (Fig. 4.2-1 B). Our results suggest that exogenous H$_2$S can suppress the productions of toxic free radicals.
Fig.4.2- 1 Effects of H₂S on mitochondrial ROS production. After treatments, isolated rabbits aorta mitochondria reacted with ROS detector dye (A) H₂DCFDA or (B) DHE. The absorbance values of the mitochondria were normalized against the values of control and presented as a percentage of control, which was set as 100%. The data shown are mean ± SEM (n=9). ## p<0.01 vs control. * p<0.05 vs vehicle + H₂O₂ group.
4.2.2 Effects of H$_2$S on mitochondrial respiration in isolated rabbits aorta

Mitochondrial respiration oxygen consumption is a prime readout of mitochondrial oxidative phosphorylation. The measurement of oxygen consumption at state 3 and respiratory control ratio (RCR) provide direct and indirect information of mitochondrial function. After incubation with indicated compounds in the respiration buffer, the state 3 and 4 respirations were analyzed. The addition of 1mM H$_2$O$_2$ lead to significant reductions of state 3 oxygen consumption and RCR ($P<0.05$, vs. control), but not in state 4 oxygen consumption. Pretreatments of NaHS (100 and 300 μM ) significantly attenuated H$_2$O$_2$–induced decreases in state 3 oxygen consumption and RCR ($P<0.05$, vs. vehicle + H$_2$O$_2$), however, this recovery effects did not show in state 4 oxygen consumption. Pretreatment of PAG (10 mM) showed stronger deduction of state 3 oxygen consumption and RCR ($P<0.05$, vs. control) and no significant effect on state 4 of mitochondrial respiration. The effects of H$_2$S on mitochondrial respiration were shown in Fig.4.2-2.
Fig. 4.2-2 Effects of H$_2$S on mitochondrial respiration. Freshly isolated rabbit aorta mitochondria were pretreated with NaHS or PAG, then incubation with H$_2$O$_2$. (A) State 3 and (B) State 4 respiratory rates were presented as nmol (O)/min/mg protein. (C) Respiratory control ratio (RCR) is defined as the ratio of state 3 to state 4 oxygen consumption. The data shown are mean ± SEM (n=9). # p<0.05 vs control. * p<0.05 vs vehicle + H$_2$O$_2$ group.
4.2.3 Effects of H$_2$S on mitochondrial ATP synthesis in isolated rabbits aorta

ATP synthesis is a major function of mitochondria, which supply cell energy. The ATP production reflects the function and status of mitochondria. The ATP/O ratio can be calculated by mitochondrial ATP synthesis rate over mitochondrial respiratory state 3 rate. The incubation of H$_2$O$_2$ resulted in a decrease in ATP synthesis and ATP/O ratio ($P<0.01$, vs. control). Pretreated with NaHS (100 and 300 μM) for 1 hour, significantly reduced the H$_2$O$_2$–induced decrease of ATP synthesis and ATP/O ratio ($P<0.05$, vs. vehicle + H$_2$O$_2$). However, pre-incubation with PAG (10 mM) for 1 hour, caused a deeper decline in ATP synthesis and ATP/O ratio($P<0.05$, vs. control). The effects of H$_2$S on ATP synthesis and ATP/O ratio were shown in Fig. 4.2-3.

![Fig.4.2- 3 Effects of H$_2$S on ATP synthesis and ATP/O ratio. After treatments, isolated rabbits aorta mitochondria reacted with ADP, pyruvate and malate as substrates. (A) were presented as nmol ATP/min/mg protein. (B) was shown as the ratio of ATP/O. The data shown are mean ± SEM (n=6). # $p<0.05$ vs control. * $p<0.05$ vs vehicle + H$_2$O$_2$ group.](image)
4.2.4 Effects of H$_2$S on mitochondrial respiration chain complex and mitochondrial matrix enzymes in isolated rabbits aorta

Oxidative phosphorylation is executed by the respiratory proteins, including mitochondrial complexes and matrix enzymes. The activities of these proteins enzymes reflect the status of mitochondrial respiration chain producing ATP and byproduct, ROS. After isolated aortic mitochondria exposed to H$_2$O$_2$, the activities of complex I, II/III and IV were significantly reduced ($P<0.05$, vs. control), whereas activities of complex I, II/III and IV were rallied by the pretreatments of NaHS ($P<0.05$, vs. vehicle + H$_2$O$_2$) and more affected to lower levels by PAG ($P<0.05$, vs. control). The effects of H$_2$S on mitochondrial respiratory chain complexes were shown in Fig. 4.2-4.

However, the same treatments did not show such strong effects on activities of PDHC and citrate synthase, but mild change in the activities of $\alpha$-KGDHC by stimulation of H$_2$O$_2$ ($P<0.05$, vs. control). Pretreatments of NaHS (300 μM) restored $\alpha$-KGDHC activity ($P<0.05$, vs. vehicle + H$_2$O$_2$) but failed to ameliorate impairments on activities of PDHC and citrate synthase. Pretreatments of PAG made the stronger reduced activities of $\alpha$-KGDHC ($P<0.05$, vs. control) and less sensitive effects on activities of PDHC and citrate synthase. The effects of H$_2$S on mitochondrial matrix enzymes were shown in Fig. 4.2-5.
Fig. 4.2 - Effects of H$_2$S on activities of mitochondrial respiratory chain complexes. The values of enzymes activities of (A) complex I, (B) complex II/III and (C) complex IV in treated mitochondria were normalized against the values for controls and expressed as a percentage of control, which was set to 100%. The data shown are mean ± SEM (n=9). # p<0.05 vs control. * p<0.05 vs vehicle + H$_2$O$_2$ group.
Fig. 4.2-5 Effects of H₂S on activities of mitochondrial matrix enzymes. The values of enzymes activities of (A) α-KGDHC, (B) citrate synthase and (C) PDHC in treated mitochondria were normalized against the values for controls and expressed as a percentage of control, which was set to 100%. The data shown are mean ± SEM (n=9). # p<0.05 vs control. * p<0.05 vs vehicle + H₂O₂ group.
4.2.5 Effects of H$_2$S on mitochondrial membrane permeability in isolated rabbits aorta

In the electron transport on the mitochondrial respiration chain, complexes I-IV pump H$^+$ out from mitochondrial matrix to mitochondrial intermembrane and complex V pump H$^+$ in, which establish an electrochemical potential ($\Delta \Psi_m$) resulting in a pH and a voltage gradient. Therefore, mitochondrial potential, $\Delta \Psi_m$, is a highly sensitive indicator of the status of mitochondrial respiration function. Addition of 1mM H$_2$O$_2$ for 30 min, the $\Delta \Psi_m$ dramatically decreased, compare to control group ($P < 0.01$). Pretreatments of NaHS (100 and 300 μM) for 1 hour, significantly prevented the H$_2$O$_2$–induced decrease of JC-1 fluorescence intensity ratio ($P < 0.01$, vs. vehicle + H$_2$O$_2$). However, pre-incubation with PAG (10 mM) for 1 hour, caused a deeper decline in JC-1 fluorescence intensity ratio ($P < 0.05$, vs. control).

Further, mitochondrial swelling was monitored by analyzing change of absorbance at 540nm (A540), which also an indicator of mitochondrial membrane permeability. The absorbance significantly increased at H$_2$O$_2$ and PAG groups ($P < 0.01$, vs. control), but reduced at NaHS (100 and 300 μM) groups in dose dependence ($P < 0.05$, vs. vehicle + H$_2$O$_2$). The effects of H$_2$S on JC-1 fluorescence intensity ratio and $\Delta$A540 were shown in Fig. 4.2-6.
Fig. 4.2- 6 Effects of H₂S on mitochondrial membrane permeability. After treatments, isolated rabbits aorta mitochondria reacted with (A) JC-1 to detect $\Delta \Psi_m$. (B) Mitochondrial swelling was detected by adding 5 mM sodium succinate and monitored at 540nm. The absorbance values of the mitochondria were normalized against the values of control and presented as a percentage of control, which was set as 100%. The data shown are mean ± SEM (n=9). ## $p<0.01$ vs control. * $p<0.05$, ** $p<0.01$ vs vehicle + H₂O₂ group.
4.3 Results of experiment III: Protective effects of Hydrogen sulfide on the development of atherosclerosis in hyperlipidemic rabbits

4.3.1 Effects of H$_2$S on the CSE/H$_2$S pathway in the hyperlipidemic rabbit

Fig. 4.3-1 shows the changes in the CSE/H$_2$S pathway during the development of atherosclerosis. Compared with rabbits fed a normal diet (sham group), high cholesterol-fed rabbits (vehicle + HCD group) showed significantly lower serum H$_2$S levels and thoracic aortic CSE activities, but higher aortic expressions of CSE mRNA ($P<0.05$, vs. sham). In HCD-fed rabbits which were injected subcutaneously with sodium hydrosulfide (NaHS) 10 and 30 μmol/kg/d for 8 weeks, serum H$_2$S levels showed dose-dependent increases ($P<0.05$, vs. vehicle + HCD). Moreover, the aortic CSE activities and mRNA levels were decreased in a dose-dependent manner, and were significantly lower with 10 and 30 μmol/kg/d NaHS ($P<0.05$, vs. vehicle + HCD). However, PAG + HCD rabbits had significantly lower serum H$_2$S and aortic CSE activities, but higher aortic CSE mRNA ($P<0.05$, vs. sham).
Fig. 4.3-1 Changes in CSE/H₂S pathway in rabbits. (A) The changes of H₂S levels in serum for each treatment group (expressed in μM). (B) CSE activities in HUVECs lysate of each group, presented as μmol/h/g. (C) CSE mRNA expression levels as determined by real-time PCR. The data shown are mean ± SEM (n=6). # p<0.05, ## p<0.01 vs sham. * p<0.05, ** p<0.01 vs vehicle + HCD group.
4.3.2 Effects of H$_2$S on body weight and serum lipids in the New Zealand white (NZW) rabbits

Fig. 4.3-2 showed the percentage increases in body weight in all rabbits. The body weight of rabbits fed a normal diet increased 15.94 ± 0.46 %, while the body weight of rabbits fed the HCD increased by 21.31 ± 2.67 % ($P<0.05$, vs. sham). Injections of NaHS or PAG did not significantly affect the percentage increases in body weight.

Fig.4.3-2 Changes in body weight, serum lipid in rabbits of each group. The changes of percentage of increasing body weight for each treatment group, expressed in %. The data shown are mean ± SEM (n=6). # $p<0.05$, vs sham group.
Fig. 4.3-3 shows the serum levels of LDL and total cholesterol (TC). Compared with sham rabbits, vehicle + HCD group showed increased serum levels of LDL and TC ($P < 0.05$, vs. sham). However, treatment with NaHS (30μmol/kg/d) suppressed the levels of LDL ($P < 0.05$, vs. vehicle + HCD); NaHS did not affect the levels of TC. The hyperlipidemic rabbits treated with PAG showed higher serum lipid levels, compared to sham rabbits ($P < 0.05$).

The cholesterol levels in serum of hyperlipidemic rabbits in each group. (A) LDL levels and (B) Total cholesterol levels were presented as mmol/L. The data shown are mean ± SEM (n=6). ## $p<0.01$ vs sham. * $p<0.05$ vs vehicle + HCD group.

The oxysterols levels were also measured, seen in Fig. 4.3-4. Compared with sham group, other treatments rabbits all showed the high levels of oxysterols, including cholesteryl ester, 7-keto cholesterol, 7-OH, 24-OH, 25-OH, 27-OH cholesterols ($P < 0.05$, vs. sham). Among them, NaHS (10 and 30μmol/kg/d) + HCD-fed rabbits showed significantly decreased sum of 24-OH, 25-OH, 27-OH cholesterols ($P < 0.05$, vs. vehicle + HCD), but did not present the lowering effects at other oxysterols.
Fig. 4.3-4 The oxysterols levels in serum of hyperlipidemic rabbits in each group. The levels of (A) cholesteryl ester, (B) 7-OH cholesterol, (C) 7-keto cholesterol and (D) sum of 24-OH, 25-OH, 27-OH cholesterols were presented as μg/ml. The data shown are mean ± SEM (n=6). # p<0.05 vs sham. * p<0.05, ** p<0.01 vs vehicle + HCD group.

The full blood count was tested in the serum of hyperlipidemic rabbits in each group. Four main indices, white blood cell (WBC), monocytes, red blood cell (RBC) and hemoglobin (HGB), were shown in Fig 4.3-5. Compared with sham group, all treatments groups showed significant differences in these indices (P< 0.05, vs. sham). Although NaHS treatments showed an increase trend in the RBC levels compared to vehicle + HCD group, the differences have little significance.
Fig. 4.3-5 The levels of blood cells in serum of hyperlipidemic rabbits in each group. (A) WBC and (B) monocytes were presented as K/μL, (C) RBC levels were presented as M/μL, and (D) HGB were presented as g/dl. The data shown are mean ± SEM (n=6). # p<0.05 vs sham. * p<0.05 vs vehicle + HCD group.

4.3.3 Effects of H₂S on atherosclerotic plaques in the thoracic aorta and carotids of the NZW rabbits

H & E staining was used to estimate plaque size and intima-media thickness (IMT) of thoracic aortas, which directly reflected the grade of the atherosclerotic lesions in aortas. It was shown that the atherosclerotic plaques were widely observed in vehicle + HCD rabbits but not in sham rabbits (Fig. 4.3-6). Compared with vehicle + HCD-fed rabbits, IMT and plaque sizes were significantly diminished in NaHS (10 and 30 μmol/kg/d) + HCD-fed rabbits and increased in PAG +HCD-fed rabbits (P<0.05).
Fig. 4.3-6 Aortic lesions by H&E staining in hyperlipidemic rabbits in each group. (A-F) The changes of aortic lesions for each treatment group, determined by H&E staining. Scale bar is shown at 100μm. (G) Itima-media thickness in impaired aorta of each group, presented as mm. (H) Plaque area in impaired aorta of each group, presented as mm$^2$. The data shown are mean ± SEM (n=3). # $p<0.05$, ## $p<0.01$ vs sham. *$p<0.05$ vs vehicle + HCD group.

Similar results were determined from HRUS of the carotids of HCD-fed rabbits, using indices of IMT, Peak flow velocity (Vp) and Relative-Sectional change (RS) (Fig. 4.3-7). Vp, a reflection of the hemodynamic status, is closely related to lumen stenosis. RS is another important index of vascular elasticity. The atherosclerotic plaques were clearly evident in the carotid arteries of vehicle + HCD-fed rabbits ($P<0.05$, vs. sham). These findings were greatly improved by injection of NaHS (10 and 30 μmol/kg/d), which decreased IMT, Vp and RS in the carotid artery of HCD-fed rabbits ($P<0.05$, vs. vehicle + HCD). In the PAG + HCD-fed rabbits, the protective efforts caused by NaHS were reversed ($P<0.05$, vs. sham).
Fig. 4.3- 7 HRUS images of carotid artery lesions in hyperlipidemic rabbits in each group. (A1-2) The changes in carotid artery lesions for each treatment group, determined by HRUS images. (A1) showed the original images, while (A2) illustrated the plaques based on the protrudes at the intima. Scale bar is shown at 1 mm. (B) Intima-media thickness in carotid artery of each group, presented as mm. (C) Peak flow velocity in impaired carotid artery of each group, presented as mm/s. (D) Relative-sectional changes in impaired carotid artery of each group.

# $p<0.05$ vs sham. * $p<0.05$ vs vehicle + HCD group.
Echocardiogram is the test to accurately assess heart function and strength. Owing to early atherosclerotic lesions, the echocardiogram did not show any significant difference in each index of heart functions among all the groups (Table 4.3-1).

Table 4.3-1 The heart function detected by echocardiogram in rabbits of each group

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Vehicle</th>
<th>HCD+NaHS 1μmol/kg/d</th>
<th>HCD+NaHS 10μmol/kg/d</th>
<th>HCD+NaHS 30μmol/kg/d</th>
<th>HCD + PAG 10mg/kg/d</th>
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<td>LVDd AVE</td>
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<td>1.089±0.015</td>
<td>1.07±0.008</td>
<td>1.053±0.002</td>
<td>1.058±0.003</td>
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<td>LVDs AVE</td>
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<td>0.555±0.017</td>
<td>0.503±0.01</td>
<td>0.568±0.004</td>
<td>0.509±0.011</td>
<td>0.517±0.018</td>
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<tr>
<td>R-R AVE</td>
<td>0.188±0.006</td>
<td>0.231±0.009</td>
<td>0.211±0.005</td>
<td>0.202±0.004</td>
<td>0.196±0.004</td>
<td>0.219±0.006</td>
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<td>FS (%)</td>
<td>46.9±1.683</td>
<td>49.067±0.839</td>
<td>53±0.069</td>
<td>46.16±0.392</td>
<td>51.92±0.958</td>
<td>51.4±1.67</td>
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<td>EF (ratio)</td>
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<td>0.844±0.008</td>
<td>0.875±0.005</td>
<td>0.817±0.004</td>
<td>0.8664±0.007</td>
<td>0.858±0.014</td>
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<td>CO (L/min)</td>
<td>0.673±0.037</td>
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<td>0.586±0.011</td>
<td>0.6386±0.006</td>
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<td>EDV (ml)</td>
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<td>2.6±0.096</td>
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<td>2.374±0.008</td>
<td>2.398±0.017</td>
<td>2.432±0.018</td>
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<td>ESV (ml)</td>
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<td>0.311±0.017</td>
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<td>SV (ml)</td>
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<td>290.4±6.554</td>
<td>301.6±5.017</td>
<td>308.2±5.257</td>
<td>396.4±7.106</td>
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4.3.4 Effects of H₂S on ultrastructure of thoracic aorta of NZW rabbits

Transmission electron microscopy (TEM) showed the ultrastructure of aorta in hyperlipidemic rabbits (Fig.4-3-8). The endothelial cells were found to be easily dislodged during sample processing. The focus was thus on the ultrastructure of the sub-endothelium and smooth muscle cells (SMCs). Sham rabbits showed clear tight intercellular junctions, uniformly distributed subendothelial tissues. SMCs were normal with intact nuclei and healthy looking organelles. Aorta from vehicle + HCD-fed rabbits showed ultrastructural disorders comprising thickened subendothelium, messy collagen, lipid droplets and vacuoles. SMCs displayed condensate chromatin, swollen mitochondria and expanding endoplasmic reticulum with cell debris and lipid droplets. The ultrastructure of aorta of rabbits treated with NaHS (1 μmol/kg/d) + HCD did not show significant difference from aorta from vehicle + HCD-fed rabbits. However, the ultrastructure of aorta of rabbits treated with NaHS (10 and 30 μmol/kg/d) +HCD was more similar to that of the sham group. It can also be seen that some SMCs showed vascular degeneration with slightly expanded mitochondria and endoplasmic reticulum; subendothelial tissues were thickened with vacuoles. Aorta from the PAG + HCD-fed rabbits showed more severe ultrastructural disorders compared to that of sham. The subendothelial layer was stacked with cell debris; the internal elastic membranes were uneven with messy collagen; SMCs were ultrastructural distorted with shrunken nuclei, unclear or corrupted organelles.
Fig. 4.3-8 Ultrastructures of thoracic aorta of rabbits. Subendothelium and smooth muscle cells of aorta in (A) the sham group; (B) vehicle + HCD group; (C) HCD + NaHS 1 μmol/kg/d group; (D) HCD + NaHS 10 μmol/kg/d group; (E) HCD + NaHS 30 μmol/kg/d group; (F) HCD + PAG 10mg/kg/d group. Bold white “N” represented nucleus of smooth muscle cells. Scale bar is shown on individual images.
4.3.5 Effects of H₂S on oxidative modification of LDL in the NZW rabbits

Serum ox-LDL (analyzed by enzyme-linked immunosorbent assay [ELISA]), was significantly higher in vehicle + HCD-fed and PAG + HCD-fed rabbits (both \( P<0.05 \), vs. sham), but significantly depressed in NaHS (30\( \mu \text{mol/kg/d} \)) + HCD-fed rabbits (\( P<0.05 \), vs. vehicle + HCD), seen in Fig.4.3-9.

![Graph showing ox-LDL levels in serum](image)

**Fig.4.3-9 Serum ox-LDL in hyperlipidemic rabbits in each group.** ox-LDL levels in serum determined by ELISA, presented as \( \mu \text{g/L} \). The data shown are mean ± SEM (n=6). \# \( p<0.05 \), ## \( p<0.01 \) vs sham. * \( p<0.05 \) vs vehicle + HCD group.

Hepatic levels of MDA, a product of lipid peroxidation, in all groups of rabbits are shown in Fig4.3-10. Vehicle + HCD-fed and PAG +HCD-fed rabbits showed higher levels of MDA while NaHS (10 and 30\( \mu \text{mol/kg/d} \)) +HCD-fed rabbits showed decreased levels of MDA (\( P<0.05 \), vs. sham)
Fig. 4.3-10  MDA levels in livers of hyperlipidemic rabbits in each group. The data shown are presented as nmol/mg and mean ± SEM (n=6). ## p<0.01 vs sham. * p<0.05, ** p<0.01 vs vehicle + HCD group.

Heme oxygenase-1 (HO-1), a cardioprotective and redox-sensitive protein, has a close relationship to lipid peroxidation. The protein and gene expressions of HO-1 in thoracic aortas of rabbits in each treatment group were shown in Fig. 4.11 A and B. In the vehicle + HCD-fed rabbits, HO-1 protein and mRNA levels were slightly increased (P< 0.05, vs. sham); while in the NaHS (10 and 30μmol/kg/d) + HCD-fed rabbits, HO-1 protein and mRNA levels were significantly increased (P< 0.05, vs. vehicle + HCD). In the PAG + HCD-fed rabbits, HO-1 protein and mRNA levels were significantly decreased (P< 0.05, vs sham).
Fig. 4.3-11 HO-1 protein and gene levels in thoracic aortas of hyperlipidemic rabbits in each group. (A) HO-1 protein expressions in thoracic aorta of each group. (B) HO-1 mRNA expressions in thoracic aorta of each group. The data shown are presented as nmol/mg and mean ± SEM (n=6). # p<0.05 vs sham. * p<0.05, ** p<0.01 vs vehicle + HCD group.

4.3.6 Effects of H\textsubscript{2}S on oxidative stress in the NZW rabbits

Fig. 4.3-12 shows the effects of different concentrations of NaHS on the redox state in the livers of hyperlipidemic rabbits. These are reflected in the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST), and levels of glutathione (GSH). Compared with sham rabbits,
vehicle + HCD-fed rabbits showed lower activities of these four antioxidant enzymes as well as levels of GSH ($P<0.05$). NaHS (1 μmol/kg/d) increased the activities of SOD, CAT and GPx ($P<0.05$, vs. vehicle + HCD), but did not significantly influence the activity of GST and levels of GSH. NaHS (10 and 30 μmol/kg/d) increased the activities of these four antioxidants and levels of GSH ($P<0.05$, vs. vehicle + HCD). PAG reversed the effects of NaHS, showing the lesser activities of SOD, CAT, GPx, GST and lower levels of GSH ($P<0.05$, vs. sham).

Fig.4.3- 12 Effects of NaHS to the redox state analyzed in livers of hyperlipidemic rabbits in each group. (A) SOD-1 activities and (B) catalase activities were presented as U/mg; (C) GPx activities and (D) GST activities were presented as μmol/min/mg; (E) GSH activities were presented as μM/mg. The data shown are mean ± SEM (n=6). # $p<0.05$, ## $p<0.01$ vs sham. * $p<0.05$, ** $p<0.01$ vs vehicle + HCD group.
These results were consistent with protein expression levels of SOD-1, CAT, GPx and GST in the aorta of the hyperlipidemic rabbits (Fig. 4.3-13). The intensities of these proteins were lighter in vehicle + HCD-fed and PAG + HCD-fed rabbits than in the sham group. NaHS (1 μmol/kg/d) increased CAT expression in the aorta. NaHS (10 and 30 μmol/kg/d) significantly increased the expressions of all four antioxidative enzymes.

![Fig.4.3- 13 Proteins expressions of antioxidants in aortas of hyperlipidemic rabbits in each group. (A)-(D) Western-blot analysis showing the intensities of SOD-1, Catalase, GST and GPx in each treatment group, were presented in bar charts indicating the different intensities of antioxidant proteins between groups. Values were normalized against the sham values. The data shown are mean ± SEM (n=6). # p<0.05 vs sham. * p<0.05 vs vehicle + HCD group.](image-url)
Gene expressions of SOD-1, CAT, GPx and GST in the aorta of the rabbits also support the protein findings, shown in Fig. 4.3-14. Lower gene expressions of these four enzymes were found in vehicle + HCD-fed and PAG + HCD-fed rabbits ($P< 0.05$, vs sham), while higher expressions were found in NaHS (10 and 30 $\mu$mol/kg/d)-treated rabbits ($P< 0.05$, vs vehicle + HCD).

Fig.4.3- 14 Gene expressions of antioxidants in aortas of hyperlipidemic rabbits in each group. (A) SOD-1, (B) catalase, (C) GST and (D) GPx mRNA expression tested by real-time PCR. The values in (A)-(D) were normalized against the sham values. The data shown are mean ± SEM (n=6). # $p<0.05$, ## $p<0.01$ vs sham. * $p<0.05$, ** $p<0.01$ vs vehicle + HCD group.
4.3.7 Effects of H$_2$S on cell adhesion in the NZW rabbits

Mechanism of cell adhesion assists the ox-LDL to stimulate vascular endothelial cells to express various adhesive cytokines, such as VCAM-1, ICAM-1, MMP-9 and MCP-1, which lead to the attachment of leucocytes to the endothelium. To examine the abilities of anti-atherogenesis by H$_2$S, firstly, we measured the soluble VCAM-1 (sVCAM-1), soluble ICAM-1 (sICAM-1), MMP-9 and MCP-1 in plasma of NZW rabbits, seen in Fig.4.3-15. ELISA showed quite low levels of sVCAM-1, sICAM-1, MMP-9 and MCP-1, but significantly increased by HCD treatment ($P<0.05$, vs. sham), NaHS (30 μmol/kg/d) significantly attenuated HCD-induced adhesion molecules expressions ($P<0.01$, vs. vehicle + HCD), while NaHS (10 μmol/kg/d) suppressed HCD-induced sVCAM-1, MMP-9 and MCP-1 ($P<0.05$, vs. vehicle + HCD). Treatment of PAG also elevated the levels of these adhesive cytokines in plasma ($P<0.05$, vs. sham).

![Fig.4.3-15 Cytokines of cell adhesions in serum of hyperlipidemic rabbits in each group. (A) sVCAM-1, (B) sICAM-1, (C) MCP-1 and (D) MMP-9 expressions in serum tested by ELISA. The values in (A)-(D) were presented μg/L. The data shown are mean ± SEM (n=6). # $p<0.05$, ## $p<0.01$ vs sham, * $p<0.05$, ** $p<0.01$ vs vehicle + HCD group.](image-url)
The similar profile was observed by analyzing aorta protein and mRNA expressions of adhesion molecules (Fig.4.3-16). Protein expressions of MMP-9 and VCAM-1 were augmented by vehicle +HCD treatment, compared to low expressions in sham (P< 0.05). Contrary, NaHS (10 and 30 μmol/kg/d) led to reduced expressions of MMP-9 and VCAM-1(P< 0.05, vs. vehicle + HCD). PAG abolished the anti-cell adhesion effects made by NaHS. Sham rabbits showed a low constitutive transcription of adhesion molecules, while intaking HCD 8 weeks caused a significant increase in mRNA levels of VCAM-1, ICAM-1, MMP-9 and MCP-1 (Fig.4.3-17). Injection of NaHS (10 and 30 μmol/kg/d) resulted in decreases of mRNA of above adhesion molecules (P< 0.05, vs. vehicle + HCD). PAG exhibited the amplified transcription of adhesion molecules (P< 0.05, vs. sham).

Fig.4.3-16 Aortic proteins expressions of cell adhesion in hyperlipidemic rabbits of each group. (A)-(B) Western-blots analysis showing the intensities of VCAM-1 and MMP-9 in aortas of each treatment group, were presented in bar charts indicating the different intensities of antioxidant proteins between groups. Values were normalized against the sham values. The data shown are mean ± SEM (n=6). # p<0.05 vs sham. *p<0.05 vs vehicle + HCD group.
Fig. 4.3-17 Aortic genes expressions of cell adhesions in hyperlipidemic rabbits of each group. (A) VCAM-1, (B) ICAM-1, (C) MCP-1 and (D) MMP-9 mRNA expressions in aortas tested by real-time PCR. The values in (A)-(D) were normalized against the sham values. The data shown are mean ± SEM (n=6). ## p<0.01 vs sham. * p<0.05, ** p<0.01 vs vehicle + HCD group.
4.4 Results of experiment IV: Protective effects of S-Propargyl-cysteine on the development of atherosclerosis in hyperlipidemic rabbits

4.4.1 Effects of SPRC on the CSE/H$_2$S pathway in the hyperlipidemic rabbit

Fig. 4.4-1 shows the changes in the CSE/H$_2$S pathway during the development of atherosclerosis. Compared with rabbits fed a normal diet (sham group), high cholesterol-fed rabbits (vehicle + HCD group) showed significantly lower serum H$_2$S levels and thoracic aortic CSE activities, but higher aortic expressions of CSE mRNA ($P<0.05$, vs. sham). In HCD-fed rabbits which were injected subcutaneously with s-propargyl-cysteine (SPRC) (10 µmol/kg/d) for 8 weeks, serum H$_2$S levels showed dose-dependent increases, which were significantly higher than in vehicle + HCD-fed rabbits ($P<0.05$, vs. vehicle + HCD). Moreover, the aortic CSE activities and mRNA levels were significantly increased in SPRC 10 µmol/kg/d ($P<0.05$, vs. vehicle + HCD). However, PAG + HCD rabbits had significantly lower serum H$_2$S and aortic CSE activities, but higher aortic CSE mRNA ($P<0.05$, vs. sham).
Fig. 4.4-1 Changes in CSE/H₂S pathway in rabbits of each group. (A) The changes of H₂S levels in serum for each treatment group (expressed in μM). (B) CSE activities in HUVECs lysate of each group, presented as μmol/h/g. (C) CSE mRNA expression levels as determined by real-time PCR. The data shown are mean ± SEM (n=6). # $p<0.05$, ## $p<0.01$ vs sham. * $p<0.05$ vs vehicle + HCD group.
4.4.2 Effects of SPRC on body weight and serum lipids in the New Zealand white (NZW) rabbits

Fig. 4.4-2A showed the percentage increases in body weight in all rabbits. The percentage of increasing body weights of rabbits fed chow diet increased 13.18 ± 0.67 %. Compared to sham group, the percentage of increasing body weights of rabbits fed HCD in all vehicle, SPRC and PAG groups significantly increased (P< 0.01, vs. sham). Injections of SPRC or PAG did not significantly affect the percentage increases in body weight, compared to vehicle+ HCD group.

Fig. 4.4-2 B-C shows the serum levels of LDL and total cholesterol (TC). Compared with sham rabbits, vehicle + HCD group showed dramatically increased serum levels of LDL and TC (P< 0.05, vs. sham). However, treatment with SPRC (10 μmol/kg/d) suppressed the levels of LDL (P< 0.05, vs. vehicle + HCD); SPRC did not affect the levels of TC. The hyperlipidemic rabbits treated with PAG showed higher serum lipid level, compared to sham rabbits (P< 0.05, vs. sham).
Fig. 4.4- 2 Changes in body weight, serum lipid in rabbits of each group. (A) The changes of percentage of increasing body weight were expressed in %. (B) Total cholesterol and (C) LDL were expressed in mmol/L. The data shown are mean ± SEM (n=6). ## p<0.01 vs sham. * p<0.05 vs vehicle + HCD group.
4.4.3 Effects of SPRC on atherosclerotic plaques in the thoracic aorta and carotids of the NZW rabbits

H & E staining was used to estimate plaque size and intima-media thickness (IMT) of thoracic aortas, which directly reflected the grade of the atherosclerotic lesions in aortas. It was shown that the atherosclerotic plaques were widely observed in vehicle + HCD rabbits but not in sham rabbits (Fig. 4.4-3). Compared with vehicle + HCD-fed rabbits, IMT and plaque sizes were significantly diminished in SPRC (10 μmol/kg/d) + HCD-fed rabbits and increased in PAG +HCD-fed rabbits (P<0.05).

Fig.4.4- 3 Thoracic aortic lesions by H&E staining in hyperlipidemic rabbits of each group. (A-E) The changes of aortic lesions for each treatment group, determined by H&E staining. (F) Itima-media thickness in impaired aorta of each group, presented as mm. (G) Plaque area in impaired aorta of each group, presented as mm². The data shown are mean ± SEM (n=3). ## p<0.01 vs sham. *p<0.05 vs vehicle + HCD group.
Similar results were determined from HRUS of the carotids of HCD-fed rabbits, using indices of IMT, Peak flow velocity (Vp) and Relative-Sectional change (RS) (Fig. 4.4-4). Vp, a reflection of the hemodynamic status, is closely related to lumen stenosis. RS is another important index of vascular elasticity. The atherosclerotic plaques were clearly evident in the carotid arteries of vehicle + HCD-fed rabbits (P< 0.05, vs. sham). These findings were greatly improved by injection of SPRC (10 μmol/kg/d), which decreased IMT, Vp and RS in the carotid artery of HCD-fed rabbits (P< 0.05, vs. vehicle + HCD). In the PAG + HCD-fed rabbits, the protective efforts caused by SPRC were reversed (P< 0.05, vs. sham).

Fig.4.4-4 HRUS images of carotid artery lesions in hyperlipidemic rabbits of each group.(A1-2) (A1) showed the original images, while (A2) illustrated the plaques based on the protrudes at the intima. Scale bar is shown at 1mm. (B) Intima-media thickness in carotid artery of each group, presented as mm. (C) Peak flow velocity in impaired carotid artery of each group, presented as mm/s. (D) Relative-sectional changes in impaired carotid artery of each group. ## p<0.01 vs sham. * p<0.05 vs vehicle + HCD group.
Echocardiogram is the test to accurately assess heart function and strength. Owing to early atherosclerotic lesions, the echocardiogram did not show any significant difference in each index of heart functions among all the groups (Table 4.4-1).

Table 4.4-1 The heart function detected by echocardiogram among rabbits

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Vehicle</th>
<th>HCD+SPRC 1 μmol/kg/d</th>
<th>HCD+SPRC 10 μmol/kg/d</th>
<th>HCD + PAG 10 mg/kg/d</th>
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<tr>
<td>LVDd AVE</td>
<td>1.069±0.004</td>
<td>1.091±0.007</td>
<td>1.114±0.024</td>
<td>1.071±0.012</td>
<td>1.078±0.005</td>
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<tr>
<td>LVDs AVE</td>
<td>0.571±0.013</td>
<td>0.562±0.014</td>
<td>0.640±0.020</td>
<td>0.503±0.006</td>
<td>0.544±0.015</td>
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<tr>
<td>R-R AVE</td>
<td>0.189±0.007</td>
<td>0.228±0.009</td>
<td>0.219±0.009</td>
<td>0.210±0.009</td>
<td>0.211±0.008</td>
</tr>
<tr>
<td>FS (%)</td>
<td>47.2±1.276</td>
<td>50.3±1.349</td>
<td>46.2±1.620</td>
<td>53.0±0.501</td>
<td>51.8±1.42</td>
</tr>
<tr>
<td>EF (ratio)</td>
<td>0.856±0.009</td>
<td>0.869±0.008</td>
<td>0.807±0.011</td>
<td>0.875±0.004</td>
<td>0.866±0.012</td>
</tr>
<tr>
<td>CO (L/min)</td>
<td>0.703±0.029</td>
<td>0.636±0.018</td>
<td>0.573±0.027</td>
<td>0.628±0.037</td>
<td>0.647±0.021</td>
</tr>
<tr>
<td>EDV (ml)</td>
<td>2.526±0.045</td>
<td>2.718±0.075</td>
<td>2.784±0.158</td>
<td>2.472±0.072</td>
<td>2.798±0.086</td>
</tr>
<tr>
<td>ESV (ml)</td>
<td>0.501±0.037</td>
<td>0.526±0.051</td>
<td>0.673±0.069</td>
<td>0.311±0.012</td>
<td>0.588±0.071</td>
</tr>
<tr>
<td>SV (ml)</td>
<td>2.166±0.048</td>
<td>2.179±0.054</td>
<td>2.106±0.031</td>
<td>2.162±0.067</td>
<td>2.112±0.063</td>
</tr>
<tr>
<td>MMHR</td>
<td>345.7±8.567</td>
<td>277.3±9.412</td>
<td>281.4±9.234</td>
<td>290.4±11.169</td>
<td>390.1±9.052</td>
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</tbody>
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4.4.4 Effects of SPRC on ultrastructure of thoracic aorta of NZW rabbits

Transmission electron microscopy (TEM) showed the ultrastructure of aorta in hyperlipidemic rabbits (Fig. 4.4-5). The endothelial cells were found to be easily dislodged during sample processing. The focus was thus on the ultrastructure of the sub-endothelium and smooth muscle cells (SMCs). Sham rabbits showed clear tight intercellular junctions, uniformly distributed subendothelial tissues. SMCs were
normal with intact nuclei and healthy looking organelles. Aorta from vehicle + HCD-fed rabbits showed ultrastructural disorders comprising thickened subendothelium, messy collagen, lipid droplets and vacuoles. SMCs displayed condensate chromatin, swollen mitochondria and expanding endoplasmic reticulum with cell debris and lipid droplets. The ultrastructure of aorta of rabbits treated with SPRC (1 μmol/kg/d) + HCD did not show significant difference from aorta from vehicle + HCD-fed rabbits. However, the ultrastructure of aorta of rabbits treated with SPRC (10 μmol/kg/d) +HCD was more similar to that of the sham group. It can also be seen that some SMCs showed vascular degeneration with slightly expanded mitochondria and endoplasmic reticulum; subendothelial tissues were thickened with vacuoles. Aorta from the PAG + HCD-fed rabbits showed more severe ultrastructural disorders compared to that of sham. The subendothelial layer was stacked with cell debris; the internal elastic membranes were uneven with messy collagen; SMCs were ultrastructural distorted with shrunken nuclei, unclear or corrupted organelles.

**Fig.4.4- 5 Ultrastructures of thoracic aorta of rabbits.** Subendothelium and smooth muscle cells of aorta in (A) the sham group; (B) vehicle + HCD group; (C) HCD + SPRC 1 μmol /kg/d group; (D) HCD + SPRC 10 μmol/kg/d group; (E) HCD + PAG 10mg/kg/d group. Bold white “N” represented nucleus of smooth muscle cells. Scale bar is shown on individual images.
4.4.5 Effects of SPRC on oxidative stress in the NZW rabbits

Fig. 4.4-6 A-E shows the effects of different concentrations of NaHS on the redox state in the livers of hyperlipidemic rabbits. These are reflected in the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST), and levels of glutathione (GSH). Compared with sham rabbits, vehicle + HCD-fed rabbits showed lower activities of these four antioxidant enzymes as well as levels of GSH ($P<0.05$). SPRC (1 μmol/kg/d) increased the activities of SOD, CAT and GPx ($P<0.05$, vs. vehicle + HCD), but did not significantly influence the activity of GST and levels of GSH. SPRC (10 μmol/kg/d) increased the activities of these four antioxidants and levels of GSH ($P<0.05$, vs. vehicle + HCD). PAG reversed the effects of NaHS, showing the lesser activities of SOD, CAT, GPx, GST and lower levels of GSH ($P<0.05$, vs. sham).

Hepatic levels of MDA, a product of lipid peroxidation, in all groups of rabbits are shown in Fig. 4.4-6 F. Vehicle + HCD-fed and PAG +HCD-fed rabbits showed higher levels of MDA ($P<0.05$, vs. sham), while SPRC (10 μmol/kg/d) +HCD-fed rabbits showed decreased levels of MDA ($P<0.05$, vs. vehicle + HCD)

These results were consistent with protein expression levels of SOD-1, CAT, GPx and GST in the aorta of the hyperlipidemic rabbits (Fig. 4.4-7). The intensities of these proteins were lighter in vehicle + HCD-fed and PAG + HCD-fed rabbits than in the sham group ($P<0.05$, vs. sham). SPRC (10 μmol/kg/d) significantly increased the expressions of all four antioxidative enzymes ($P<0.05$, vs. vehicle + HCD).
Fig. 4.4- 6 Effects of SPRC to the redox state analyzed in livers of hyperlipidemic rabbits of each group. (A) SOD-1 activities and (B) catalase activities were presented as U/mg; (C) GPx activities and (D) GST activities were presented as μmol/min/mg; (E) GSH activities were presented as μM/mg; (F) levels of MDA were shown are presented as nmol/mg. The data shown are mean ± SEM (n=6). ## p<0.01 vs sham. * p<0.05, ** p<0.01 vs vehicle + HCD group.
Fig. 4.4-7 Proteins expressions of antioxidants in aortas of hyperlipidemic rabbits of each group. (A)-(D) Western-blot analysis showing the intensities of SOD-1, Catalase, GST and GPx in each treatment group, were presented in bar charts indicating the different intensities of antioxidant proteins between groups. Values were normalized against the sham values. The data shown are mean ± SEM (n=6). # p<0.05, ## p<0.01 vs sham. * p<0.05, ** p<0.01 vs vehicle + HCD group.

Gene expressions of SOD-1, CAT, GPx and GST in the aorta of the rabbits also support the protein findings, shown in Fig. 4.4-8. Lower gene expressions of these four enzymes were found in vehicle + HCD-fed and PAG + HCD-fed rabbits (P< 0.05, vs sham), while significantly higher expressions were found in SPRC (10 μmol/kg/d)-treated rabbits (P< 0.05, vs vehicle + HCD).
Fig. 4.4-8 Gene expressions of antioxidants in aortas of hyperlipidemic rabbits of each group (A) SOD-1, (B) catalase, (C) GST and (D) GPx mRNA expression tested by real-time PCR. The values in (A)-(D) were normalized against the sham values. The data shown are mean ± SEM (n=6). # p<0.05, ## p<0.01 vs sham. * p<0.05, ** p<0.01 vs vehicle + HCD group.

4.4.6 Effects of SPRC on cell adhesion in the NZW rabbits

Various adhesion cytokines, such as VCAM-1, ICAM-1, MMP-9 and MCP-1, induce leucocytes binding to endothelial cell. To examine the abilities of anti-atherogenesis by H2S, firstly, we measured the soluble VCAM-1 (sVCAM-1), soluble ICAM-1 (sICAM-1), MMP-9 and MCP-1 in plasma of NZW rabbits, seen in Fig. 4.4-9. ELISA showed quite low levels of sVCAM-1, sICAM-1, MMP-9 and MCP-1, but significantly increased by HCD treatment (P< 0.05, vs. sham). SPRC (10 μmol/kg/d) significantly attenuated HCD-induced adhesion molecules expressions (P< 0.01, vs. vehicle + HCD). Treatment of PAG also elevated the levels of these adhesion
cytokines in plasma ($P<0.05$, vs. sham).

The similar profile was observed by analyzing aorta mRNA expressions of adhesion molecules (Fig. 4.4-10). Sham rabbits showed a low constitutive transcription of adhesion molecules, while intaking HCD 8 weeks caused a significant increase in mRNA levels of VCAM-1, ICAM-1, MMP-9 and MCP-1. Injection of SPRC (10 μmol/kg/d) resulted in decreases of mRNA of above adhesion molecules ($P<0.05$, vs. vehicle + HCD). PAG exhibited the amplified transcription of adhesion molecules ($P<0.05$, vs. sham).
Fig. 4.4- 10 Aortic genes expressions of cell adhesions in hyperlipidemic rabbits of each group. (A) VCAM-1, (B) ICAM-1, (C) MCP-1 and (D) MMP-9 mRNA expressions in aortas tested by real-time PCR. The values in (A)-(D) were normalized against the sham values. The data shown are mean ± SEM (n=6). ## $p<0.01$ vs sham. * $p<0.05$ vs vehicle + HCD group.
Chapter 5
Discussion
5.1 Discussion on experiment I:

This part study explored the cardiovascular protective effects by H$_2$S on H$_2$O$_2$ induced injured in HUVECs as assessed by measurements of mitochondrial integrity and antioxidative systems. It is well known that oxidative stress is a potent atherogenic factor during the initiation of atherosclerotic lesions. Oxidized cholesterol particles promote inflammation, the formation of plaques and the migration of smooth muscle cells, which all contribute to the development of atherosclerotic lesions. Moreover, at the cellular level, these toxic free radicals can also damage mitochondria. Identifying chemical agents and drugs that can protect mitochondria is a rational means to prevent severe cellular damages. However, the current treatments for atherosclerosis are limited and with strong adverse drug reactions occurring in some patients. Previous studies on H$_2$S, have found it to be a vasodilator in the cardiovascular system, yet few studies have determined the effects of H$_2$S on the endothelium nor the mechanisms by which it can protect mitochondrial function and suppressing oxidative damage. To test our hypothesis that H$_2$S has a protective effect on the endothelium against cell damage, we used HUVECs as a model system. Oxidative injury was induced by H$_2$O$_2$ treatment and the protective effects of H$_2$S assessed using the donor compound, NaHS. Considering the important roles of mitochondria in cell energy production, free radical generation and cell survival, this research unveils for the first time the mechanisms involved in mitochondria protection by H$_2$S in endothelial cells.

We first evaluated the toxicity of H$_2$S in HUVECs. Cell viability studies indicated that H$_2$S was non-toxic at the μM levels. In addition, we also demonstrated a concentration dependent protective effect of H$_2$S against H$_2$O$_2$ induced loss of cell viability. Moreover, the protective effect of H$_2$S against H$_2$O$_2$ may be regulated by inhibition of early apoptosis. Interestingly, H$_2$S is synthesized by the enzymes CSE and cystathionine β-synthase (CBS) [266]. CSE is expressed in the cardiovascular system, liver, kidney, stomach and uterus; while CBS is chiefly located in the
nervous system, liver, kidney, placenta and pancreatic islets [23]. In our study, we found that CSE gene and protein expression levels were suppressed by H$_2$O$_2$ treatment. This observation has been further confirmed by the noted reduction in H$_2$S levels in cell lysates following H$_2$O$_2$ treatment. Parallel studies also found that CBS gene and protein expression levels were not significantly influenced by H$_2$O$_2$, H$_2$S or PAG treatments. The trends in H$_2$S levels, CSE and CBS gene and protein expressions correspond well with our investigations on the changes of mitochondria functions and redox status. Furthermore, as an irreversible inhibitor of the H$_2$S enzyme CSE, PAG is a key enzyme involved in glutathione synthesis [267] and the metabolic trans-sulfuration pathway which regulates homocysteine concentration and mediates cysteine synthesis [268]. NaHS and PAG on CSE showed increasing and decreasing effects, respectively, which may resulted from the weaker or stronger lesions in endothelial cells, with tender feedbacks induced by the acute damage (within 4 hours) to this sulfide-producing enzyme, CSE. In a study design, necessity of an inhibitor (PAG) can help to observe the effects of imposed drug (H$_2$S). The adverse effects of the targeted drug (H$_2$S) induced by the inhibitor (PAG), may indicate that the results of an experiments may be due to influences of the drug (H$_2$S). Therefore, in our experiments, PAG was introduced through all the studies. Consequently, our observations correspond with previous findings about the localization of H$_2$S synthesis enzymes, suggesting the mitochondria protective effects by exogenous H$_2$S are probably though CSE/H$_2$S pathway.

We further explored the mechanisms of action of H$_2$S on H$_2$O$_2$ induced cells viability. Since mitochondrial function is linked to cell redox status, and that H$_2$S is a known redox active molecule, we conducted a series of studies on mitochondria. The primary function of mitochondria is to generate ATP by the process of oxidative phosphorylation [269]. ATP synthesis is driven via the transfer of electrons through complex I to V generating a concentration gradient of protons across the inner mitochondrial membrane thus maintaining membrane potential [270]. During stress
induction electron transportation and ATP synthesis often fails leading to the accumulation of free radicals and mitochondrial dysfunction [271]. In our study, ATP synthesis was increased following pre-treatment with H$_2$S. In contrast, H$_2$O$_2$ abolished ATP synthesis. This result suggests that exogenous H$_2$S stimulates efficient oxidative phosphorylation rates thus raising mitochondrial energy metabolism. This leads us to speculate that one of the mechanisms of H$_2$S protection against oxidants may be directly through improved rates of ATP synthesis.

An additional factor involved in mitochondrial functions is the integrity of the mitochondrial membrane structure, which is responsible for the transmembrane proton gradient and ATP energy production [243]. During a severe stress insult, mitochondrial membrane depolarization occurs leading to organelle swelling resulting in ROS production, ATP hydrolysis and apoptosis [272]. Several lines of evidence have demonstrated that atherosclerotic lesions are promoted by mitochondrial depolarization [273] and cytochrome c redistribution [274]. Under our experimental conditions, preconditioning with NaHS attenuated the discontinuity of the outer mitochondrial membrane, stabilized mitochondrial membrane permeabilization and controlled cytochrome c release from the mitochondria to the cytosol. According to our study, H$_2$S significantly inhibited cytochrome c release and preserved endothelial ultrastructure. One possible mechanism may be directly through the preservation of mitochondrial inner and outer membranes, like the cristae, and indirectly through the inhibition of matrix remodeling. Our results also showed that mitochondria dysfunction occurred as early as 4 hours following H$_2$O$_2$ treatment, and stimulated an increased production of ROS [205]. Therefore, protective intervention in these early stages may contribute to the amelioration of atherosclerosis damage.

The second and most important factor that should be highlighted is the interplay between mitochondrial ROS production and antioxidants. Although oxidation
reactions are crucial for physiological functions, elevated levels of ROS can be
damaging and toxic [275]. It has been widely acknowledged that ROS are involved
in the initiation and progression stages of atherogenesis [276]. We observed that
H2O2 (600μM) strongly reduced endothelial cell viability however, pretreatment with
H2S (300 μM NaHS), preserved cell viability. This finding correlated with a
significant decrease in MDA, DPPP and endogenous ROS. Interestingly, the
protective effects of H2S could be reversed by PAG. One possible explanation for
this observation is that H2S is a strong reducing agent and may readily react with
labile molecules, particularly those derived from reactive oxygen and nitrogen
species, like the superoxide radical anion [77], hydrogen peroxide [75], peroxynitrite
[78] and hypochlorite [79]. All these compounds are highly reactive and their
reactions with H2S may contribute to the protection of mitochondria in endothelial
cells. Moreover, our results also found that pretreatment with NaHS elevated the
protein expressions and activities of the antioxidant enzymes SOD, catalase and
glutathione peroxidase. These findings further support an antioxidant role for H2S.
Our observations agree with recent studies that found H2S was cardioprotective by
virtue of its antioxidant properties [136]. In this case, our results suggest that the
third underlying mechanism of mitochondrial protective by exogenous H2S is though
decreasing the toxicity of ROS via increasing the expression levels of antioxidants
enzymes.

The third possible mechanism by which oxidative stress may trigger cellular toxicity
in HUVECs may be the induction of the mitochondrial apoptotic pathway. It has
reached a consensus that mitochondria do not merely supply cellular energy, are also
involved in a range of other processes, such as cellular differentiation, program cell
death (apoptosis), as well as the control of the cell cycle and cell growth [277]. In the
mitochondrial pathway of apoptosis, mitochondrial outer membrane
permeabilization has a close relationship with release of cytochrome c, the
dissipation of mitochondrial inner transmembrane potential (ΔΨm) and regulation of
The break of inner and outer membranes of mitochondria will result in cellular apoptosis or even necrosis in several pathologies of neurological disorders, ischemic diseases and cardiac dysfunction, such as atherosclerosis, stroke, ischemia/reperfusion (I/R), cardiomyopathy, and congestive heart failure [279-281]. Members of the Bcl-2 family of proteins, including pro-apoptotic (e.g. Bax) and anti-apoptotic (e.g. Bcl-2 and Bcl-XL) members, are major regulators of mitochondrial cytochrome c release and down-stream caspase activation and as such play an important role in the regulation of cardiomyocyte apoptosis [62, 282]. Bcl-2 is regarded as an important cellular component and it could guard against apoptotic cell death [283]. Bcl-2 prevents the release from mitochondria into the cytoplasm of apoptogenic factors, including cytochrome c and apoptosis inducing factor [251]. Meanwhile, its antagonists, such as Bax, caspase enzymes, are also involved in mitochondrion-dependent apoptosis. For example, it was recently investigated that lacking caspases 3 and 7 were highly resistant to mitochondrial apoptosis, delay of Bax translocation and cytochrome c release [284]. In our studies, NaHS increased expression levels of Bcl-2 and Bcl-XL and decreased levels of Bax and caspase-3, indicating that the mitochondrial protection by pharmacological preconditioning with exogenous H₂S could be though enhancing cell anti-apoptotic effect.

In a separate set of experiments, we also evaluated whether the exogenous H₂S used in the current study activated the Akt signaling pathway. Akt, a serine/threonine kinase, have been reported in a number of studies that the activity of this protein can be regulated through redox stress [285]. The Akt pathway is activated not only by various growth factors and cytokines but also by G protein–coupled receptor agonists, such as bradykinin, through transactivation of receptor tyrosine kinase or activation of non-receptor tyrosine kinase, such as Src, in a ROS-dependent manner [286]. It has also been reported that Akt antagonized mitochondrial apoptosis though inhibiting cytochrome c release [287], regulating mitochondrial hexokinases [288] and interacting with phosphorylates X-linked inhibitor of apoptosis protein [289]. In
In our study, we found increased expression of phospho-Akt (activated) in NaHS–treated groups. PAG could abolish this redox-sensitive signaling. This suggested that exogenous H$_2$S may negatively regulate mitochondrial apoptosis by stimulated growth factor though Akt pathway.

In conclusion, this part study describes the cellular protective effects of H$_2$S against H$_2$O$_2$-induced toxicity, as shown in **Fig 5-1**. This protection was associated with the preservation of mitochondria function and stimulation of cellular antioxidants and anti-apoptosis defenses. Collectively, the ability of the CSE/H$_2$S pathway to alter oxidative conditions suggests that the modulation of CSE expression and H$_2$S production may provide a novel therapeutic avenue for the treatment of atherosclerosis. This is the first attempt to link H$_2$S treatment with mitochondria protection in endothelial cells and provides a new insight into the cellular protective mechanisms of H$_2$S. The availability of H$_2$S donors should facilitate further studies on its cardiovascular protective roles in tissue-, animal- and patient-specific studies.
Chapter 5

Fig. 5-1 Conceptualization of the way in which H₂S may influence on H₂O₂-induced cell damage by preserving mitochondrial functions and displaying antioxidative and anti-apoptosis abilities though CSE/H₂S pathway.
5.2 Discussion on experiment II:

Atherosclerosis is a chronic inflammatory disease, which is initially induced by high level of serum lipid and oxidative stress [1]. ROS is mainly produced by mitochondria in the cells [111]. Once mitochondria are been attacked and damaged, the respiration chain will lose biological function and generate extreme ROS, which is toxic to cell and contributed to cell death [290]. Therefore, mitochondrial dysfunction has very close relationship to the early stage of atherogenesis. Increased mitochondrial ROS generation and dysfunction play vital roles in most cardiovascular disease, such as ischemia–reperfusion [18], peripheral vascular disease [291], atherosclerosis [205] and myocardial infarction [292]. Aortic samples collected from atherosclerotic patients were observed stronger mtDNA damage than that of nonatherosclerotic patients in age-matched transplant donors [293]. Meanwhile, NO, another gasotransmitter, has been found that NO releaser - bradykinin, modulate the canine myocardial mitochondrial respiration [294]. However, the effects of H2S on aortic mitochondrial functions are still unknown. Therefore, in this part of study, we hypothesized that H2S may protect thoracic aortic mitochondria of NZW rabbits and reserve the aortic mitochondrial function against the damage by H2O2. To test our hypothesis, we examined that respiration chain function and enzymes activities, mitochondrial matrix enzymes activities, ATP production, ROS generation and mitochondrial membrane permeability as the major indice of mitochondrial function. Importantly, this is the first time revealing the protective effects by H2S on rabbit aortic mitochondria from the several main mitochondrial function directions.

Considering oxidative stress is highly related with atherogenesis, free radical and related agents is the first factor we interest. In tranquil status, mitochondria produce ROS as the natural byproduct of the normal metabolism of oxygen. The cellular internal antioxidant defense system can maintain the cellular inner environment
redox homeostasis by scavenging the toxic free radicals. However, various pathological conditions can break this balance and raise the formation of ROS [208]. The diseases influenced by oxidative stress can be divided into 2 groups. The first group is characterized by prooxidants altering the thiol/disulfide redox state and deteriorating glucose tolerance, including cancer and diabetes mellitus. The other group is characterized by inflammatory oxidative condition and increased activity of either NAD(P)H oxidase (such as atherosclerosis and chronic inflammation) or xanthine oxidase-induced formation of ROS (such as ischemia and reperfusion injury) [295]. The outcomes of excessive ROS stimuli were observed at two favorite atherosclerotic cell lines, human aortic smooth muscle cells (HASMCs) and HUVECs, that increase of mtDNA mutation, decrease of steady-state levels of mtDNA encoded mRNA transcription, suppression of mitochondrial enzymes synthesis, reduction of membrane potential and diminution of ATP pools[296]. Due to the antioxidative effects of H2S on our previous study, in this part of thesis we also detected the effects of H2S on H2O2-induced mitochondrial ROS generation in isolated rabbit aortic mitochondria. Our results showed that exogenous H2S significantly reduced the generation of oxygen free radicals in isolated rabbit aortic mitochondria injured by H2O2, indicating that the ability of H2S to prevent oxidative stress probably involves protection of mitochondrial function. Moreover, this effects likely to stem from a restoration of the oxidative-antioxidative balance, according to our previous part study that H2S can antagonist the damage of H2O2 and elevate the expressions of SOD, catalase, GPx and GST.

The main function of mitochondria is ATP production by a process of oxidative phosphorylation. In this process, electrons are delivered from NADH/FAD(2H) via complexes on the electron transport chain (respiration chain) [205]. At the end of this chain, the final electron acceptor is oxygen and ultimately forms H2O. The energy from the reduction of O2 to H2O is utilized to synthesize ATP by ATP synthase [217]. During electron transportation, the complex I, III and IV push protons from
mitochondrial matrix out to the mitochondrial intermembrane space and complex V pumps protons back to matrix[206]. This creates a concentration gradient of protons that ATP synthase utilize the power to produce the energy carrier molecular ATP. Therefore, to study the above mentioned mitochondrial events, ATP synthesis rate and respiratory control ratio were analyzed. We found that the H$_2$O$_2$ can significantly ameliorate the ATP synthesis rate, ATP/O and mitochondrial active respiration. This result indicates that the negative effects of excessive ROS on mitochondria will in turn inhibit oxidative phosphorylation and may mainly related to the impairment of the electron transportation process. In agreement with our cellular study in previous part, exogenous H$_2$S significantly attenuated mitochondrial toxicity induced by H$_2$O$_2$. H$_2$S treatments were found significantly inhibited the reduction of the ATP synthesis rate, ATP/O and caused by H$_2$O$_2$. The mitochondrial protective effects of H$_2$S on mitochondrial active respiration may be partly owing to the direct promotion of the ATP synthesis rate, which help to control the rate of electron flow and regulate the consumption of oxygen. Therefore, our results suggest that H$_2$S may directly enhance the efficiency of oxidative phosphorylation and improve mitochondrial energy metabolism.

Mitochondrial respiration chain is considered as the main site of the ROS production. In order to produce ATP, complex I transfer the electrons from NADH to ubiquinol, delivering O$_2^-$ to mitochondrial matrix[216]. Complex III accept electrons from ubiquinol and cytochrome c, deliver O$_2^-$ to mitochondrial matrix and intermembranous space[217]. Although ROS is the byproduct of ATP generation, under oxidative stress the impairment of mitochondrial respiration chain will release excessive ROS, which may directly decrease the efficiency of oxidative phosphorylation and aggravate ROS toxicity. On this respiration chain, there is a series of concurrent protein complexes located at the mitochondrial inner membrane and matrix. In order to ascertain the degree of impairment of these enzymes, we measured the activities of mitochondrial respiration chain complexes and matrix
enzymes. Our results showed that exogenous H$_2$S significantly attenuated damage of mitochondrial complex and matrix enzymes activities induced by H$_2$O$_2$, implying that the development of the activities of these key enzymes probably attributes to the preservation of mitochondrial active respiration by H$_2$S.

The forth important factor for the oxidative phosphorylation is the integrity of the mitochondrial membrane structure, whose impairment not only demolishes transmembrane proton gradient and interferes the ATP synthesis, also indirectly induced an augmentation of ROS generation and deprivation of antioxidants activities, finally may release cytochrome c and trigger apoptosis process[297]. Excessive ROS can devastate mitochondrial membrane lipids and mitochondrial proteins, open mitochondrial permeability transition (MPT) pores, even lead to the release of apoptotic proteins, like cytochrome c, etc [230, 231]. Our studies found that exogenous H$_2$S significantly attenuated H$_2$O$_2$–induced mitochondrial swelling and abatement of mitochondrial membrane potential. Generally, it is accepted that mitochondrial swelling is because the growth of inner membrane permeability and opening of MPT pores, a high conductance channel with multiple macromolecular components including ATP/ADP translocase [272]. The induction of MPT, abolish the mitochondrial membrane potential leading to mitochondria further depolarized, and hydrolyzing ATP synthase leading to the decline of ATP production [243]. Therefore, the beneficial action of H$_2$S on the mitochondrial membrane permeability should be through the inhibition of opening of MPT pores, which is also supported by the recovery effects of H$_2$S on ATP synthesis rate and ATP/O values.

In this study, we have several innovations. First, we have demonstrated for the first time that, in aortic mitochondria of NZW rabbit, H$_2$S can attenuate mitochondrial dysfunction induced by H$_2$O$_2$. Importantly, it is also the first attempt to link H$_2$S mitochondrial protection with its antioxidative effects and mitochondrial function reservation. Our study highlights the mechanism of H$_2$S modulate the oxidative
phosphorylation through the conservation of membrane integrity and amelioration of energy metabolism. Our findings draw attention to a novel mitochondrial therapeutic target for the treatment of atherosclerosis in the organelle level.

In conclusion, this study demonstrated the protective effects of H$_2$S on the isolated mitochondria from thoracic aortas of NZW rabbit. The protection of H$_2$S was associated with inhibition of H$_2$O$_2$–induced the growth of ROS generation, decline of ATP production, loss of respiration chain function, mitochondrial swelling and recession of mitochondrial membrane potential. This new attempt may provide a potential template for the development of anti-atherosclerosis drugs. The conceptualization of the way in which H$_2$S may influence on H$_2$O$_2$–induced rabbits aortic mitochondrial damage by preserving mitochondrial membrane permeability, protecting respiration chain and matrix enzymes, displaying antioxidation and reserving ATP production abilities is shown in Fig. 5.2.

![Conceptualization of the way in which H$_2$S may influence on H$_2$O$_2$–induced rabbits aortic mitochondrial damage by preserving mitochondrial membrane permeability, protecting respiration chain and matrix enzymes, displaying antioxidation and reserving ATP production abilities.](image)

OM= mitochondrial outer membrane, IM= mitochondrial inner membrane
5.3 Discussion on experiment III:

Atherosclerosis is a chronic inflammatory disease ascribed to high fat diet, lipid oxidation, oxidative stress and inflammation in the early phase of atherogenesis. Prior work has documented the cardiovascular protective effects of H\textsubscript{2}S on several types of hypertension, myocardial injury and vascular structural remodeling. However, the studies were mainly in vitro studies or focused on a single factor, like intercellular adhesion molecule-1 (ICAM-1) [15, 16]. In this study we examined the anti-atherosclerotic effects of H\textsubscript{2}S (with respect to inhibition of oxidative modification of LDL, vascular protective effects by diminishing atherosclerotic plaques and antioxidative ability) in NZW rabbits rendered hypercholesterolemia by HCD. To test our hypothesis that H\textsubscript{2}S may attenuate the early atherosclerotic lesions, we investigated the effects of NaHS - the donor of H\textsubscript{2}S, and of PAG - the potent and irreversible inhibitor of CSE (an H\textsubscript{2}S-producing enzyme), in this rabbit model. Importantly, our study for the first time reveals the effects by H\textsubscript{2}S on inhibition of ox-LDL, rebalance of redox status, suppression of cell adhesion and prevention of atherosclerotic plaque development in hyperlipidemic rabbits.

To demonstrate the CSE/H\textsubscript{2}S pathway involved in manipulation of atherosclerotic progression, we determined serum H\textsubscript{2}S levels, serum CSE activities and aortic CSE mRNA in all groups. H\textsubscript{2}S is produced from L-cysteine and synthesized by CSE and cystathionine β-synthase (CBS) [266]. The concentrations of these enzymes have different ratios in different parts of the human system. For example, in the cardiovascular system, CSE is the main H\textsubscript{2}S synthesis enzyme; while in the nervous system, CBS is the dominant enzyme that produces H\textsubscript{2}S. In our studies, HCD-fed rabbits showed significantly decreased H\textsubscript{2}S levels in serum and CSE activities in thoracic aorta, which may lead to a positive feedback, resulting in a compensatory growth of CSE gene expression. Due to the CSE distribution in each system, the
reduced H₂S levels in serum may be attributed to the decline of CSE activity not by CBS. Additionally, 3-MST together with AAT, present as the third H₂S synthesis enzymes in brain and vascular cells. In mitochondria, L-cysteine and α-ketoglutarate as substrates, can be converted to 3-mercaptopropionate by AAT; then the intermediate product is converted to H₂S by 3-MST [42]. However, endothelial cells exist the two enzymes, 3-MST and AAT, while smooth muscle cells only 3-MST. Therefore, 3-MST concentration may not directly indicate H₂S producing rate, which will be related to AAT and enzymes locations. These interesting works will be investigated in future studies. Above discussion indicates that the CSE/H₂S pathway may participate in early atherosclerotic development, which is in agreement with peer studies. Moreover, the HCD-fed rabbits treated with NaHS showed dose-dependent increases in serum H₂S levels and decreased CSE activities and CSE mRNA in aorta. This can be explained by the fact that exogenous H₂S administration raised serum levels, causing a positive feedback, lowering CSE gene expression. Conversely, treatment with PAG + HCD led to significant decrease in serum H₂S levels and aortic CSE activities thus up-regulation in CSE mRNA expression in aorta. This is explained by PAG inhibition of CSE activities, resulting in much lower H₂S levels in serum and a positive feedback to augment CSE gene expression. However, although high levels of homocysteine in the sera is considered as an biomarker of cardiovascular disease, the debate was ongoing over does this biomarker can really suggest any cardiac events or not. Especially, a multicenter, randomized, controlled trial and a vitamin study for vascular disease showed that homocystine levels was reduced by vitamin treatments without any improvement of cardiovascular morbidity and mortality[298, 299]. Due to this issue, homocystine was not measured in our studies. These effects of exogenous H₂S (from NaHS) and the CSE inhibitor, PAG, provide more evidence that the CSE/H₂S pathway is probably involved in early atherosclerotic progression, and are demonstrated for the first time in the hyperlipidemic rabbit model.
The lipoproteins play essential physiological roles as vehicles for the delivery of cholesterol between livers and peripheral tissues. The lipid abnormalities, especially LDL, are closely associated with atherogenesis [300]. The exaggerated levels of this lipoprotein increase the risk of atherosclerotic vascular disease as well as progressive and cumulative complications. Rabbits fed HCD showed higher percentage increases in body weight, serum levels of TC and LDL than rabbits on chow diet. This is in accord with previous studies [301]. Hyperlipidemic rabbits treated with NaHS and PAG had decreased serum LDL or highly increased lipids in serum, respectively. Additionally, although blood cells, especially monocytes are involved in atherogenesis, the blood panel did not show significant changed by treatments of H2S. This can be interpreted that stimulation pathway of aggregation of monocytes may not be influenced by H2S. However, sustained high level of monocytes may be a healing mechanism of scavenging toxic and redundant ox-LDL.

Many lines of evidence suggest that oxidative modifications of LDL drive the initial formation of fatty streaks [300]. We found that the highly induced serum ox-LDL in HCD-fed rabbits was inhibited by exposure to H2S. Meanwhile, the atherosclerotic plaques detected in aorta and carotids were both shown to be smaller with H2S treatment, indicating that H2S may retard the development of atherosclerotic lesions, likely via an antioxidative effect.

A number of antioxidants, which can inhibit LDL oxidation, have been tested for their ability to attenuate atherosclerotic lesions in animal models of the disease. 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-tert-butylbenzofuran (BO-653), a synthetic antioxidant with structural components of vitamin E [302], inhibited the serum lipid peroxidation in rat [303] and reduced atherosclerotic lesions in hyperlipidemic mice [304]. Similarly, N,N-diphenyl-phenylenediamine (DPPD) attenuated atherosclerosis in the aorta of cholesterol-fed rabbits and ApoE-deficient mice [142]. In addition, 3,3’5,5’-tetrabutyl-1,1’-biphenyl-4,4’-diol, a lipophilic
bisphenol, inhibited atherosclerosis in mice deficient in apolipoprotein E and the LDL receptor [305]. These findings indicate a common feature of inhibiting oxidation of LDL in reducing atherosclerosis, and are in line with our findings. Furthermore, we also found that H₂S decreased MDA, which was stimulated by HCD. The findings of decreased ox-LDL and MDA suggest that the oxidative lipids can be degraded by H₂S. H₂S, as a strong reducing agent, may readily react with labile and highly reactive molecules, particularly those derived from reactive oxygen and nitrogen species, like superoxide radical anion [77], hydrogen peroxide [75], peroxynitrite [78] and hypochlorite [79], and thus contribute to vascular protection. Moreover, we found that the levels of 24-OH, 25-OH, 27-OH cholesterols, indicating biomarker of lipid oxidation, were decreased by treatments of H₂S. Cholesterol oxidation products induced by iron and LDL modified by macrophages may be inhibited by H₂S, possibly owing to binding of S⁻ anion or HS⁻ anion with iron from negatively charged binding sites in lipids and proteins. These data indicated that H₂S may act as an indirect antioxidant by competing with prooxidant metals such as iron and cooper for strategic binding sites and prohibiting the formation of reactive oxygen species, then in turn reducing oxidative damage and delaying progress of atherosclerosis. However, unstable S⁻ anion or HS⁻ anion lead to weak bingdings of metal ion, which in turn decreased one biomarker of lipid oxidation not all.

Oxidative modification of lipoprotein has also been found to be catalyzed by hemoglobin-derived heme [306]. To further elucidate how H₂S regulates oxidative modification of LDL, we investigated the changes in the level and expression of the cardioprotective protein, heme oxygenase (HO)-1. We found that H₂S increased the protein expression of HO-1 in hyperlipidemic rabbits. Hemin (Fe³⁺-protoporphyrin IX) has been shown to bind to LDL thereby inducing lipid peroxidation, like LDL oxidation [307-309]. HO, the first rate-limiting enzyme, degrades the hemin analog - heme (Fe²⁺-), to generate equimolar amounts of carbon monoxide (CO), free
ferrous iron, and biliverdin (BV) [310, 311]. Induction of HO-1 (inducible HO) or HO over-expression also exerts protective effects via attenuation of ROS-mediated endothelial damage [312], a reduction of neointimal formation [313, 314], and inhibition of plaque lesions [315, 316]. In our study, the regulatory effect on oxidative modification of LDL by H₂S may be mediated by elevated levels of HO-1, leading to increased degradation of hemin, and in turn, suppression of hemin-mediated oxidation of LDL. This is the first time that lipidomic and proteomic evidence from in vivo studies indicate that H₂S can ameliorate atherogenesis through inhibiting oxidative modification of LDL.

It is important to highlight the role of oxygen radicals and antioxidative agents. Oxidative stress is associated with increased formation of ROS that modifies lipids and proteins leading to peroxidation and oxidation of thiol groups [317]. The assaults by ROS lead to changes in LDL, the oxidizing forms of which are the potent inducers in the initiation and progress of atherogenesis [276]. The action of H₂S on redox status observed in our study was a direct activation of the antioxidant enzymes (SOD, CAT, GPx, GST) and the non-enzyme (GSH). These findings indicate that the antioxidative effects of H₂S are not only from the reactions with highly reactive free radicals, but also the elevation of both enzymatic and non-enzymatic antioxidants. Taken with the effects on MDA, the finding of antioxidative abilities of H₂S is in agreement with that in our previous studies on acute myocardial ischemia [136]. This effect, in combination with a large increase in HO-1 expression, results in the protection of the vasculature and is important in preventing atherogenesis.

The inflammatory process mediated by cell adhesive molecules plays roles in all phases of atherosclerosis [195]. VCAM-1 and ICAM-1 are endothelial adhesion molecules belonging to Ig superfamily that participate in the progress of atherosclerosis by promoting the accumulation of monocytes to endothelial cells in arterial intima [318]. Some studies showed the dominant role of VCAM-1 in the
initiation of atherosclerosis[179]. ICAM-1 was also found upregulation in serum and atherosclerotic aortas of apo E-/mice and human atherosclerotic plaques [15, 319]. Matrix metalloproteinases (MMPs) are zinc-dependent proteases, which are secreted in a latent proform and require activation for proteolytic activity, and play functions as degradation of extracellular matrix components at neutral PH [320]. The activities of MMPs are increased in the process of intimal lesion formation and overall geometrical remodeling[199]. Monocyte chemotactic protein-1 (MCP-1) is a chemokine involved in atherogenesis due to its chemotactic activity for monocytes [321]. It has been reported that NO can modulates the MCP-1 expression in endothelial cells [322]. Although H₂S and NO are all the gasotransmitter, whether H₂S exerts the regulatory effects on expressions of these cellular adhesion molecules in the development of atherosclerosis is unknown. Therefore, we tested sVCAM-1, sICAM-1, MMP-9 and MCP-1 in serum and relative molecules proteins and gene expressions in thoracic aortas. Exogenous H₂S attenuated the levels of these cell adhesion molecules, which is consistent with the results that NaHS diminished the size of atherosclerotic plaques in thoracic aortas and carotids. These data indicate that H₂S inhibited secretion and proteins and genes expressions of adhesive molecules in H₂S-induced reduction of atherosclerotic lesions. Thereafter, this may provide a mechanism of H₂S anti-atherogenesis effects that may through suppression of cell adhesion.

In this study, we have several innovations. First, we have demonstrated for the first time that, in hyperlipidemic rabbits, H₂S can attenuate atherosclerotic lesions at the early developmental stage. Importantly, it is also the first attempt to link H₂S cardioprotection with its antioxidative effects and ox-LDL-modulating effects. Our study highlights the molecular mechanism of how H₂S activates HO-1 to regulate the oxidative modification of LDL. Interestingly, these H₂S actions are mediated via the CSE/ H₂S pathway. Our findings draw attention to a novel therapeutic target for the treatment of atherosclerosis.
In conclusion, this study demonstrated the anti-atherosclerotic effects of H\textsubscript{2}S in the hyperlipidemic NZW rabbit. The protection was associated with inhibition of oxidative modification of lipoprotein and antioxidation. The involvement of the CSE/H\textsubscript{2}S pathway in regulating ox-LDL and oxidative stress indicate that the modulation of CSE expression and H\textsubscript{2}S production may be a novel therapeutic target for the treatment of atherosclerosis. This is the first attempt to link H\textsubscript{2}S treatment with oxidative modification of LDL and antioxidation in hyperlipidemic rabbits, providing a new insight into the cardiovascular protective roles of H\textsubscript{2}S. The availability of H\textsubscript{2}S donors should facilitate further studies on the mechanism(s) of its cardiovascular protection. The conceptualization of the way in which H\textsubscript{2}S may attenuate atherosclerotic lesions in hyperlipidemic rabbits by inhibiting lipid oxidation and displaying antioxidative abilities through the CSE/H\textsubscript{2}S pathway is shown in Fig. 5.3.

**Fig. 5.3.** Conceptualization of the way in which H\textsubscript{2}S may attenuate atherosclerotic lesions in hyperlipidemic rabbits by inhibiting lipid oxidation,
displaying antioxidative abilities and suppression inflammatory cell adhesion through the CSE/H₂S pathway.
5.4 Discussion on experiment IV:

Garlic, also called *Allium sativum*, has been consumed by humans over 7,000 years \[107\]. It is widely used as a seasoning, as well as traditional medicine and a functional food to maintain physical health\[56\] [9]. The beneficial effects of garlic have been found to prevent cancer and cardiovascular disease, such as atherosclerosis, high serum cholesterol and hypertension\[323\]. A variety of studies on garlic used aged garlic extract (AGE) as the effective extracted compound \[324-326\]. S-Allylcysteine (SAC), the water-soluble chemical, is the major composition in AGE, showing its cardioprotection in ischemic rat heart and antioxidative effects in endothelial cells and rat brain ischemia \[10, 63\], [327\]. S-Propargyl-cysteine (SPRC), a structural analog of SAC, presents its cardioprotective roles in both in vitro and in vivo studies, and showed as a novel pharmacological agent modulating H\(_2\)S level \[64, 65, 328, 329\]. Although some investigations showed SAC or AGE can prevent atherogenesis, the analog SPRC did not exhibit its role on the development of atherosclerosis. To verify our hypothesis that SPRC may attenuate the early stage development of atherosclerosis, we tested the serum cholesterol, aorta and carotid atherosclerotic lesions, free radicals, antioxidants and inflammatory adhesive molecules in rabbit models. Importantly, this part study for the first time discovers the effects by SPRC on inhibition of atherosclerotic plaque development, rebalance of redox status and suppression of inflammatory adhesive molecules in hyperlipidemic rabbits.

SPRC shares a common cysteine-containing structure with SAC, which has been found its cardioprotection and anti-cancer effects \[64, 330\]. SAC, a substrate of CSE, induce CSE expression that lead to an increase of H\(_2\)S production\[10\]. As a structural analog of SAC, SPRC is also shown as a slow H\(_2\)S-releasing compound that provides cysteine. L-cysteine is the source of producing H\(_2\)S that is synthesized by CSE and cystathionine \(\beta\)-synthase (CBS) \[266\]. CSE and CBS express differently in the organs and systems of human being. In the cardiovascular system, CSE is the main H\(_2\)S
synthesis enzyme; while in the nervous system, CBS is the dominant H₂S-producing enzyme [32-34]. To demonstrate the CSE/H₂S pathway involved in vascular protection of SPRC in atherosclerotic progression, we determined serum H₂S levels, CSE activities and aortic CSE mRNA in all groups. First, in our studies, HCD-fed rabbits showed significantly decreased H₂S levels in serum and CSE activities. Due to the CSE distribution in each system, the reduced H₂S levels in serum may be attributed to the decline of CSE activity. The CSE mRNA expression in thoracic aorta of HCD-fed group was significantly increased, due to the positive feedback between circulating H₂S levels and CSE gene expression. This indicates that the CSE/H₂S pathway may participate in early atherosclerotic development, which is in agreement with peer studies. Moreover, the HCD-fed rabbits treated with SPRC showed dose-dependent increases in H₂S serum levels, CSE activities and aortic CSE mRNA. This can be explained by the fact that SPRC is an endogenous H₂S donor, which can produce cysteine and in turn to raise CSE expression and activity, finally may reflect the increased H₂S serum levels. Interestingly, the changes of CSE activity and gene expression influenced by endogenous H₂S donor, SPRC, is quite different from exogenous H₂S donor, like NaHS. This may be caused that SPRC supply cysteine, the source of H₂S production, not like NaHS which transfer to H₂S directly. This characteristic of SPRC may result in the growth of CSE not the compensatory suppression by NaHS. Lastly, HCD-fed rabbits treated with PAG, the inhibitor of CSE, showed significant decrease in H₂S serum levels and CSE activities, and up-regulation of CSE mRNA expression in aorta. There is a limitation that current CSE antibody in Western Blot protein detecting experiment cannot be attached to rabbit tissue. Fortunately, the CSE protein expression in aorta can be speculated by aortic CSE activities. Therefore, much lower H₂S levels by the suppression of CSE by PAG may lead to a positive feedback to augment CSE gene expression. These effects of endogenous H₂S – SPRC and the CSE inhibitor – PAG on the change of H₂S levels and CSE expressions, provide the evidence that the CSE/H₂S pathway is probably involved in cardioprotection of SPRC in early atherosclerotic progression, and are
demonstrated for the first time in the hyperlipidemic rabbit model.

The inhibition of atherosclerotic aggression by SPRC is further verified by H&E staining of aortas and HURS in carotids. As the result shown, the plaque sizes and IMT were diminished in SPRC treated hyperlipidemic rabbits. Moreover, the index of Vp and RS in carotids are also shown the positive results supporting the cardioprotection of SPRC. Vp is the velocity of peak blood flow. If there is a plaque leading to the narrow of the vessel, the Vp will increase. RS, Relative-Sectional change is calculated as: \( RS = \frac{(Dd^2 - Ds^2)}{Dd^2} \) [aortic end-diastole diameter (Dd) and end-systolic diameter (Ds)]. RS is considered as an index of vascular elasticity in HRUS analysis. The arteries with plaques have higher pressure to the vascular wall, so the elasticity of the vessels becomes worse. Vp and RS were found an increase in HCD-fed rabbits and drops in SPRC treated rabbits, indicates that SPRC may improve the arterial elasticity and inhibit the plaque formation. These solid evidences from aortas and carotids indicate that the vascular protection and prohibition of plaque lesions may be caused by intaking the endogenous H₂S. These changes is in line with the previous results regarding H₂S effect on hyperlipidemic rabbits and peer studies of H₂S effect on apo-E KO mice. However, little change was found in echocardiogram showing the heart function and strength. This may be due to the early stage of atherosclerotic lesions, which usually deposit the lipids in big arteries, like aorta. The complications of atherosclerosis, such as stroke or heart attack, happen at the late stage that influent the small arteries. The little change of echocardiogram indicated the coronary arteries were not eroded by lipid plaques. The effects on heart function by SPRC could be investigated in another animal model presenting late atherosclerosis or the ischemia of heart.

An important role in atherogenesis is oxidative stress, which generates toxic free radicals to induce lipids oxidation and initiate atherosclerotic progress. To investigate the effects on oxidative stress by SPRC, we first tested hepatic levels of MDA (a
product of lipid peroxidation), hepatic activities of non-enzyme antioxidant (GSH) and antioxidant enzymes (SOD, catalase, GST, GPx), and aortic protein and gene expression of antioxidant enzymes. Our positive results indicated that SPRC may inhibit the oxidative stress in atherogenesis. This effect by SPRC may be through two mechanisms: 1. H$_2$S produced by SPRC have the ability to react with labile and highly reactive molecules, particularly those derived from reactive oxygen and nitrogen species, like superoxide radical anion [77], hydrogen peroxide [75], peroxynitrite [78] and hypochlorite [79], and thus contribute to vascular protection. 2. SPRC, itself can be easily oxidized and inevitably react with active radicals to produce new transient species, which may alleviate atherosclerotic aggression[331].

It is highlight that the inflammatory cell adhesion plays vital roles in all phases of atherosclerosis [195]. Endothelial adhesion molecules, like VCAM-1 and ICAM-1, stimulating the adhesion of monocytes to endothelial cells in arterial intima, participate in the process of intimal atherosclerotic lesion formation [318]. Peer studies have found that the dominant role of VCAM-1 and ICAM-1 in the early atherosclerosis [15, 179, 319]. MMPs and MCP-1, due to chemotactic activity, were found in early lesion formation and overall geometrical remodeling[199] [321]. Our results showed that SPRC decreased the protein and gene expressions of inflammatory cell adhesive molecules which can be abolished by the inhibitor PAG, indicating the SPRC may suppress the atherosclerotic progression by inhibition of inflammation mediated by cell adhesive molecules. The similar results were found in some investigations that SAC decreased breast tumor cell adhesion[332]. As the structural analog of SAC, SPRC having the stronger chemical bonds, may through such mechanism to show its anti-atherosclerosis.

In conclusion, this study demonstrated in first time that SPRC attenuated atherosclerotic lesions in the hyperlipidemic NZW rabbit. The vascular protective effects were associated with inhibition of oxidative stress and inflammatory cell
adhesion. As the structural analog of SAC and endogenous H$_2$S, SPRC shows the cardiovascular protection through the CSE/H$_2$S pathway, indicating that the modulation of CSE expression and H$_2$S production may be a novel therapeutic target for the treatment of atherosclerosis. The contribution and innovations of this part study are: the first attempt to link endogenous H$_2$S with anti-oxidation and anti-inflammatory; first time to discover SPRC having the ability of inhibition of atherosclerotic lesions at the early developmental stage in hyperlipidemic rabbits, providing a new insight into the cardiovascular protective roles of H$_2$S. The conceptualization of the way in which SPRC may attenuate atherosclerotic lesions in hyperlipidemic rabbits by inhibiting oxidative stress and inflammatory cell adhesion through the CSE/H$_2$S pathway is shown in Fig. 5.4.

![Diagram](image)

**Fig. 5-4.** Conceptualization of the way in which SPRC may attenuate atherosclerotic lesions in hyperlipidemic rabbits by inhibiting aortic plaques, displaying antioxidative abilities and suppression inflammatory cell adhesion through the CSE/H$_2$S pathway.
Chapter 6

Conclusion and Future Perspective
6.1 Conclusion

Due to long term high lipid diet, formation of multiple plaques leads to arteries narrow and harden, called atherosclerosis, which is initiated by oxidative stress and inflammatory processes. Under oxidative stress, circulating lipids are oxidized, such as ox-LDL, which become the potent inducer of atherosclerosis. From the cellular level, reactive free radicals will lead to mitochondrial dysfunction, which may cause severe oxidative stress. Meanwhile, inflammation is thought to link hyperlipidemia with atherosclerosis, which process mediated by cell adhesion leads to the attachment of leucocytes to endothelium. Therefore, the mitochondrial protection and intervention of oxidative stress and inflammation could be the approach of the atherosclerotic therapy. Previous studies have been found H$_2$S has the cardioprotection and antioxidative ability. These encouraging results prompt us to further our studies on the effects of H$_2$S and SPRC in vitro and in vivo.

First, the pilot study in experiment I have achieved the first objective in this thesis that to verify the possible therapeutic potential of exogenous H$_2$S on HUVECs against H$_2$O$_2$-induced mitochondrial dysfunction, oxidative stress and apoptosis. This part study shows that the cellular protective effects of H$_2$S act through CSE/ H$_2$S pathway by observation of modulation of H$_2$S level and CSE protein and gene expressions. Exogenous H$_2$S showed no toxic to HUVECs in μM level, as well as the growth of cell viability reduced by H$_2$O$_2$. The mitochondrial protection of H$_2$S could be observed from the mitochondrial function by increase of ATP production and mitochondrial integrity by growth of ΔΨ$_m$ and inhibition of cytochrome C release. The anti-oxidation of H$_2$S was obtained by reduction of ROS production, MDA and enhancement of antioxidative enzymes, such as SOD, catalase, GST and GPx. Moreover, the cell viability survived by H$_2$S, observed by Annexin V/PI staining and counted by flow cytometry, may be through its anti-apoptosis from inhibition of pro-apoptotic related proteins expressions, like Bax and caspase-3, and augmentation of anti- apoptotic related proteins expressions, like Bcl-2 and Bcl-xl, and modulation
of Akt pathway. These findings are the first time to demonstrate in endothelial cells that H$_2$S presents its cellular and mitochondrial protection by and suppression of oxidative stress and apoptosis.

Then, in experiment II, the rabbit aortic mitochondria were isolated for investigating the mitochondrial function protected by H$_2$S in the largest artery with atherosclerotic high-risk area. The findings of a greater extent of ROS production and less ATP biosynthesis in H$_2$O$_2$ treated mitochondria are in line with the phenomenon that mitochondria are the both of contributors and targets of toxic free radicals in stressful condition. Under oxidative stress stimulated by H$_2$O$_2$, an augmentation of ROS in aortic mitochondria was prohibited by exogenous H$_2$S in a dose dependent manner. Major mitochondrial function is ATP synthesis, which decreased by oxidative stress and recovered by treatment of H$_2$S. This finding suggests that H$_2$S may have the effect of metabolic arrest to the aortic mitochondria, resulting in the cytoprotective barrier to the stressful mitochondria. As the location of ATP generation, mitochondrial respiration chain was evaluated that reduced oxygen consumption was ascended by H$_2$S, showing the mild uncoupling effect to the mitochondrial respiration. Meanwhile, the activities of mitochondrial respiration chain complex and matrix enzymes were partially increased by H$_2$S, reflecting another mechanism of mitochondrial protection by H$_2$S may stabilize mitochondrial respiration chain to achieve the mitochondrial function. Moreover, the detection of $\Delta\Psi_m$ and mitochondrial swelling revealed mitochondrial protection by H$_2$S may be through the maintenance of mitochondrial integrity. It is the first attempt, in rabbit aortic mitochondria, to link H$_2$S mitochondrial protection with its antioxidative effects and mitochondrial function reservation. The findings of H$_2$S conferring the modulation of the oxidative phosphorylation through the conservation of membrane integrity and amelioration of energy metabolism, provide a novel mitochondrial therapeutic target for the treatment of atherosclerosis.
Next, in vivo experiment III achieved the 3rd objective in this project that the inhibition of atherogenesis in New Zealand White rabbit by H$_2$S. 8 weeks high cholesterol feeding was applied to induce hyperlipidemic model, which showed severe plaques in aortas. The injection of exogenous H$_2$S may diminish the plaques sized and eroded IMT, indicating the potential therapeutic application of H$_2$S on atherosclerosis. For the mechanism investigation, firstly, these findings of mild inhibition of serum cholesterol, suppressed oxysterols levels and reduced ox-LDL indicated that the inhibited effect of lipid oxidation of H$_2$S. Moreover, augmented HO-1 protein and gene expression, prohibited ROS and increased antioxidants suggested that anti-oxidative lipid effect of H$_2$S acts through activating HO-1 expression and antioxidative ability. Furthermore, inflammation mediated by cell adhesion was observed by several important proteins expressions, such as VCAM-1, ICAM-1, MCP-1 and MMP-9, reflecting the anti-inflammatory effect of H$_2$S. It is the first time to demonstrate, in hyperlipidemic rabbits, H$_2$S may attenuate atherosclerotic lesions at the early developmental stage, through its antioxidative effects and inhibition of lipid oxidation and inflammation, and provide encouraging evidences to atherosclerotic therapeutic applications.

Last, the 4th objective was achieved in experiment IV. To higher degree of similarity, SPRC, as the analog of garlic extraction (SAC) and sulfide-containing chemical, showed profound protective effect on atherosclerotic aortas. NZW rabbits were also applied to set up the hyperlipidemia model by feeding 1% cholesterol diet 8 weeks, which profound atherosclerotic lesions can be observed in rabbit aortas. Injection of SPRC 8 weeks with high cholesterol diet lead to diminished plaques size, decreased IMT and improved ultrastructure by, reflecting the effect of anti-atherogenesis by SPRC. The protein and gene expressions of antioxidant enzymes and inflammatory cell adhesive molecules were inhibited by SPRC, which revealed the undergoing mechanisms of anti-atherogenesis of SPRC. These encouraging findings suggest that the therapeutic effects of SPRC act through anti-oxidative and anti-inflammatory
targets. It is the first time to link garlic extraction analog with the therapeutic target of atherosclerosis that SPRC may inhibit atherogenesis, which serves a novel treatment of atherosclerosis.

All in all, this project demonstrates H$_2$S and sulfide-containing chemical (SPRC) may confer the anti-atherosclerotic effect in both *in vitro* and *in vivo* experiments. Our studies is the first time to elucidate the inhibited effect of H$_2$S and (or) SPRC on atherosclerotic related cells or animal model. This is a good attempt to associate the anti-atherosclerotic effect of H$_2$S and SPRC with their abilities of improving mitochondrial function, challenging undergoing apoptosis, balancing redox status and prohibiting inflammation. These evidences prompt researchers to further their studies on drug development of atherosclerosis.
Chapter 6

6.2 Limitation of study

Study on human disease-atherosclerosis is not an easy job, because this disease is the outcome of long term effects, which is influenced by multiple factors, such as, oxidative stress, inflammation, program cell death and cell migration. Our in vitro study focused on endothelial cells and in vivo study used NZW rabbit to set up the hyperlipidemic model, due to the investigation on early atherosclerosis. Nonetheless, none of these models fully reflects all human atherosclerotic phenomena. This limitation contributes to countless abortions of drug development on therapeutic agents which have showed their anti-atherosclerotic effect, owing to the strong drug adverse reaction or narrow therapeutic dosage range. For these reasons, deeper understanding on pathophysiology of atherosclerosis and more evidences for supporting the therapeutic effect will be helpful for peer studies and clinical trials. However, it cannot undermine the value and importance of current studies that provide useful information of a new identified gasotransmitter, H$_2$S on treatment of this tough cardiovascular disease.

Although H$_2$S is a hot issue in recent years, the pharmacological effects on every human system are still under investigation. Some of these findings even are conflicting, ascribed to the different study models, different H$_2$S form or donors, etc. Nonetheless, as a gasotransmitter sharing several characteristics with NO, H$_2$S may present its pharmacological effect on human public health.
6.3 Future perspective

The current therapies of atherosclerosis mainly focusing on the lowering the serum lipid with strong adverse drug reaction and low patients tolerance remain a challenging task. Experimental models of atherosclerosis have provided us treasure insights that atherosclerosis is a chronic inflammatory process involving a series of complex signaling interacting the atherogenesis. Therefore, in long term research, multifaceted molecular pathways are needed to investigate that can help to deeply understand atherogenesis and identify the drug development target.

As one of the mechanism in pathophysiology of atherosclerosis, oxidative stress interplay with other factors among different signaling pathways. Considering oxidative stress initial this chronic disease, its effects on activating other molecules and cells become very important. Therefore, researches on this field could be an interesting issue that inspires peers to pay more passions on drug development of antioxidant strategy.

Research on H$_2$S is a hot issue with abundant unknown characteristics. For example, it is still debating that the effects of H$_2$S on inflammation, cancer and Alzheimer’s disease are prohibition or promotion. Considering atherosclerosis is a chronic inflammatory disease, future perspective can deeply investigate the effects of H$_2$S on inflammation and immune system. Hence, the studies on pharmacology of H$_2$S on human system could further the knowledge of this gasotransmitter that may present excellent performances as its brother, NO.
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