The Cross-talk of Hydrogen Sulfide and Nitric Oxide in Vascular Endothelial Cells

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ABSTRACT

Gasotransmitters, like hydrogen sulfide (H_2S) and nitric oxide (NO), are small gaseous molecules that can be generated in different types of mammalian cells by enzymatic catalyzation. Cystathionine γ-lyase (CSE) and endothelial NO synthase (eNOS) are responsible for the majority of endogenous production of H_2S and NO in vascular endothelium, respectively. H_2S and NO maintain different vascular functions. Here we show that H_2S interacts with eNOS to increase NO release from endothelial cells (ECs). Two mechanisms are involved in this interaction. Firstly, H_2S indirectly induces eNOS phosphorylation. Secondly, H_2S directly modifies one cysteine residue of eNOS through *S*-sulfhydration. Stimulation of eNOS phosphorylation and *S*-sulfhydration by H_2S subsequently increases NO release. The phosphorylation of eNOS by H_2S is p38 and Akt-dependent. eNOS *S*-sulfhydration is partially affected by *S*-nitrosylation but not by phosphorylation. We further found that knockdown of CSE gene by siRNA technique, or blockage of CSE enzyme activity by PPG (dl-propargylglycine), attenuates NO production. CSE overexpression or L-cysteine (a substrate of H_2S) supplementation stimulates NO production.

The level of eNOS *S*-sulfhydration in aortic tissue from CSE knockout (CSE-KO) mice was lower than that from wild type (WT) mice. L-cysteine treatment increases *S*-sulfhydration of eNOS in ECs isolated from WT mice, but not in ECs isolated from CSE-KO mice. GSNO (a NO donor) induces, but NaHS reduces, eNOS *S*-nitrosylation. However, GSNO does not alter eNOS *S*-sulfhydration whereas NaHS alters *S*-nitrosylation. Site-directed mutagenesis of one cysteine residue Cys-443 in eNOS (Cys-443-eNOS) completely eliminates eNOS *S*-sulfhydration and partially decreases eNOS *S*-nitrosylation. Although the mutation of serine 1179 (Ser-1179)

completely abolishes eNOS phosphorylation, it does not affect eNOS *S*-sulfhydration. The dominant configuration of vascular eNOS proteins purified from WT mice is dimer, whereas in CSE-KO mice it is monomer. In the presence of GSNO, more monomers are found with WT-eNOS, which is reversed by a subsequent treatment with NaHS. Cys-443-eNOS manifests itself as monomers, which is not changed by either GSNO or NaHS treatments. The production of NO is decreased but superoxide is increased in CSE-KO ECs in comparison with WT ECs.

H_2S treatment increases EC proliferation, tube formation, angiogenesis, and accelerates wound healing. With an *in vitro* aortic ring angiogenesis assay, we found a reduction in the number of microvessels formed by culturing aortic rings from CSE-KO mice, even in the presence of VEGF (vascular endothelial growth factor). We further found that wound healing is faster in WT mice when compared with CSE-KO mice, and H_2S promotes wound healing recovery. Blockade of NO production by eNOS-specific siRNA or L-NAME (L-NG-nitroarginine methyl ester) reverses, but eNOS overexpression potentiates the proliferative effect of H_2S. In contrast, CSE knockdown attenuates the pro-proliferative effect of NO.

Overall, our studies demonstrate that H_2S increases NO release in ECs through phosphorylation and *S*-sulfhydration of eNOS. Thus, H_2S and NO are required for the physiological control of angiogenesis and superoxide production. Mechanistic understanding of H_2S-NO interaction in vascular endothelium will help advance novel therapeutic strategies for EC dysfunction related vascular diseases.

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DEDICATION

I dedicate this thesis to my beloved parents Nayef and Aminah,

Who inspired me and sparked my interest in pursuing higher education, giving me the help and encouragement every moment.

To the memory of my late beloved uncle Nawaf who died of Cancer in Jordan throughout the process of writing this thesis my thesis, I will never ever forget him and he will be always missed.

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LIST OF ABBEREVIATIONS

BSA – Bovine serum albumin

cAMP – Cyclic adenosine monophosphate

CBS – Cystathionine β-synthase

CSE – Cystathionine γ-lyase

CO – Carbon monoxide

DFO – Deferoxamine

DMEM – Dulbecco's modified eagle medium

DNA – Deoxyribonucleic acid

ECL – Enhanced chemiluminescence

ECs – Endothelial cells

eNOS – Endothelial nitric oxide synthase

ERK – Extracellular signal-regulated kinases

FBS – Fetal bovine serum

G418 –Geneticin

PPG– D, L-propargylglycine

H_2O_2 – Hydrogen peroxide

H_2S – Hydrogen sulfide

HO – Heme-oxygenase

iNOS – Inducible nitric oxide synthase

JNK – Jun n-terminal kinase

K_{ATP} – Adenosine triphosphate-sensitive potassium channel

K_{Ca} – Calcium- activated potassium channel

KO – Knockout

L-NAME – L-NG-nitroarginine methyl ester

LPS – Lipopolysaccharide

LT-PAGE – Low temperature polyacrylamide gel electrophoresis

MAPK – Mitogen-activated protein kinase

mRNA – Messenger ribonucleic acid

NAD+/NADH – Nicotinamide adenine dinucleotide

NF-kB – Nuclear factor-kappa B

nNOS – Neuronal nitric oxide synthase

NO – Nitric oxide

NOS – Nitric oxide synthase

O_2^- – Superoxide anion

$ONOO^-$ – Peroxynitrite

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PI3K – Phophoinositide 3-kinase

PTMs – Post-translational modifications

ROS – Reactive oxygen species

NADPH – Nicotinamide adenine dinucleotide phosphate

SDS-PAGE – Sodium docecyl sulfate-polyacrylamide gel electrophoresis

SMC – Smooth muscle cell

SOD – Superoxide dismutase

TNF – Tumour necrosis factor

WT – Wild-type

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Part (1. 12) in Chapter 1 is currently under review in *Current Medicinal Chemistry* "**Hydrogen sulfide and endothelial dysfunction: relationship with nitric oxide**"

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1. Gasotransmitters

Hydrogen sulfide (H_2S), nitric oxide (NO), and carbon monoxide (CO) are the members of gasotransmitter family [1, 2]. The term "gasotransmitter" was first defined by Dr. Rui Wang in 2002: *i*) It is a small molecule of gas; *ii*) It is freely permeable to membranes; *iii*) Their production are endogenously and enzymatically regulated *v*) They have well-defined and specific functions at physiologically relevant concentrations; *vi*) Their cellular effects may or may not be mediated by second messengers but should have specific cellular and molecular targets" [2]. During the following years, divergent physiological functions of gasotransmitter were discovered. Gasotransmitters signal in multiple ways to modulate numerous cellular proteins, thus affecting cellular metabolism and functions. However, each gasotransmitter has its unique chemical modification. For instance, H_2S modulate cellular activity *via* *S*-sulfhydration and opening K_{ATP} channels, NO *via* *S*-nitrosylation and soluble guanylyl cyclase activation, whereas CO by direct binding to the heme group [3]. The following brief overview provides further context of H_2S and NO gasotransmitters.

1. 1. Hydrogen Sulfide

H_2S is a colorless and flammable gas with rotten-egg smell, often produced by the breakdown of waste material [3]. H_2S is found in nature in volcanic gases and hot springs, or as an introduced contaminant in the environment from petroleum industry [3]. The oxidation of H_2S can form sulfur dioxide (SO_2), sulfates such as sulfuric acid, and elemental sulfur [3]. H_2S is slightly soluble in aqueous solution and weakly acidic with pKa 6.76 [4]. In water, H_2S will dissociate to form hydrosulfide anion (HS^-) (pKa 7.04), and sulfide anion (S_2^-), (pKa11.96) according to the following sequential reactions [4]: $\mathbf{H_2S \longrightarrow H^+ + HS^- \longrightarrow 2H^+ + S^{2-}}$

In physiologic saline at 37°C and pH 7.4, approximately 18.5% of the total sulfide exists as the undissociated acid and 81.5% as the HS^- anion [4]. However, it is difficult to determine which form of H_2S (H_2S, HS^-, or S_2^-, the mix of free inorganic sulfides) is active, therefore, the term "Hydrogen Sulfide" has been used to describe the total free sulfides" [3, 5]. The toxic effects due to H_2S inhalation in natural environment are not common. In addition, H_2S does not accumulate easily in the body [3]. Once H_2S gets into the human body it will be rapidly oxidized to sulfate and thiosulfate, which can be easily excreted in the urine [3]. However, acute exposure to H_2S leads to severe health complication [3]. For example, exposure to high concentration around 800 ppm for 5 minutes could be lethal to human, whereas exposure to concentration up to 1000 ppm causes immediate loss of breathing and death [3]. The toxic effects of H_2S are attributed mostly to mitochondrial poisoning, this toxic effects due to its high affinity to cytochrome *c* oxidase [6]. H_2S can potentially binds to cytochrome and inhibits cellular respiration, thus blocking the regulator of cellular oxygen consumption and ATP synthesis [7].

1. 2. Enzymatic biosynthesis of H_2S

H_2S is produced in a significant amount in most tissue in the human body [2]. The biosynthesis of H_2S in mammalians is generated *via* enzymatic and non-enzymatic pathway [2]. Three enzymes, including cystathionine γ-lyase (CSE), cystathionine beta-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (MST), are responsible for endogenous production of H_2S in mammalians [3]. Both CBS and CSE are pyridoxal 5'-phosphate (PLP) dependent, while MST enzyme is considered zinc-dependent [3]. In general, the expressions of these enzymes are tissue specific. For example, CBS and CSE enzymes are expressed in liver and kidney, and are required for generation of H_2S in these tissues. CSE is mostly responsible for H_2S production in the

vascular system, while CBS is the major source of H_2S in brain [2]. At cellular levels, CSE and CBS enzymes are exclusively localized in the cytosol [2], while MST is localized in both mitochondria and cytosol [3, 8, 9] in normal conditions.

1. 2. 1. Cystathionine β -synthase (CBS)

CBS gene is located on chromosome 21 in humans or 17 in mouse [10, 11]. The CBS protein contains 551 amino acids with a molecular weight of 63kDa [10, 11]. CBS enzyme (EC 4.2.1.22) catalyzes homocysteine to produce cystathionine, which is then turned (cleaved) by CSE into cysteine, α-ketobutryate and H_2S as shown in Figure 1. 1. The active form of CBS enzyme is a homotetramer, containing one heme (protoporphyrim 1X) group and one pyridoxal 5'-phosphate group per subunit [2]. Besides liver and kidney, CBS is also mainly expressed in central nervous system and brain tissue [12]. CBS enzyme is essential for homocysteine metabolism [13]. Human CBS deficiency is caused by inherited rare disorder [13] and affects homocysteine catabolism, and leads to hyperhomocystinuria [14]. CBS deficiency can also lead to a multi-systems disorder such as dislocated lenses, mental deficiency, and osteoporosis, etc. [15].

1. 2. 2. Cystathionine γ-lyase (CSE)

CSE gene is located on chromosome 1, and its protein contains 398 amino acids with a molecular weight of 43 kDa [3]. CSE enzyme (E.C 4.4.1.1) is mainly distributed within the cytosol and requires pyridoxal-5′-phosphate (vitamin B_6) as a co-factor [2]. The two major CSE substrates to produce H_2S are cysteine and cystathionine [2, 16]. CSE enzyme lyses cystathionine into cysteine, α-ketobutryate, and then produces H_2S as shown in Figure 1. 1 [3]. Likewise, CSE lyses cysteine to thiocysteine to produce H_2S (Figure 1. 1). CSE enzyme is

expressed in many tissues such as the cardiovascular system, respiratory system, liver, kidney, placenta and pancreatic islets [17-19]. Human CSE deficiency is caused by autosomal recessive inheritance, and leads to cystathioninuria [20]. The hypercystathioninemia increases the risk of development of atherosclerosis and many types of cancer [21, 22].

1. 2. 3. 3-mercaptopyruvate sulfurtransferase (MST) and cysteine aminotransferase (CAT)

Another recently discovered enzymes involved in H_2S synthesize are MST (EC 2.8.1.2) and CAT (EC 2.6.1.3) [23, 24]. MST and CAT are found in the mitochondria and the cytosol [23, 24]. The MST enzyme is considered zinc dependent, whereas CAT is considered pyridoxal-5′-phosphate dependent [9]. MST and CAT have been shown to produce H_2S in different tissues such as brain and vascular endothelium of thoracic aorta [23, 24]. Both MST and CAT catalyze the sulfur transfer reactions from 3-mercaptopyruvate (3MP) to sulfite or other sulfur acceptors or form elemental sulfur as shown in Figure 1. 1. Human deficiency of MST or CAT is rare, and it is not life-threatening [25].

1. 3. Non-enzymatic synthesis of H_2S

Non-enzymatic production of H_2S is considered a minor source of total endogenous H_2S generation in mammalian systems [2]. H_2S is produced by non-enzymatic reduction of elemental sulfur, which is derived from the reducing equivalents of the oxidized glucose during glycolysis as shown in the equation [26]: $\mathbf{2C_6H_{12}O_6 + 6S0 + 3H_2O \rightarrow 3C_3H_6O_6 + 6H_2S + 3CO_2}$

Garlic and garlic-derived organic polysulfide's can also produce H_2S in a thiol dependent manner [27]. The non-enzymatic oxidation of sulfide will produce thiosulfate that will be subsequently converted to sulfite by the enzymes thiosulfate reductase or/and thiosulfate sulfurtransferase [2].

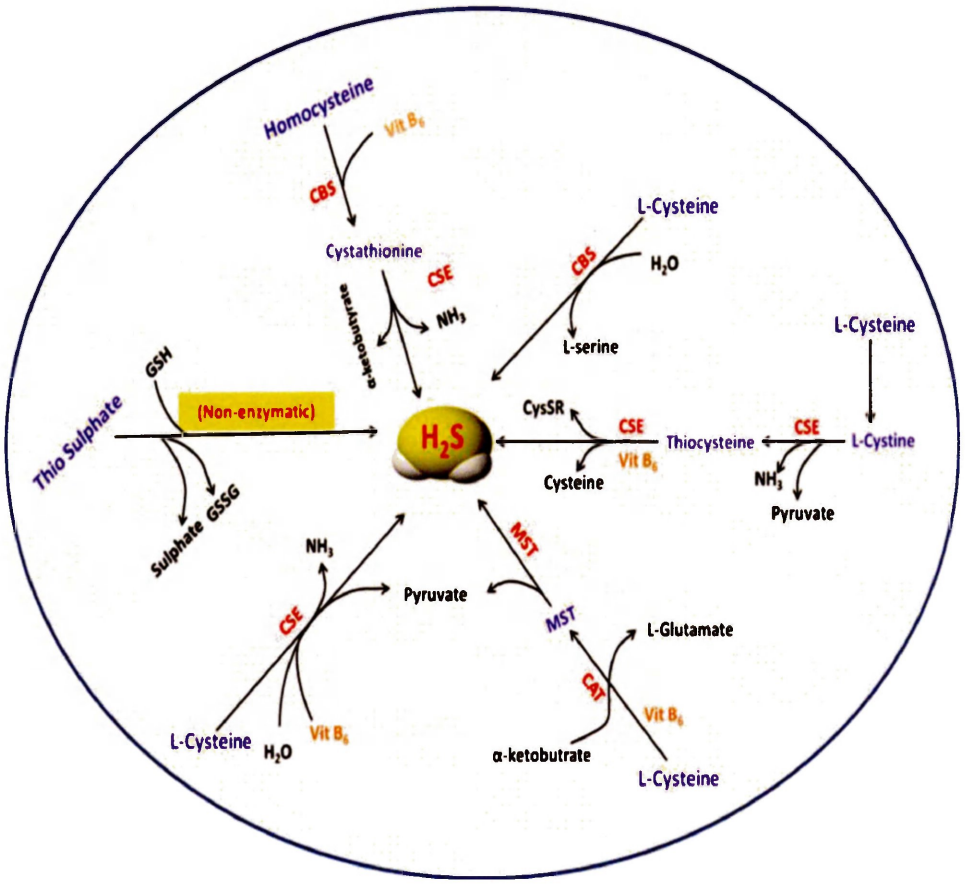


Figure 1. 1. H_2S biosynthesis in mammalian cells. The enzymatic production of H_2S by CSE, CBS and MST in mammalians. This Figure has been modified from [3].

1. 4. Metabolism of H_2S

The metabolism of H_2S maintains the physiological balance and prevents excess accumulation of H_2S. The rapid metabolism of H_2S *in vivo* occurs through many pathways as shown in Figure 1. 2 [2, 3, 28]. H_2S can be exhaled [3], and excreted in the urine primarily as sulfate (either free sulfate or thiosulfate) and in feces and flatus unchanged as free sulfide [3]. In addition, H_2S can be oxidized in the mitochondria of liver cells to produce sulfate [2, 29]. H_2S can be methylated in the cytosol by thiol S-methyltransferase (TSMT) enzyme to produces methanethiol (CH_3SH) and methanethiol, which also can be further methylated to produce dimethylsulfide (CH_3SCH_3). Finally, H_2S can be scavenged by methemoglobin or metallo- or disulfide-containing molecules such as oxidized glutathione (GSSG) [2, 28, 30].

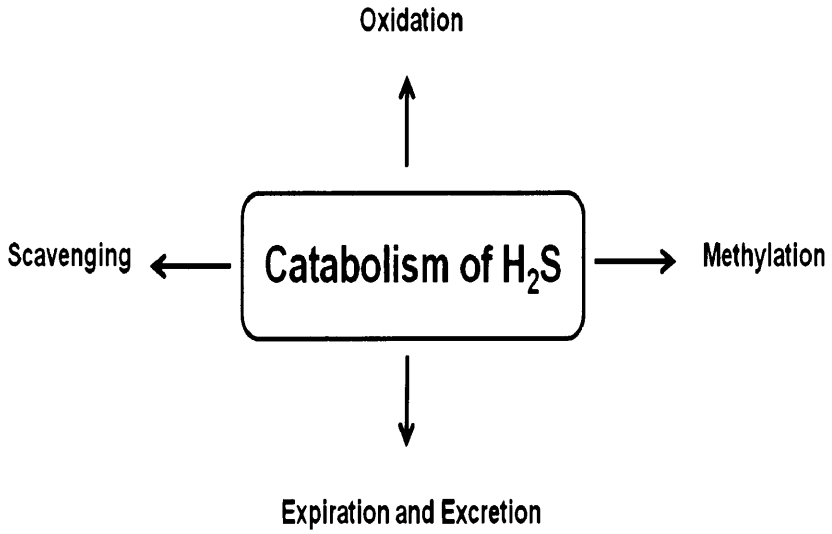
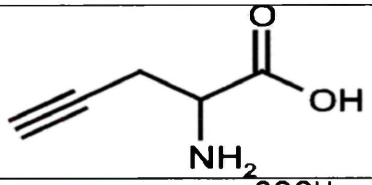
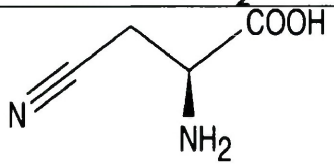
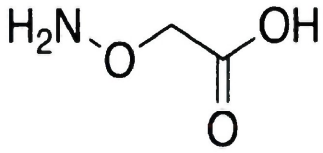
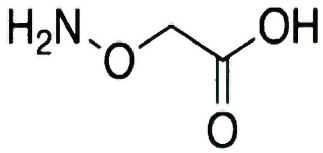
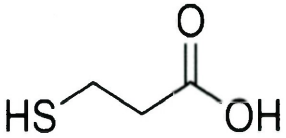


Figure 1. 2. Metabolism of H_2S *in vivo*. The metabolism of H_2S *in vivo* occurs through many pathways such as expiration and excretion, oxidation, methylation and scavenging.

1. 5. Pharmacological inhibitors of H_2S producing enzymes

The development of pharmacological inhibitors to block H_2S biosynthesis is a widely accepted strategy to study the effect of H_2S in different physiological and pathophysiological events. Each enzyme has its unique inhibitor as shown in Table 1. 1. However, the specificity of these inhibitors are not absolute, and varies greatly depending on concentrations and treatment period [3].

Table 1. 1. Pharmacological inhibitors of H_2S producing enzymes

Enzyme	Inhibitor	Structure	Reference
CSE	Propargylglycine (PPG)		[4]
CBS	β-cyanoalanine (BCA)		[31]
	Carboxymethoxylamine (AOA)		[3, 32]
CAT	Carboxymethoxylamine (AOA)		[32]
3-MST	3-mercaptopropionic acid		[33]

1. 6. The discovery of NO

NO is the first discovered gasotransmitter in 1970s [34]. Two decade after discovery of NO, Science magazine chose NO as "molecule of the year" in 1992 [35]. Seven years later, Drs. Robert Furchgott, Louis Ignarro and Ferid Murad were awarded the *Nobel Prize in Physiology or Medicine* in 1998 for their discoveries of the NO [35, 36]. NO acts through several mechanisms such as *i)* activation of soluble guanylate cyclase (sGC) *ii*) binding to a haem proteins (*e.g.* β-globin) [37] *iii)* binding to protein cysteines in a process called *S*-nitrosylation (*e.g.* GAPDH) [38]. The following brief overview provides further context on NO in details.

1. 7. The chemistry of NO

NO is a free radical with a molecular weight of 30 and vapor density (d) of 1.249. The half-life of NO *in vivo* is very short, close to five seconds [39, 40]. However, single molecule of NO can readily moves between cells many times within this time span [41]. The lipophilic nature and small size of NO enables it to diffuse over cell membranes within a millisecond without channels or receptors [40, 42]. The concentration of NO is determined by the rate of formation and catabolism. Three major mechanisms are shown in Figure 1. 3 for NO metabolism. These mechanisms include the reaction of NO with molecules that have unpaired electrons, *i.e.* superoxide anion to produces peroxynitrate ($ONOO^-$) [43], binding of NO with transition metals ions, *i.e.* CuNO [43]. Finally, oxidation of NO to generate elemental nitrogen, *i.e.* nitrite (NO_{2-}) and nitrate (NO_{3-}) in aqueous systems or air [44, 45].

Although NO is reported to have potentially different toxic effects, many of them are more likely mediated by its oxidation products rather than NO itself [41]. The reaction of NO

and superoxide occurs rapidly and forms peroxynitrite anion ($ONOO^-$), which has a powerful oxidant ability and leads to several cellular/tissue injury [41]. Furthermore, excessive peroxynitrite formation leads to nitrated proteins, inhibition of mitochondrial respiration, DNA damage, apoptosis and necrotic cell death [46].

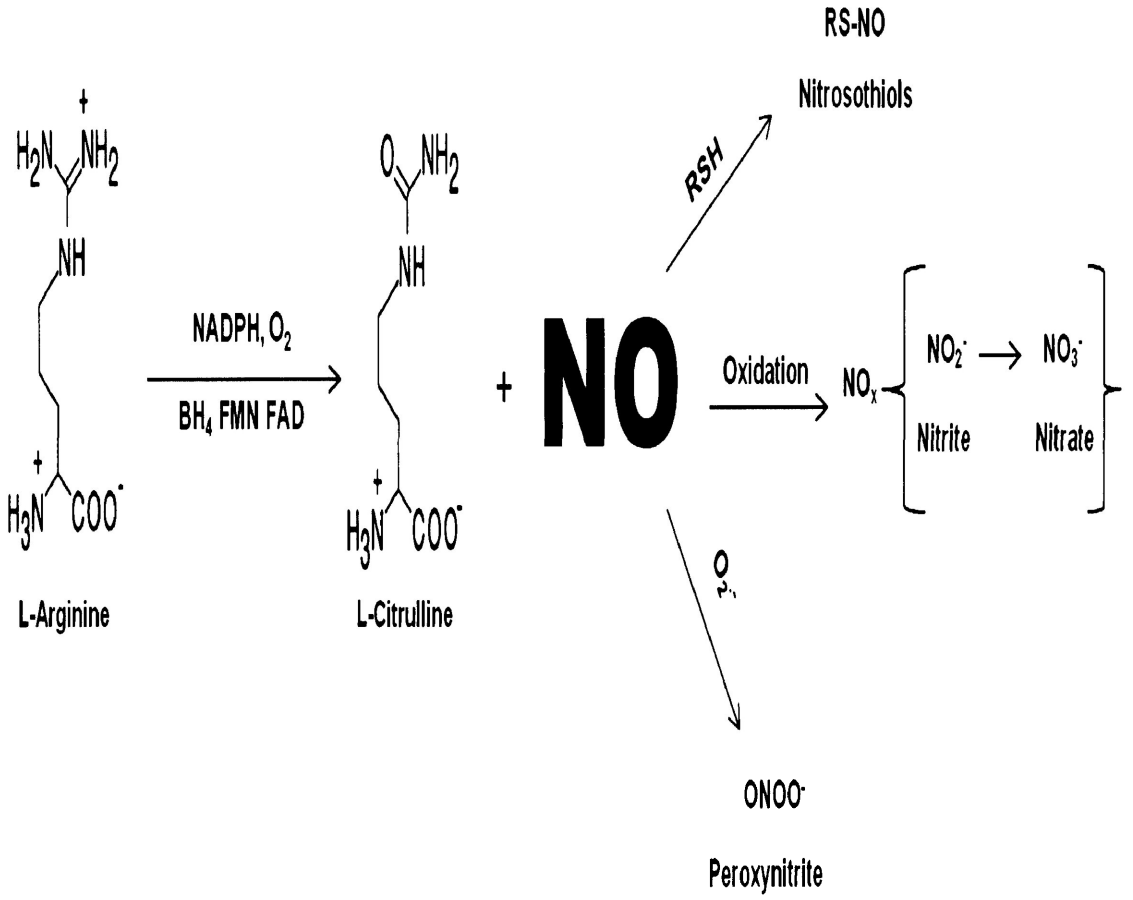


Figure 1. 3. NO production and metabolism in mammalian cells. L-arginine is catalyzed by NOS enzymes to produce L-citrulline and NO. Oxygen and NADPH are essential co-factors for NOS enzymes. Three major ways for *in vivo* NO metabolism are oxidation, binding with thiols, and superoxide.

1. 8. Enzymatic biosynthesis of NO

NO is synthesized from l-arginine by NOS, of which there are three isoforms featuring different tissues distribution and functions: endothelial (eNOS; 130 kDa), neuronal (nNOS; 160 kDa) and inducible (iNOS; 130 kDa) [36, 47]. The homology among NOS isoforms are about 51-57%. The classification of NOS isoforms depends on several factors including gene origins, localization within the cells, and their mechanisms of regulation [48, 49]. For example, eNOS protein is located in plasma membrane rather than cytosol [50-52]. iNOS and nNOS are located in the cytosol and considered as soluble proteins [52]. An increase in intracellular Ca^{2+} levels will stimulate the binding of calcium to calmodulin (CaM) [53], and activation of eNOS and nNOS [36]. Whereas, iNOS is described as Ca^{2+}-insensitive isoform, likely due to its tight non-covalent interaction with CaM and Ca^{2+} [36]. Moreover, iNOS isoform is not constitutively expressed under normal condition, and its expression requires induction by additional stimulus such as inflammatory agents [54]. iNOS isoform is expressed with CaM bound to the protein, and thus making iNOS insensitive to intracellular Ca^{2+} concentration changes [48].

1. 8. 1. Neuronal NOS (NOS I)

nNOS was isolated from brain tissue in rat and porcine cerebellum [55, 56]. The termed neuronal NOS or NOS I is based on the order of their first purification and their location [55, 56]. The nNOS isoform can be expressed sometimes in non-neuronal cell types including SMC, skeletal muscles, kidneys, lung epithelial cells, skin and other cells or tissues [57-59]. NO produced from nNOS participates mainly in neurotransmission and neuromodulation [60, 61]. NO acts as a neuromodulator that regulates synaptic or postsynaptic plasticity, both NOS inhibitors N-nitro-L-arginine (L-NNA) or L-NAME, blocks the long-term potentiating of

synaptic transmission [62]. NO is also involved in neurodevelopment and in late phase differentiation of motor neuron [63]. Moreover, NO acts as a regulator of various genes in neurons cells. Up to 63 genes were found to be NO dependent, and these genes may be required for neuron survival and development [64].

Human nNOS gene is located on chromosome 12q24.21, and considered to be the largest isoform [58, 65]. nNOS protein has a total of 1439 amino acids, compared with another 2 isoforms, nNOS has extra 300 amino acids located at the N-terminus [58, 65]. The nNOS shares a high sequence homology between species, with 93% amino acid identity between rat and humans, with a unique regions termed PDZ domain as shown in Figure 1. 4 [66]. PDZ domain is a very common domain and occurs in a variety of dissimilar enzymes [67]. The PDZ domain interacts with several proteins that contain similar domain and creates PDZ-PDZ interaction [68], and modulate its function. For example, PDZ domain of nNOS binds to a similar domain of a post-synaptic density protein 95 (PSD-95) [69], and this PDZ-binding mediates synaptic association of nNOS and regulates the formation of macromolecular signaling complexes [69]. The sequenced human genome boasts over 150 PDZ containing proteins [67, 70]. However, only nNOS has PDZ domain, but not eNOS and iNOS [71-73].

1. 8. 2. Inducible NOS (NOS II)

iNOS was first purified from murine and rat macrophages in 1991 [74]. Human iNOS gene is located on the chromosome 17q11.2 -q12, and iNOS protein has 1153 amino acid [65, 75, 76]. The iNOS is considered as Ca^{2+}-independent [49, 77-79] and stimulated under inflammatory conditions [49, 77-79]. For example, various pro-inflammatory cytokines such as interleukin-1beta (IL-1β), interleukin -6 (IL-6), or tumor necrosis factor-α (TNF-α) will evoke

iNOS expression [77]. As a result, NO will be produced in high concentration up to micromolar range, which can last comparatively for a long period until the enzyme is degraded [77, 80]. The NO levels released from iNOS were suggested to reach up to 100 - 1000 fold more than NO released from eNOS isoform [81, 82], suggesting the correlation of iNOS and the pathological conditions of numerous diseases [77, 83, 84].

1. 8. 3. Endothelial NOS (NOS III)

eNOS was first purified from bovine aortic ECs [85]. Human eNOS gene is located on the chromosome 7q36, and eNOS protein has a 1203 amino acid [65]. The expression of eNOS gene is predominantly restricted in EC, however, other reports have detected eNOS in other cells such as cardiomyocytes, hepatocytes, thrombocytes, and lung epithelial cells [86]. NO is present in all blood vessels and mainly acts as a vasodilator in the vascular system. The produced-NO from ECs dilates all types of blood vessels [79, 87]. NO diffuses across the ECs into the underlying SMC and activates sGC, thus increasing the cyclic guanosine monophosphate (cGMP) and triggering SMC relaxation and subsequent dilation of the blood vessel [79, 87].

Genetically engineered mice in which eNOS gene was knockout have been developed [88]. These mice demonstrated the absence of eNOS mRNA and enzymatic activity, but are fertile and have normal anatomy [88, 89]. eNOS deficiency leads to lower endothelium-derived relaxing factor (EDRF) activity and development of hypertension [88, 89], increased vascular SMC proliferation [90], platelet aggregation [91], leukocyte–endothelial adhesion [92], abnormalities in mitochondrial function and biogenesis [93], insulin resistance [94], development of severe strokes [95] and atherosclerosis [96].

1. 9. NOS structure

The three NOS isoforms in human exhibit a common bi-domain structure as shown in Figure. 1. 4. The first domain structure is called oxygenase domain located on the N-terminal [48]. The oxygenase domain contains binding sites for iron haem, H_4B and L-arginine [48]. The second domain is called reductase domain located on the C-terminal [48]. Reductase domain includes binding sites for the redox cofactors nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) respectively [79]. The inter-domain linker is located in the center of NOS enzyme, between the reductase and oxygenase domain, and contains the calmodulin (CaM)-binding sequence [97]. The CaM-binding site acts as a switch to regulate electron flow between the reductase and oxygenase domain to produce NO as will be discuss latter in details.

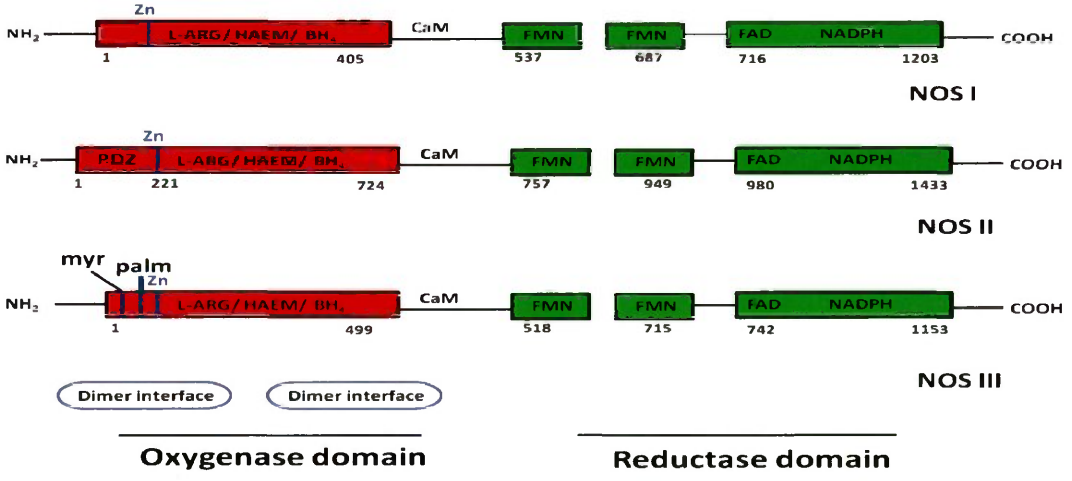


Figure 1. 4. Structural domains of human NOS isoforms. Oxygenase domain contains binding sites for (L-arginine, haem, and BH_4) and important for NOS dimerization. Reductase domain contains binding sites for redox cofactors (NADPH, FMN, and FAD). This figure is modified from [48].

1. 10. NOS coupling and dimerization

The dimerization or coupling state of NOS enzyme is essential for its activation [53]. The two NOS dimer interfaces are located in the oxygenase domain (Figure. 1. 4) [48, 98]. When NOS is coupled, the electrons donated from NADPH will flow from the reductase domain of one monomer to the oxygenase domain of the other monomer as shown in Figure. 1. 5 [99, 100]. These electrons will precede one by one *via* the redox carriers FAD and FMN, to bind with heme group and BH_4 [53, 100]. This binding will catalyze the reduction of oxygen with L-arginine to generate citrulline and NO [100].

However, in various pathological conditions, NOS becomes uncoupled, and it will no longer use the enzymatic reduction of oxygen to produce NO [53]. The uncoupled NOS will generate superoxide instead of NO [100]. The link between NOS activity and its conformation status have been shown in vascular system. For example, ECs treated with peroxynitrite were associated with reduced eNOS activity and disruption of eNOS dimers [101]. ECs exposed to high glucose or in organs from diabetic mice disrupt eNOS dimers [101]. The reduction of eNOS dimer/monomer ratio is combined with increased oxidative stress and reduced NO formation in hyperglycemic ECs [102]. *In vitro* studies have shown that L-arginine and BH_4 were essential for dimer stability [48]. The present of sufficient substrate L-arginine, and co-factor BH_4, allow NOS dimers to couple with the heme and oxygen reduction, contributing to the synthesis of NO [103]. In contrast, reduction in BH_4 bioavailability induces uncoupling of NOS, and leads to superoxide production [104]. More evidence has shown the importance of zinc in NOS dimer stability as well [105-107]. Zinc in NOS has a structural rather than a catalytic function [107]. NOS may use zinc binding to maintain the conformational stability of the dimer interface. Each NOS proteins contain a zinc-thiolate cluster (ZnS_4) formed by a zinc ion that is tetrahedrally

coordinated to two CysxxxxCys motifs (one contributed by each monomer) at the NOS dimer interface [98, 107, 108], mutation of these binding sites prevent zinc binding, and eliminates NOS dimer [105-107].

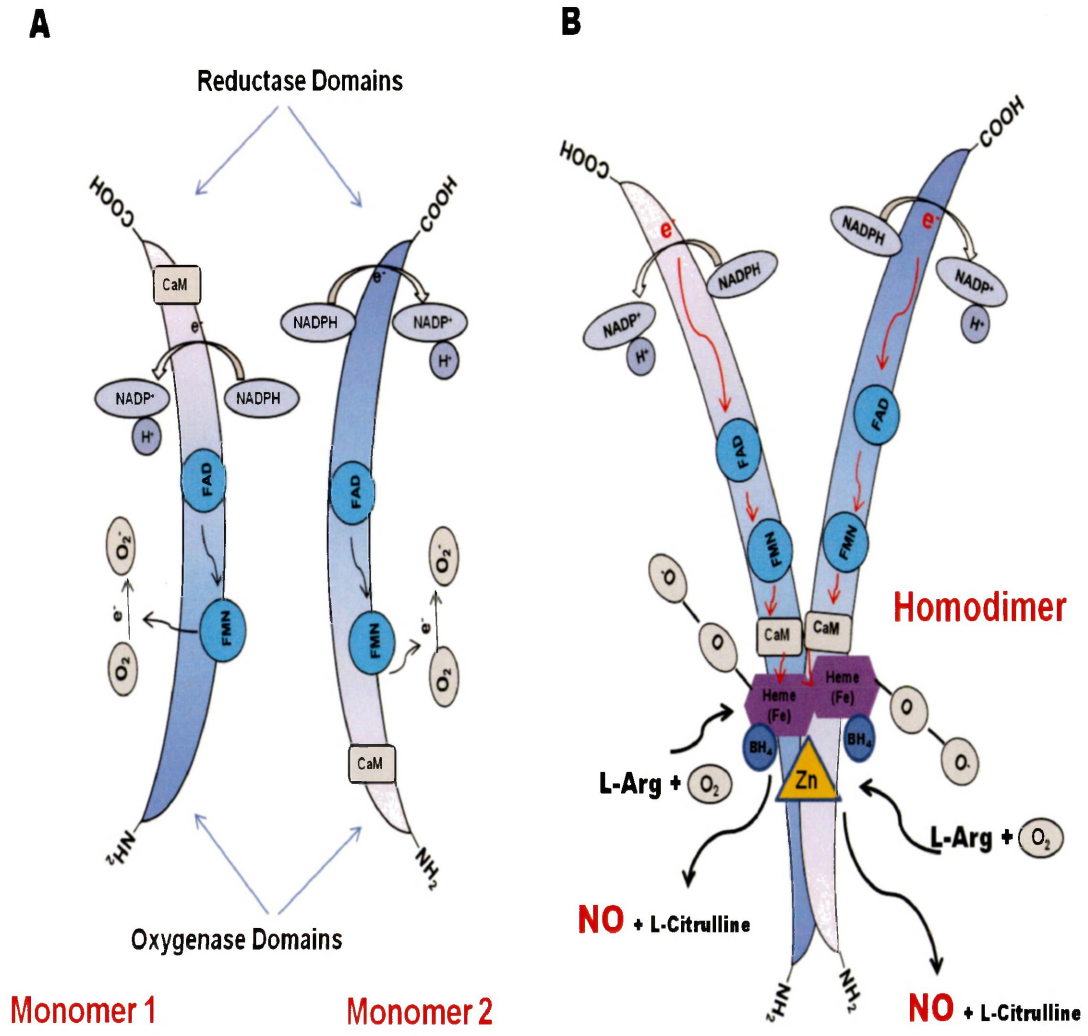


Figure 1. 5. NOS dimeric structure. Functional NOS protein contains two identical sub-units of the reductase and oxygenase domain. The dimeric conformation allows electrons to flow in the NO synthase reaction to produce NO as following: NADPH -- > FAD -- > FMN -- > heme -- > O_2. The picture was modified from [100].

1. 11. The roles of H_2S and NO in post-translational modifications

H_2S and NO contribute to cell signaling by inducing posttranslational modifications of proteins, and these are summarized briefly in Table 1. 2. Posttranslational modifications not only affect the structure of individual proteins but also modify protein-protein interaction and function. This review provides an introduction of current knowledge of H_2S and NO proteins modifications promoting or inhibiting cell functions with special attention in ECs and eNOS.

1. 11. 1. Phosphorylation

Protein phosphorylation is the attachment of a phosphate (PO_4^-) group to a protein. The new negatively charged phosphorus group makes allosteric conformational changes and can alter the role of eNOS: it can activate, deactivate, or cause a change in function [53]. Phosphorylation is the most widespread type of post-translational mechanism for eNOS regulation; eNOS protein can be phosphorylated at multiple sites such as serine (Ser-114, Ser-617, Ser-635, and Ser-1177/1179), theronine (Thr-495) and tyrosine (Tyr81 and Tyr567) residues [109, 110] as shown in Figure. 1. 6.

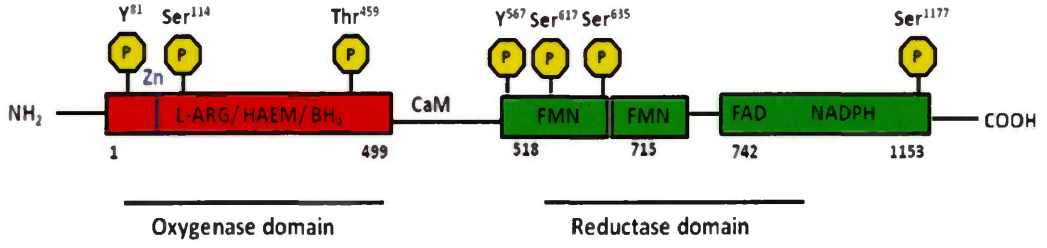


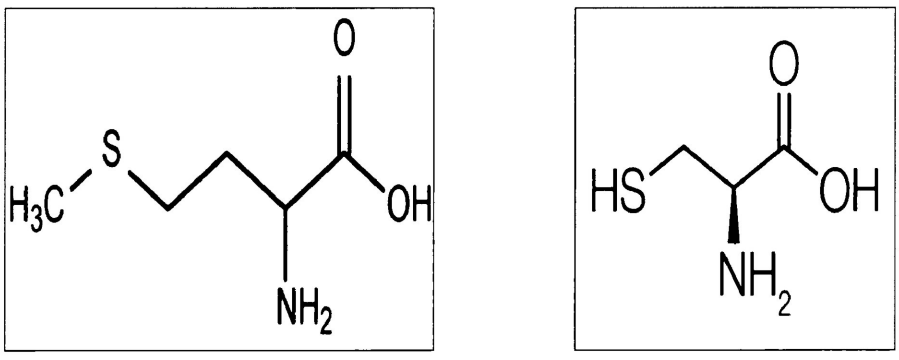
Figure 1. 6. Multiple phosphorylation sites of eNOS protein

As mention earlier eNOS phosphorylation at different site may have different regulatory effect. For example, phosphorylation at Ser-114 inhibits eNOS activity [111]. Whereas, phosphorylation of Ser-1179, Ser-617 and Ser-635 stimulate eNOS activity [112]. The phosphorylation Tyr-81 stimulates eNOS activity [113], and Tyr-567 attenuates eNOS activity [114]. Among the numerous potential phosphorylation sites in eNOS, perhaps the best studied is Ser-1179. The activation of eNOS at Ser-1179 is primarily dependent on CaM [100, 115]. The eNOS activity can be acutely regulated by increasing the intracellular Ca^{2+} concentrations [116]. The increase in intracellular Ca^{2+} levels will activates CaM which in turn activates CaM kinase II and phosphorylates Ser-1179 [117]. The general mechanism of CaM regulation is that CaM binds to the CaM-binding motif and displaces the adjacent auto-inhibitory loop on eNOS, thus facilitating NADPH-dependent electron flux from the reductase domain of the protein to the oxygenase domain [118].

Indeed, many studies have proven the ability of H_2S and NO as inducers of phosphorylation. For example, H_2S induces p38 MAPK, Akt and eNOS phosphorylation in EC, and thus maintains its proliferation [119]. H_2S increases ERK1/2 phosphorylation in SMC, and thereby regulates its proliferation and apoptosis [120]. H_2S increases the phosphorylation of vasodilator-stimulated phospho-protein (VASP- Ser-239) in EC, and maintains angiogenesis [121]. Similarly, NO induces p38 MAP kinase, JNK, and Erk1/2 phosphorylation in neural progenitor cells [122], JNK phosphorylation in neuroblastoma cell line (SH-Sy5y) [123], and thereby mediate apoptosis. NO induces protein kinase C (PKC)-ɣ and p53 phosphorylation in, mouse embryonic substantianigra-derived cell line (SN4741), and thereby contributes to apoptosis [124]. NO induces TNF phosphorylation in murine fibroblast cell line (L929), and contributes to L929 cell death [125].

1. 11. 2. Cysteine modification: *S*-nitrosylation and *S*-sulfhydration

Cysteine is a highly unique amino acid due to its thiol side chain reactivity. The pKa of the free cysteine thiol is between 8 – 9 [126]. The reactivity of thiols is correlated with its pKa value [127]. Structurally cysteine and methionine belong to the sulfur amino acids, because of sulfur atom appearing in its side chain as shown in Figure 1. 7. However, cysteine differs from methionine which has a methyl group attached to the sulfur, so methionine is more hydrophobic, sterically larger and much less reactive than cysteine [128]. Numerous reactions are known to occur on cysteine thiol side chains that affect protein structure and function [129], such as the formation of disulfide bond between two sulfur atoms derived from two or multiple thiol groups in the present of oxidizing agent [129], reaction with several metal ions, including zinc, copper and iron [130]. Considering the remarkable reactivity of cysteine thiol group, cysteine can play a key biological role in catalysis and serve as an important site for many posttranslational modifications such as *S*-nitrosylation and *S*-sulfhydration. The following brief overview provides further context on cysteine modification induced by H_2S and NO.



Methionine **Cysteine**

Figure 1. 7. Structure of sulfur containing amino acids: methionine and cysteine

1. 11. 2. 1. *S*-nitrosylation

S-nitrosylation is a principle mechanism of NO-based signaling, and refers to the binding of NO group to thiol group (-SH) of protein cysteine residues resulting in the formation of *S*-nitrosothiol (S-NO) [131]. The analysis of *S*-nitrosylation shows that not all protein cysteines that remain in the free-thiol state become *S*-nitrosylated [132]. The molecular mechanisms for *S*-nitrosylation are still unclear. However, many factors make the sulfhydryl in cysteine more reactive or accessible to *S*-nitrosylation agent including, *i)* amino acid microenvironment and the present of the acid-base motif *i.e*, cysteine residue between Aspartic acid (acid)-Histidine(base) motif becomes a valid cysteine for *S*-nitrosylation in many proteins. *ii)* nucleo-philicity (pKa), *iii)* presence of different metal ions (such as Mg_2^+ or Ca_2^+) [131].

The three NOS isoforms (nNOS, iNOS and eNOS) are all capable of driving *S*-nitrosylation, which is direct evidence that NO can undergo redox reactions necessary to form SNO. *S*-nitrosylation can influence protein activity, by either inhibition or activation. A study done by Santhanam *et al* [133] showed that *S*-nitrosylation of arginase1 in aging rats is mediated by iNOS, and enhances arginase1 activity, which, in turn, reciprocally reduces eNOS-dependent NO production, and contributes to EC dysfunction in aging rats [133]. *S*-nitrosylation of H-Ras is mediated by nNOS, which inhibits calcium ionophore-mediated extracellular-signal-regulated kinase activity [134]. Finally, eNOS itself can be *S*-nitrosylated, treatment with GSNO nitrosylate eNOS and inhibits its activity, whereas, de-nitrosylation leads to eNOS activation [135].

S-nitrosylation can be reversed by de-nitrosylation [136]. Importantly, the specificity and reversibility of *S*-nitrosylation enables cells to dynamically modify function in response to redox alteration in their environment. Generally de-nitrosylation occurs enzymatically and non-

enzymatically [137]. The non-enzymatic de-nitrosylation can be accomplished by removing the reduced environment of protein, this can be accomplished by several factors such as reducing agent, transition metals and UV [137]. Furthermore, de-nitrosylation can be accomplished enzymatically [112] by two enzymes *S*-nitrosoglutathione reductase (GSNOR) and thioredoxin systems (Trx) [112]. GSNOR removes -NO groups from cysteine thiols in proteins (SNO-proteins) through metabolism of S-nitrosoglutathione (GSNO, which is in equilibrium with SNO-proteins) [138]. Whereas, Trx is a small redox protein widely expressed in most organisms and known for its disulfide reductase activity. Trx has highly conserved and active cysteinemotif (CXXC), located on its exterior [136]. These cysteine motifs promote electron and disulfide exchange between Trx and its substrates, and thus mediates de-nitrosylation [139], as shown in Figure. 1. 8.

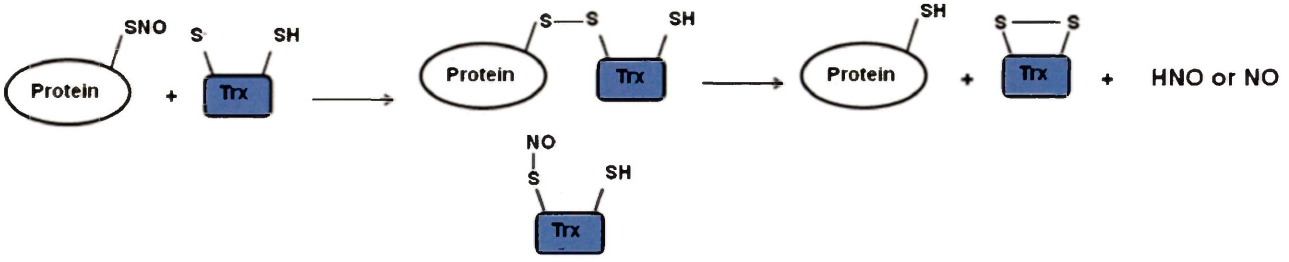
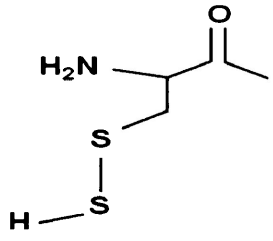
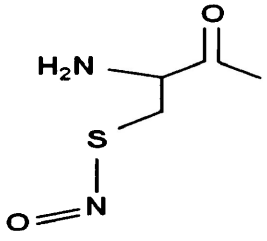
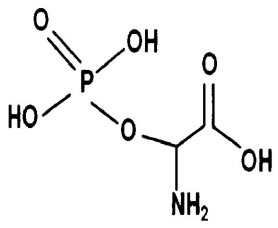


Figure 1. 8. Proposed mechanisms of de-nitrosylation by thioredoxin (Trx). Two reaction mechanisms are involved in de-nitrosylation mechanism. First, formation of an intermolecular disulphide intermediate (in which Trx is covalently linked to the substrate protein through a disulphide bridge). Second, transnitrosylation (in which Trx is transiently S-nitrosylated). This Figure has been modified from [139].

1. 11. 2. 2. *S*-sulfhydration

S-sulfhydration is increasingly recognized as an important signaling mechanism for H_2S [140]. *S*-sulfhydration refers to the conversion of cysteine (-SH) groups to hydropersulfides (-SSH) [140]. The first report on *S*-sulfhydration was published in 2009, Mustafa et al, showed that about 10 to 25% of expressed proteins in liver tissue can be sulfhydrated under physiological conditions [140]. They showed that *S*-sulfhydration of these proteins changes their functions. For example, *S*-sulfhydration of GAPDH elicits a seven fold increase in its activity [140]. *S*-sulfhydration of actin enhances its polymerization and reveals rearrangement of the actin cytoskeleton [140]. H_2S *S*-sulfhydration has also been reported to regulate other physiological events. For example, H_2S *S*-sulfhydrates ATP-sensitive potassium (K_{ATP}) channel in vascular SMCs, and thus induces relaxation of the vascular tissues [141]. H_2S leads to *S*-sulfhydrates and hyperpolarization of ECs through intermediate (IK_{Ca}) and small conductance (SK_{Ca}) calcium-dependent potassium channels [142]. H_2S *S*-sulfhydrates the nuclear factor κB (NF-κB), and physiologically determines its antiapoptotic transcriptional activity [143]. H_2S *S*-sulfhydrates protein tyrosine phosphatases (PTP1B), and alters endoplasmic reticulum stress response in HEK-293T cells [144]. H_2S *S*-sulfhydrates Kelch-like ECH-associated protein 1 (Keap1), and attenuates oxidative stress and delays cellular senescence in mouse embryonic fibroblasts cells [145]. H_2S *S*-sulfhydrates parkin and enhances its E3 ligase activity [146]. Collectively, these studies show that H_2S *S*-sulfhydrates a large number of proteins in different tissues under physiological condition. However, it is still far away to fully understand the physiological and pathological implications of *S*-sulfhydration as well as its enzymatic regulation.

Table 1. 2. Posttranslational modification effects of H_2S and NO in ECs

	***S*-sulfhydration**	***S*-nitrosylation**	**Phosphorylation**
Gasotransmitters	H_2S	NO	H_2S and NO
Modification type	Non-catalyzed chemical modification	Non-catalyzed chemical modification	Enzyme driven
Effect on enzyme activity	Increase	Mostly decrease	Increase or decrease depending on the phosphorylated site
Reversible mechanism	Not known	Reversible: *i.e* Thioredioxine, GSNO reductase	Reversible: *i.e* Phosphatases
Energetic mechanism	No ATP needed	No ATP needed	Bio - energetic and need ATP
Bond-dissociation energy	60 kcal/mole	12 - 20 kcal/mole	5 kcal/mole
Detection method	Modified biotin switch assay/ Immunoblot, LC MS/MS	Biotin switch assay + Ascorbate, LC MS/MS	Phospho-specific antibody/ Immunoblot
Known modified proteins	GAPDH, actin, eNOS	GAPDH, Akt, eNOS	Akt, ERK1/2, p38, eNOS
Bond	Hydropersulfide (-SSH)	S-nitrosothiol (-SNO)	Phosphate ($-PO_4^{3-}$)
Amino acid residue	Cysteine	Cysteine	Serine, theronine, alanine
Modification			

1. 12. Pathophysiological roles of H_2S and NO in ECs

The H_2S and NO bioavailability in the vascular system, especially in endothelium, are important determinant for many pathophysiological events, which affect various vascular functions. Here we briefly reviewed the roles of H_2S and NO in regulation of different EC function, and their precise role in diseases related to EC dysfunction.

1. 12. 1. The roles of H_2S and NO in vasodilation

H_2S, released from sodium hydrogen sulphide (NaHS), dilates blood vessels both *in vitro* and *in vivo*, albeit distinct vascular beds display different sensitivities to the gasotransmitter [2, 3]. The vasorelaxing action of NaHS, administrated at concentrations ranging from 50 μM to 100 μM, has been observed in rat aorta and hepatic artery [17, 147], as well as in resistance mesenteric arteries [148], gastric artery and gastric mucosal circulation [149], cerebral arterioles and artery [150, 151], pulmonary artery [152], and coronary artery [153]. Since NaHS is five-to-nine fold more potent in relaxing mesenteric arteries than thoracic aorta and pulmonary artery (EC_{50} 25 μM *vs.* 125 μM and 233 μM, respectively), it has been proposed as a key regulator of peripheral resistance arteries and, therefore, of blood pressure [3]. H_2S has been shown to dilate other isolated mammalian blood conduits *in vitro*, such as human internal mammary artery [154], mouse aorta and ear microcirculation [155, 156], and newborn pig cerebral arterioles [157]. Consistent with these data, the systemic injection of NaHS at 10-50 μM kg^{-1} causes a transient decrease in mean arterial pressure (MAP) *in vivo* by 12-40 mmHg [17, 158, 159], whereas GYY4137 (26-133 μM Kg^{-1}), a slow H_2S-releasing donor, produces a slowly developed fall in MAP that persists long after drug administration [159]. These observations have been corroborated by the CSE-knockout (CSE-KO) mouse model. CSE-KO mice develop an age-

dependent hypertension starting at 7 weeks of age, and peaked at 12 weeks [19]. This significant elevation in blood pressure is associated with the lack of H_2S leading to reduced endothelium-dependent and elevated resting-membrane-potential of VSMCs[19], but not that those of aorta [160]. These observations are compatible with the notion that H_2S serves as an endothelium-derived relaxing factor (EDRF) [161]. It has been proposed that acetylcholine activates CSE in a Ca^{2+}/CaM-dependent manner in ECs [162, 163]. Once produced, H_2S may diffuse to the adjoining vascular SMCs (VSMCs), where it activates K_{ATP} channels, promoting membrane hyperpolarization and reducing vascular tone by counteracting the activation of voltage-gated Ca^{2+} channels [17, 161]. H_2S may also act as an EDHF, as recently show in the mesenteric arteries of CSE-KO mice. H_2S causes endothelium-dependent hyperpolarization of VSMC by stimulating intermediate- and small-conductance K_{Ca} (IK_{Ca} and SK_{Ca}) channels [142, 160]. H_2S has been suggested to mimic NO effect on cGMP level but through different mechanisms. While NO stimulates cGMP production, H_2S decreases cGMP degradation by inhibiting phosphodiesterase 5 (PDE-5) activity in VSMCs, which leads to vasorelaxation in a PKG-dependent manner [115, 121, 164]. In aortic ECs isolated from WT mice, H_2S activates the PI-3K/Akt signaling pathway following increased eNOS phosphorylation in Ser-1177, thereby increasing NO production [119]. Likewise, NO-induced cGMP accumulation and vasorelaxation were attenuated by the genetic knockout of CSE [165], suggesting that both gasotransmitters may act on cGMP to reduce blood pressure.

1. 12. 2. The roles of H_2S and NO in apoptosis

H_2S affects programmed cell death, or apoptosis, in a cell-specific manner. For instance, NaHS has been shown to prevent apoptosis in both neuronal and non-neuronal cells,

cardiomyocytes, colon cancer cells, and 3T3 fibroblasts, at concentrations lower than 300 μM [3]. The anti-apoptotic action of H_2S is related to its capability to prevent the mitochondrial membrane potential dissipation by the activation of multiple mechanism, but all through K_{ATP} channels [3]. H_2S stimulates VSMC apoptosis [166]. The endogenous production of H_2S derived from CSE overexpression induces apoptosis in human VSMCs by activating ERK1/2 and capsase-3 [166]. Interestingly, VSMCs isolated from CSE-KO mice were more susceptible to apoptosis induced by exogenous H_2S at 100 μM. The pro-apoptotic effects of H_2S are mediated by the phosphorylation of ERK1/2 and expression of cyclin D1 and p21 (Cip/WAF-1) [120]. As for vascular endothelium, NaHS pretreatment has recently been demonstrated to decrease SA β-gal (cell senescence-associated β-galactosidase) positive rate and cellular apoptosis in HUVECs [167]. Similarly, NaHS triggers an anti-oxidative stress mechanism which protects primary HUVECs from apoptosis challenged with high glucose [168]. The anti-apoptotic action of H_2S in macrophages cells is mediated by *S*-sulfhydration of NF-kB [143]. H_2S sulfhydrates the p65 subunit of NF-κB at cysteine-38, which promotes its binding to the co-activator ribosomal protein S3 (RPS3) [143]. H_2S inhibits NO production, iNOS gene expression and NF-κB activation in LPS -stimulated macrophages cells *via* a mechanism involving the action of heme oxygenase-1 (HO-1) and CO [169]. Finally, the pro-apoptotic or anti-apoptotic effects of NO depends on the concentration of NO employed, *i.e.* nano-molar range affects Akt phosphorylation and hypoxia inducible factor (HIF)-1α stabilization (pro-survival pathways) and prevent apoptosis, whereas micromolar range triggers phosphorylation of p53 and induces apoptosis [170].

1. 12. 3 The roles of H_2S and NO in oxidative stress

H_2S is a strong reducing agent and may easily interact with oxidative species [3]. Several studies have shown that H_2S displays anti-oxidant activity at 10 - 100 μM [158, 171, 172], which would protect the luminal surface of blood vessel from the oxidative stress caused by ischemia/reperfusion injury, as well as from the development of atherosclerotic lesions [173, 174]. H_2S rescues CSE- KO mice from the injury and mortality associated with renal ischemia. Moreover, CSE overexpression reduces the amount of reactive oxygen species (ROS) produced during stress in renal tissues [173]. NaHS has been shown to mitigate the methionine-induced production of free radicals in mouse brain ECs (bEnd3) and to enhance the inhibitory action of reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), L-NAME on ROS production [175]. NaHS (10-500 μM) preserves mitochondrial function by reducing the deleterious effects of oxidative stress on the antioxidants enzymes, SOD, catalase, glutathione peroxidase and glutathione-*S*-transferase [176]. Furthermore, H_2S delays EC senescence by attenuating oxidative stress [167]. A study show that late passages (e.g. 12) HUVEC have lower SOD activity and higher H_2O_2 level as compared with younger HUVECs (e.g. 4), whereas NaHS pretreatment reverses the changes of SOD activity and H_2O_2 level [167]. Similarly, the expression levels of both xanthine oxidase (XO) and subunits p67 (phox) of NADPH oxidase are increased in the old group relative to the young one, whereas manganese-superoxide dismutase (Mn-SOD) expression levels are decreased. NaHS treatment results in the up-regulation of both XO and p67(phox) levels but down-regulation of Mn-SOD expression [167], thereby slowing cell aging. Another study shows that H_2S delays HUVEC senescence and prevents H_2O_2-induced damage *via* sirtuin 1 activation [177]. In addition, H_2S-releasing drugs, NaHS and ACS6 (both at 10 μM), inhibit superoxide formation and gp91 (phox) (a catalytic subunit of NADPH oxidase)

expression in porcine pulmonary arterial ECs by increasing cAMP levels and recruiting PKA upon the inhibition of phosphodiesterase type 5 (PDE-5) [178]. The cytoprotective effect of H_2S in vascular endothelium might involve an increase in the intracellular levels of GSH, one of the most abundant and effective components of the defense system against free radicals [145]. Studies conducted on mouse embryonic fibroblasts isolated from CSE-KO mice (CSE-KO-MEFs) shaw an increase in oxidative stress and acceleration in cellular senescence compared with MEFs isolated from wild-type mice (WT-MEFs) [145]. Incubation of CSE-KO-MEFs with NaHS significantly increases GSH levels and rescues KO-MEFs from senescence [145]. H_2S sulfhydrates Keap1, which regulates the antioxidant response, thereby inhibiting Nrf2 activity [145]. *S*-sulfhydration of Keap1 at cysteine-151 induces Nrf2 dissociation from Keap1, thus enhancing the nuclear translocation of Nrf2 and stimulating mRNA expression of Nrf2-targeted downstream genes, such as glutamate-cysteine ligase and glutathione reductase [145]. In addition, H_2S might scavenge $ONOO^-$ and suppress tyrosine nitration in VSMCs in the presence of homocysteine (Hcy) [179]. The anti-oxidant properties of H_2S could therapeutically be exploited to prevent lung endothelium damage caused by oxygen therapy [180], particulate air pollution or tobacco smoke [181]. Similarly, regulation of NO production during conditions of oxidative stress is very important for cell survival. Previous studies have shown that increased oxidative stress is often associated with decreased NO levels [182]. Additionally, as mention before NO generation, together with increased superoxide generation, leads to elevated peroxynitrite formation, which is a powerful oxidant, and related to oxidative injury of blood vessels [182].

1. 12. 4. The roles of H_2S and NO in inflammation

The vascular protective action of H_2S involves its anti-inflammatory effects on the innermost lining of blood vessels. H_2S, released from either NaHS (10-100 μM) or S-propargyl-cysteine (SPRC; 1-10 μM), a novel sulfur-containing amino acid, inhibits the expression of adhesion molecules, including ICAM-1, VCAM-1, P-selectin, and E-selectin, induced by pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), in HUVECs [183, 184]. H_2S prevents the complication of nonsteroidal anti-inflammatory drugs (NSAIDs) and acetylsalicylic acid (ASA) therapy [185]. H_2S attenuates the gastric mucosal injury, TNF-α, ICAM-1 in rat mesenteric venules challenged with ASA or non NSAIDs [185]. Conversely, pharmacological blockade of CSE with β-cyanoalanine (BCA) enhanced leukocyte adhesion and rolling [186]. In agreement with these data, carrageenan-induced paw edema is suppressed by either NaHS (EC_{50} = 35 μM kg^{-1}) or Na_2S (EC_{50} = 28 μM kg^{-1}) and boosted by BCA [185]. The anti-inflammatory action of H_2S involves the activation of K_{ATP} and BK_{Ca} channels [185, 187] and the up-regulation of HO-1 [184], as well as the inhibition of p38 and NF-kB signalling pathways. Furthermore, H_2S hampers TNF-α-induced monocyte-endothelial interactions by down-regulating the expression of the monocyte chemoattractant protein-1 (MCP-1) through the inhibition of disintegrin and metalloproteinase metallopeptidase domain 17 (ADAM17) [188]. The ADAM17-dependent TNF-converting enzyme (TACE) activity is, in turn, essential for soluble TNF-α shedding and up-regulation of MCP-1 levels in HUVECs [188]. NaHS administration at 100 μM kg^{-1} restores gastric microcirculation in a K_{ATP} channels-dependent manner [185]. NO also plays a key role in the pathogenesis of inflammation as an anti-inflammatory mediator under normal physiological conditions. On the other hand, NO is considered as a pro-inflammatory mediator that induces inflammation due to over production in

pathological conditions [189]. NO inhibits adhesion of inflammatory cells to the endothelial surface as well [190].

1. 12. 5. The roles of H_2S and NO in angiogenesis

H_2S has recently been recognized as an important regulator for angiogenesis both *in vitro* and *in vivo* under physiological conditions [191, 192]. Silencing of CSE reduces migration and sprouting of HUVECs in *vitro* [191]. In agreement with these data, the BrdU assay disclosed that the proliferation rate of primarily isolated ECs was dramatically suppressed by knockdown of CSE but restored by exogenously H_2S treatment [119]. Moreover, the *ex vivo* mouse aortic ring angiogenesis assay reveals a marked decrease in neovascularization when the aortic tissue from CSE-KO was cultured *in vitro* [119]. Conversely, NaHS promotes blood vessel growth of aorta tissues from both WT and CSE-KO mice [119]. Intraperitoneal administration of NaHS for 7 days at 10 - 50 $mmol \cdot kg^{-1} \cdot day^{1}$ increases neovascularization in the mouse *in vivo* [193]. Consistently, treatment with Na_2S increases the vascular length in chicken chorioallantoic membranes (CAMs) at doses of 0.24 - 240 pmole/egg [193]. PPG treatment for 48 hr at increasing concentrations (3, 30, and 300 μmole/egg) decreases vascular network length and branching of CAM [191]. NaHS at a dose of 100 $\mu mol \cdot kg^{-1} \cdot day^{-1}$ restores blood perfusion in a rat model of hindlimb ischemia by promoting local vessel growth [192]. Therapeutic angiogenesis of occluded peripheral arteries has also been reported in C57BL/6J mice supplemented with Na_2S at 0.5 mg/kg and 1 mg/kg [194]. Likewise, NaHS (30 μM/l in drinking water for 4 weeks) attenuates cardiac remodeling promoting *in vivo* angiogenesis in a rat model of myocardial infarction [195]. The signal transduction pathways underlying the pro-angiogenic role of H_2S are not fully unraveled [119, 196]. Activation of PI-3K/Akt by H_2S has been shown

to regulate tube-like structure formation in the retinal EC line (RF/6A) by inducing the up-regulation of the adhesion molecules, integrin α2 and β1, and survivin [193]. Accordingly, integrin α2 and β1 maintain angiogenesis by controlling EC adhesion to the underlying substrate [192]. H_2S increases Akt phosphorylation and improves regional blood flow in rat unilateral hindlimb ischemic model [192]. Mitogen-activated protein kinases (MAPK) and ERK1/2 are not activated by H_2S in RF/6A cells [193], but the activation of MAPKs, such as ERK1/2 and p38, by H_2S was shown to play a key role in HUVEC migration [191]. The use of different concentrations of H_2S donors, of multiple EC types, and of diverse assay systems could explain the heterogeneity observed in the molecular underpinnings of the pro-angiogenic effects of H_2S. Importantly, the K_{ATP} channel blocker glibenclamide abolished H_2S-induced p38 phosphorylation and HUVEC motility [191]. Therefore, K_{ATP} channels are located upstream of MAPKs in the signaling pathway contributing to H_2S-induced angiogenesis.

Another putative mechanistic link between H_2S and the molecular decoders of its pro-angiogenic effect is the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [196, 197]. In fact, many studies have provided the evidence that NaHS affects intracellular Ca^{2+} signals in a variety of ECs, such as human saphenous vein ECs [198], human microvascular dermal ECs (HMVECs) [199], and rat aortic ECs (RACEs) [200]. The Ca^{2+} response to NaHS in human ECs is shaped by the interaction between intracellular Ca^{2+} release from the endoplasmic reticulum reservoir, which is mainly mediated by inositol-1,4,5-trisphosphate ($InsP_3$) receptors, and store-operated calcium entry (SOCE) across the plasma membrane [198]. Conversely, Ca^{2+} inflow patterned H_2S-evoked elevations in $[Ca^{2+}]_i$ in both HMVECs and RAECs [196, 199]. Ca^{2+} entry in rat aortic endothelium is supported by the reverse mode of the Na^+/Ca^{2+} exchanger (NCX) and sustained by K_{ATP} channels-dependent membrane hyperpolarization [200]. Similar to

vasorelaxation, NO may be involved in the pro-angiogenic action of H_2S [115] Accordingly, pharmacological blockage of protein kinase G (PKG) prevented H_2S-induced bEnd3 proliferation and migration *in vitro*, whereas NaHS-elicited neo-vascularization of Matrigel plugs *in vivo* was absent in eNOS- KO mice [165]. In addition, CSE deficiency abolished the pro-angiogenic role of NO donors both *in vitro* and *in vivo* [119]. Treatment with either L-NAME or siRNA to knockdown eNOS attenuates the H_2S-angiogenic effect [119]. Furthermore, L-arginine (the substrate of NOS) induces neo-vessel growth in cultured ring isolated from wild type mice but not from CSE-KO mice [119]. Therefore, both H_2S and NO are required for optimal angiogenic activity, yet angiogenesis still proceeds in the presence of either H_2S or NO alone albeit to a reduced degree. However, the exact molecular mechanism underlying H_2S-mediated NO pro-angiogenic response remain unclear.

Vascular endothelial growth factor (VEGF) is the most powerful stimulator of angiogenesis. The down-regulation of CSE gene led to a decrease in VEGF-induced MAPK activation and HUVEC motility [191]. The *ex vivo* aortic ring angiogenesis assay further showed that VEGF-elicited elevation in microvessels formation is dramatically hampered in CSE-KO mice [191]. Similarly, the genetic suppression of CSE attenuated VEGF-induced proliferation in bEnd3 micro-vascular ECs, whereas it does not impair the mitotic effect of basic fibroblast growth factor (bFGF) [165]. The influx of Ca^{2+} triggered by VEGF into ECs might serve as a suitable signal to initiate CSE activation and H_2S synthesis [163, 199, 201, 202].H_2S also stimulates angiogenesis of ischemic tissues. NaHS activated the hypoxia inducible factor 1-α (HIF-1α), the transcription factor driving the expression of numerous growth factors [162], in capillary ECs harvested from mouse skeletal muscle [194]. This process was mediated by H_2S-induced NO and VEGF release and subsequent increase in EC proliferation under hypoxia [194].

These data have been supported by the increase in VEGF expression reported in both ischemic heart and peripheral limbs and related to the pro-angiogenic effect exerted by NaHS treatment in mice [192]. However, we recently found that NaHS represses hypoxia-induced HIF-1α protein translation in ECs (EA.hy926) [203]. H_2S enhances eukaryotic initiation factor 2-α (eIF2-α) phosphorylation, and the consequent fall in HIF-1α accumulation resulted in the down-regulation of VEGF expression, thus EC proliferation [203]. This discrepancy might be due to the differences in ECs isolated from distinct vascular beds and animal species.

In addition to the therapeutic angiogenesis, H_2S might be involved in controlling the neovascularisation of growing tumours. NaHS induces Ca^{2+} influx in higher levels in tumour ECs (TECs) harvested from breast cancer (B-TECs) as compared to their healthy counterparts [199]. Importantly, NaHS causes a dose-dependent stimulation of B-TEC proliferation but had little effect on the control healthy cells [199]. VEGF-induced Ca^{2+} entry was prevented by CSE blockade by PPG, albeit the physiological meaning of this process was not further investigated [199]. In consideration of the endothelial phenotypic changes observed in ECs isolated from tumor samples, the involvement of H_2S in supporting tumour vascularisation should, therefore, be assessed by focussing on TECs rather on their normal counterparts [199].

1. 12. 6. The roles of H_2S and NO in atherosclerosis

EC dysfunction, hypertension, VSMC proliferation and migration, dyslipidemia, oxidative stress, and recruitment of inflammatory cells are the important factors involved in the development of atherosclerotic lesions [174, 204]. NO and H_2S share several athero-protective actions, including blood vessel relaxation and regulation of vascular tone, endothelial regeneration, inhibition of leukocyte adhesion, inhibition of platelet clumping to make the blood

thinner and prevention of VSMCs proliferation and migration [174, 205-208]. The role of NO in atherosclerosis development has been studied in apolipoprotein E knockout (apoE-KO) mice, where L-NAME treatment significantly decreased NO-mediated vascular response, and increased atherosclerosis development [209]. NOS gene overexpression reduced adhesion molecules expression, reduced the inflammatory process, and inhibited VSMC proliferation and migration [210, 211]. Another study proved that decreased NO bioavailability in high cholesterol-induced vascular dysfunction of rabbit aortic rings was corrected by eNOS gene transfer, which improved vascular relaxation in response to acetylcholine [212]. Kuhlencordt *et al.* [208] studied whether eNOS deficiency affects atherosclerosis development using apoE/eNOS double knockout (DKO) mice and found that both male and female apoE/eNOS DKO mice showed significantly increased lesion, when compared to apoE KO mice [208].

The anti-atherosclerotic role of H_2S was explored in recent years. CSE expression and H_2S production were significantly decreased during the development of balloon injury-induced neointimal hyperplasia in the rat carotid artery, and that exogenous H_2S significantly reduced neointimal lesion formation [213]. Treatment of apoE-KO mice with NaHS decreased, and with PPG increased, atherosclerotic lesion size [214]. NaHS (100 μmol/L for 12 hrs) inhibited ICAM-1 expression in TNF-alpha-induced HUVECs *via* the NF-kappaB pathway [214]. Moreover, NaHS administration concentration-dependently (50-200 μM) reduced CX3CR1 and CX3CL1 expression in mouse peritoneal macrophages, as well as CX3CR1-mediated chemotaxis [215]. In fat Apo-E mice, NaHS (1 mg/kg, i.p., daily)- attenuated, and PPG (10 mg/kg, i.p., daily) exacerbated, the extent of atherosclerotic plaques [215]. H_2S against apoliprotein accumulation, H_2S (50 μM) attenuated H_2O_2 and oxidized LDL (oxLDL)-mediated endothelial cytotoxicity in HUVECs [216]. GYY4137 (a slow H_2S releasing molecule) decreased the atherosclerotic plaque

formation and partially restored the endothelium-dependent relaxation of apoE-KO mouse aorta [207]. The direct and sold evidence for the anti-atherogenic role of H_2S was reported in 2013, demonstrating that CSE-KO mice fed with atherogenic paigen-type diet, but not WT mice, developed early fatty streak lesions in the aortic root, increased aortic intimal proliferation and aortic adhesion molecule expression, and enhanced oxidative stress [174]. These animals also showed increased plasma total cholesterol and LDL-cholesterol levels compared to WT mice fed with the same atherogenic diet. NaHS supplementation to, atherogenic diet-fed CSE-KO mice improved plasma lipid profile and decreased atherosclerotic lesions [174]. Collectively, these studies suggest that NO and H_2S play similar roles in the prevention of atherosclerosis development. The crosstalk between NO and H_2S in the development of atherosclerosis, however, has not been explored in details, so far.

1. 12. 7. The roles of H_2S and NO in aging

Aging is related to dramatic structural and functional alterations in both heart and blood vessels, which can explain the age-related increase in cardiovascular risk [217, 218]. During aging, ECs undergo cellular senescence, which leads to a reduced angiogenic activity [219, 220]. Cellular senescence, an irreversible arrest of cell cycle [221], can be triggered by the reduced bioavailability of both H_2S and NO. For instance, senescent human aortic ECs (HAECs) exhibited higher ICAM-1 expression and lower eNOS activity [222]. Accordingly, the eNOS protein isolated from mesenteric arteries in young mice has the dimer configuration, whereas eNOS protein from aged mice was mostly uncoupled to monomers [223]. The same study revealed that eNOS uncoupling contributed to augmented superoxide levels in aged vessels and was due to a reduced BH_4 availability [223]. The aging-related EC dysfunction is largely

ascribed to oxidative stress and inflammation [224, 225]. An excess of the ROS, superoxide, and hydrogen peroxide compromises the vasodilator activity of NO and facilitate the formation of the deleterious radical [224]. Basal NO production and sensitivity to acetylcholine-mediated vasodilation in thoracic aorta rings were reduced, whereas the pro-inflammatory reaction of ECs was increased in aged mice compared to young mice [226]. In addition, arginase-II (Arg-II) expression/activity was higher in senescent ECs, and its silencing suppressed eNOS-uncoupling and several senescence markers such assenescence-associated-β-galactosidase activity, p53-S15, p21, and expression of VCAM1 and ICAM1 [227]. Over-expression of Arg-II in non-senescent EC promoted eNOS-uncoupling and enhanced VCAM1/ICAM1 levels [227]. Protein nitrotyrosine formation is accompanied with eNOS uncoupling in mesenteric arteries of aged mice [223]. Chronic increase in shear stress in mesenteric arteries of aged rats restored EC function through increasing NO production and antioxidant capacity, and thus decreasing superoxide levels [228]. In soil nematode *Caenorhabditis elegans*, which is a prominent model organism for studying aging [229], after exposure to H_2S it survive longer and are more thermotolerant than untreated controls [230]. NaHS treatment protects against HUVEC senescence by modulating SIRT1 activity, and against fibroblast aging *via S*-sulfhydration of Keap1 and Nrf2 activation in association with oxidative stress [145]. A recent study revealed that CSE protein expression was increased in the aorta of aging rats maintained on an ad libitum (AL) diet, but CSE expression was unchanged in rats maintained on a caloric restriction (CR) diet. Furthermore, CR-fed animals at ages of 18, 29, and 38 months had lower CSE expression than in AL-fed animals, whereas CSE expression at 8 months was not affected by diets [231], suggesting that CR diet may help to stabilize the H_2S signaling system during aging.

1. 12. 8. The roles of H_2S and NO in diabetes

H_2S treatment or CSE over-expression protected ECs from the deleterious consequences of hyperglycemia induced enhancement of ROS formation, attenuated nuclear DNA injury [232]. H_2S preserved the development of EC dysfunction in aortic tissues incubated in medium with elevated glucose concentration (*in vitro* "hyperglycemia") and reduced the bio-energetic derangements in ECs [232]. On the other hand, CSE knockdown deteriorated hyperglycemia-induced ROS production and led to more severe loss of endothelium-dependent relaxant function [232]. Oxidative stress plays a major role in the development of diabetic microvascular complications [233]. Non-obese diabetic (NOD) mice, which gradually develop type-1 diabetes, exhibited reduced of H_2S levels in plasma and aortic tissue [234]. The administration of exogenous H_2S relieved vascular abnormalities by upregulating connexin 40 and connexin 43 and normalizing NADPH oxidase and PKCε in streptozotocin (STZ)-injected rats [235]. These results have been corroborated by the finding that the circulating levels of H_2S were lower in STZ-diabetic rats [232]. The same study showed that, under hyperglycaemic condition bEnd3, microvascular ECs accelerated H_2S consumption due to the mitochondrial formation of ROS, which severely affected cell viability and caused nuclear DNA damage and switched cell metabolism from oxidative phosphorylation to glycolysis[232]. As expected, these features were associated with the impairment of endothelium-dependent relaxations of rat aortic rings exposed to high glucose *in vitro*. EC functionality was restored either by over-expressing CSE or by supplying NaHS (100 - 300 μM) [232]. The observation that ECs in diabetes failed to produce sufficient amount of NO has been documented in animal model of the disease [236]. Similarly, loss of insulin signalling in the vascular endothelium led to EC dysfunction due to the decrease in NO synthesis, which impaired endothelium-dependent vasodilation and accelerated the

progression of atherosclerotic lesions in apoE KO mice [237]. Likewise, hyperglycemia inhibited eNOS activity in cultured BAECs and in the aorta of diabetic rats [238]. All together, these data illustrate the importance of H_2S and NO in protecting ECs from deleterious vascular consequences of diabetes.

Objective of the study

NO is a well-known gasotransmitter. H_2S is now considered a third member of the gasotransmitter family. In the vascular system, CSE is mainly responsible for endogenous production of H_2S. CSE enzyme is expressed in SMCs. However, our group in 2008 was the first to report that CSE can be expressed in ECs as well. This discovery has triggered more attention towards the interaction of H_2S and NO in the vascular system and precisely in ECs. Here we hypothesized that H_2S acts as a modulator of eNOS and NO generation in ECs. The objectives of this thesis are to:

1. Examine the effects of both exogenous and endogenous H_2S on eNOS expression, phosphorylation and NO production in vascular tissue.
2. Evaluate the interaction between H_2S and NO and their roles in the regulation of different vascular function such as endothelium superoxide production and angiogenesis.
3. Investigate the role of *S*-sulfhydration as a new regulatory mechanism for eNOS, as well as the interplay between *S*-sulfhydration and other posttranslational modifications.
4. Examine the effect of H_2S on eNOS structure and function of dimer uncoupling.

CHAPTER 2

Crosstalk between Hydrogen Sulfide and Nitric Oxide in Endothelial Cells

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2.1. Abstract:

Hydrogen sulfide (H_2S) and nitric oxide (NO) are major gasotransmitters produced in endothelial cells (ECs), contributing to the regulation of vascular contractility and structural integrity. Their interaction at different levels would have a profound impact on angiogenesis. Here, we showed that H_2S and NO stimulated the formation of new micro-vessels. Incubation of human umbilical vein endothelial cells (HUVECs-926) with NaHS (a H_2S donor) stimulated the phosphorylation of endothelial NO synthase (eNOS) and enhanced NO production. H_2S had little effect on eNOS protein expression in ECs. L-cysteine, a precursor of H_2S, stimulated NO production whereas blockage of the activity of H_2S-generating enzyme, cystathionine gamma-lyase (CSE), inhibited this action. CSE knockdown inhibited, but CSE overexpression increased, NO production as well as EC proliferation. LY294002 (Akt/PI3-K inhibitor) or SB203580 (p38 MAPK inhibitor) abolished the effects of H_2S on eNOS phosphorylation, NO production, cell proliferation and tube formation. Blockade of NO production by eNOS-specific siRNA or L-NAME reversed, but eNOS overexpression potentiated, the proliferative effect of H_2S on ECs. Our results suggest that H_2S stimulates the phosphorylation of eNOS through a p38 MAPK and Akt-dependent pathway, thus increasing NO production in ECs and vascular tissues and contributing to H_2S-induced angiogenesis.

Keywords: Hydrogen sulfide; Nitric oxide; endothelial cells; eNOS; CSE; cystathionine gamma-lyase.

2. 2. Introduction

Hydrogen sulfide (H_2S) and nitric oxide (NO) are known gasotransmitters that contribute to many physiological functions [2]. These gaseous messengers can be produced endogenously to respond to diverse physiologic and patho-physiologic stimuli [2]. In endothelial cells (ECs), H_2S can be generated from L-cysteine by the enzymatic action of cystathionine gamma-lyase (CSE; EC 4.4.1.22) [19]. H_2S-induced relaxation of vascular tissue was partially reduced by the removal of the vascular endothelium or in the presence of L-NAME (an inhibitor of NO synthase) [17]. NO can be generated in ECs from L-arginine by endothelial nitric-oxide synthase (eNOS; EC 1.14.13.39) [239].Being a homo-dimeric protein, the activation of eNOS is dependent on intracellular calcium (Ca^{2+}) level and other cofactors like nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin, flavin adenine dinucleotide, and flavin mononucleotide [240].The activity of eNOS is affected by many posttranslational modification mechanisms, such as phosphorylation on multiple amino acids like Ser-1179/1177 (bovine/human) and Thr-495 residues [241, 242], whereas eNOS protein can be self-inhibited by high concentrations of NO through *S*-nitrosylation [135]. Due to its reducing capability, H_2S may reduce NO to form a thiol-sensitive molecule *S*-nitrothiols (RSNO) [243] . Conversely, H_2S has been found to reduce RSNO to release NO from GSNO (S-Nitrosoglutathione) [244]Moreover, H_2S and NO interact on each other's catalyzing enzymes; NO donor increases the expression and activity of CSE in cultured aortic smooth muscle cells (SMCs) [17]. In rat vascular SMCs, H_2S had no direct effect on NO production, but it augmented interleukin-induced NO production, and this effect was related to increased iNOS expression (inducible NOS) [245]. NaHS (a H_2S donor) treatment reduced eNOS activity and expression but not of nNOS (neuronal NOS) and iNOS in isolated rat aortas and human umbilical vein endothelial cells (HUVECs) [246]. NaHS inhibited

eNOS-catalyzed conversion of [^{3}H]-arginine to [^{3}H]-citrulline [247]. NaHS also inhibited iNOS expression and NO production in macrophages cells (RAW264.7) [169]. Na_2S selectively augmented NO production in chronically ischemic tissues, by influencing iNOS and nNOS expression and stimulating nitrite reduction to NO *via* xanthine oxidase (XO) under hypoxic condition [194]. The angiogenic crosstalk between H_2S and NO in ECs has been unclear. Our present study showed that the pro-angiogenic effect of H_2S appears to be regulated by both a NO-dependent and an independent mechanism, whereas NO effect on angiogenesis is partially dependent on H_2S. We demonstrated that H_2S stimulated NO release by increasing eNOS phosphorylation *via* a p38 MAPK and Akt-dependent mechanism, which contributes to the stimulatory effect of H_2S on EC proliferation and angiogenesis.

2. 3. Materials and Methods

Cell culture and chemicals

HUVEC-derived EA.hy 926 cells were kindly provided by Dr. Cora-Jean S. Edgell [248] (University of North Carolina, USA). The cells were cultured in Dulbecco's modified eagles medium (DMEM) without ferric nitrate (Sigma, Oakville, Canada), containing penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% (v/v) fetal bovine serum. The primary aortic endothelial cells were isolated from the aorta of 10-12 week-old C57BL/6J/129 mice, as previously described [249]. Aortic endothelial cells were cultured in a medium containing 20% FBS, 100 U/ml penicillin-G, 100 μg/ml streptomycin, 2 mM L-glutamine, 25 mM HEPES (pH 7-7.6), 100 μg/ml heparin, 100 μg/ml endothelial cell growth supplement (ECGS), and DMEM (Sigma). The nature of ECs was confirmed using endothelial-specific markers CD31 (Santa Cruz Biotechnology, Santa Cruz, CA) and eNOS (Cell Signaling Technologies, Beverly, MA, USA)

by Western blot, and endothelial tube formation using Matrigel assay (BD Biosciences, Mississauga, ON, Canada) (data not shown). The culture medium was changes every 2 days and ECs between passages 3-5 were used.

Measurement of NO production

Total nitrate/nitrite concentrations were measured by conversion of nitrate to nitrite after incubating supernatants with nitrate reductase (10 U/ml) and NADPH (5 mM) for 1 hr at 37°C. The total nitrite was measured with a Griess assay kit (Promega, Madison, WI, USA) using a reference sodium nitrate standard curve [250]. The results obtained with the Griess assay have also been validated by the diaminofluorescein fluorophore system (DAF-FM), which can be deacetylated by intracellular esterases and further reacts with NO to form a fluorescent benzotriazole (DAF fluorescence) (Invitrogen, Burlington, ON, Canada). ECs were incubated with 5 μM DAF-FM for 30 min at 37°C. The cells were washed to remove excess dye, replaced with fresh medium and observed under a fluorescent microscope as previously described [251]. To detect the production of NO in aortic tissues, isolated aortas were incubated with DAF-FM (5 μM) at 37°C in Kreb's buffer and then rapidly removed and frozen at −20°C. Aortic tissue samples were embedded in optimal cutting temperature (OCT) compound until frozen, and sectioned using Leica CM1850 UV microtome-cryostat (Leica Biosystems, Concord, Ontario, Canada). The tissue blocks were cut into 10 μm-thick sections and observed under a fluorescent microscope [252].

Gene knockdown and overexpression

ECs were seeded in 6-well plates and cultured until they reached 70-80% confluence. The cells were then transfected with specific siRNA to knockdown CSE or eNOS gene (50 nM). Negative siRNA was used as transfection control (50 nM), using Lipofectamine™ RNAi-MAX

transfection reagent according to the manufactory instruction (Invitrogen). Overexpression experiments were carried out with plasmid DNA containing CSE cDNA (pIRES2-EGFP, 4.0 μg) or eNOS cDNA (pcDNA 3.1 eNOS-GFP, 4.0 μg). Mock empty vector was used as transfection control (Addgene, Cambridge, MA, USA) [109, 120, 253] using Lipofectamine™ 2000. Forty-eight hr after transfection, the cells or media were collected and evaluated by Western blot or Griess assay analysis.

Western blot analysis

Cultured cells were collected and incubated in a lysis buffer containing 0.5 M EDTA, 1 M Tris-Cl (pH 7.4), 0.3 M sucrose, and a protease inhibitors mixture (Sigma). The cell extracts were sonicated three times (5-10 seconds/each) on ice using a cell sonicator (Sonic Dismemrator Model 100, Fisher Scientific) [120]. Cellular extracts were separated by centrifugation at 14,000 × g for 15 min at 4°C. Supernatants were collected, and the same amounts of proteins were separated on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes (Pall Corporation, Pensacola, FL, USA). All primary antibody incubations were performed at 4 °C overnight. The antibody dilution for phospho-eNOS (Ser1177), eNOS, phospho-ERK, ERK, phospho-p38 MAPK, p38 MAPK, phospho-Akt (S473) and Akt was at 1:1000 (Cell Signaling Technologies). Anti-CSE antibody was used at 1:5000 (Proteintech Group, Chicago, IL, USA), and anti-β-actin antibody was at 1:10000 (Sigma). The membranes were stripped using a buffer containing 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl (pH 6.8) at 50°C for 30 min. Membranes were visualized using enhanced chemiluminescence western blotting system (GE Healthcare, Piscataway, NJ, USA). Densito-metric quantification was performed using Alpha Digi Doctor Software (Richardson, TX, USA). The protein bands were quantified and normalized against either β-actin or total form levels of the target protein, and expressed as a

percentage relative to the controls (equals 100%). The phosphorylation level is defined as the ratio between the phosphorylated target proteins and their total forms and expressed in the summarized bar graphs as the percentage of the untreated controls.

Capillary-like tube formation assay

The Matrigel matrix gel was thawed overnight at 4°C on ice and then added to pre-chilled culture dishes and allowed to polymerize at 37°C for 1 hr. ECs (2×10^4 cells) were incubated with different agents in 500 μl DMEM and then seeded onto the surface of Matrigel (BD Biosciences). After 12 hrs, the formation of capillary-like structure was imaged by light microscope. The total lengths of tube-like structures per field were measured using image analysis software (NIH Image software- Image J).

Cell proliferation assay

Cells were counted using automated cell counter TC10^{Tm} from BioRad (Mississauga, ON, Canada) and seeded into 96-well plates (1×10^4 cells/well). After 24 hrs of initial seeding, cells were incubated with DMEM serum-free medium for overnight [120]. The proliferation rates were evaluated by 5-bromo-2'-deoxyuridine (BrdU) incorporation assay according to manufacturer's instructions (EMD Biosciences, San Diego, CA, USA).

Micro-vessel formation assay

CSE knockout (KO) mice were generated as described previously [19]. Eight-week old male CSE-KO and wild type (WT) mice were sacrificed, and aorta were rapidly cleaned off adipose tissues and blood. Aorta were cut in to rings (length, ~3 mm) and implanted in a fibrin gel obtained by adding 400 μl of a fibrinogen solution (3 mg/ml) and thrombin (1.5 U/ml) (Sigma). After 30 minutes, 500 μl of DMEM was added with the treatment. As a control, the

effect of medium alone was assayed, and quantitative evaluation of new micro-vessels was carried out after 72 hrs [191]. All animal experiments were conducted according to the Care and Use of Laboratory Animals Guide (NIH Publication No. 85-23, revised 1996) and approved by Lakehead University Animal Care Committee, Canada.

Statistical analysis

All data were expressed as mean ± SEM. Each data point represented at least three to four independent experiments. Statistical comparisons were evaluated using Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

2. 4. Results

H_2S induced NO production in ECs

Stimulation of ECs with NaHS for 30 min increased NO production over a concentration range from 10 μM to 100 μM (Figure 2. 1A). The effect of NaHS on NO production was blocked when cells were pre-treated with NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) (Figure 2. 1B). NaHS-induced increase in NO production was further confirmed in primarily cultured mouse ECs (Figure 2. S1). L-NAME treatment also significantly reduced NO production. We next determined the effect of L-cysteine (H_2S precursor) on NO production. L-cysteine pre-treatment stimulated NO production in ECs (Figure 2. 1B). However, blocking of CSE activity by PPG reversed L-cysteine effect (Figure 2. 1B). CSE knockdown using CSE-specific siRNA significantly reduced CSE protein level and attenuated NO production in comparison with the cells transfected with negative siRNA. Moreover, CSE overexpression significantly elevated CSE expression level and resulted in an increase in NO level (Figure 2. 1C

and 2.1 D). NO data was further confirmed by DAF-FM fluorescence dye showing that NaHS treatment stimulated NO release in aortic tissues and ECs (Figure 2. 1E and 2.1 F).

NaHS (50 μM and 100 μM) treatment markedly increased the phosphorylation of eNOS in ECs (Figure 2. 2A). The stimulatory effect of NaHS on eNOS phosphorylation was time dependent, and the increase in phosphorylated eNOS appeared at 10 min, peaked at 30 min, and gradually declined to base-line over the period of 1-hr NaHS exposure (Figure 2. 2B). NaHS treatment up to 36 hrs had no significant effect on eNOS expression level (Figure 2. 2C).

The role of p38 MAPK/Akt in H_2S-induced eNOS phosphorylation and NO production

Diverse kinases such as Akt, p38-MAPK kinase, and ERK are important for NO production and signaling activation [254-256]. To elucidate the signaling pathways involved in H_2S-induced eNOS phosphorylation and the NO production, we examined the roles of Akt, ERK and p38 MAPK in H_2S-stimulated NO production. Treatment with NaHS at 100 μM enhanced the phosphorylation of p38 MAPK, Akt, and ERK to different levels (Figure 2. 3). SB202190 (a p38 MAPK inhibitor) and LY294002 (a PI3K/Akt inhibitor), but not U0126 (an inhibitor of ERK), significantly reduced H_2S-induced phosphorylation of eNOS (Figure 2. 4). We further found that the stimulatory effect of NaHS on NO production was decreased by the same treatments (SB202190 or LY294002), and neither SB202190 nor LY294002 alone had any detectable effect on NO production (Figure 2. 5A). Additionally, p38 MAPK inhibition by SB202190 attenuated the NaHS-induced phosphorylation of Akt (Figure 2. 5B), indicating that p38 MAPK might regulate the upstream signaling cascade that leads to Akt activation. These results suggest that p38 MAPK and Akt are required for NO activation by H_2S.

The role of NO in H_2S-induced EC proliferation and angiogenesis

NaHS significantly induced EC proliferation (Figure 2. 6A). To show the effect of endogenously produced H_2S, CSE knockdown with a siRNA approach attenuated cell proliferation. The knockdown of CSE significantly attenuated the proliferation of EC by about 25% compared with the control group (Figure 2. 6B). We also found that CSE knockdown significantly decreased, but NaHS induced a similar and comparable increase, in the proliferation of primarily cultured mouse ECs (Figure 2. S2). The CSE overexpression stimulated EC proliferation (Figure 2. 6B). Next we study the effect of NO on proliferation. The overexpression of eNOS stimulated cell proliferation, which was strengthened by NaHS treatment (Figure 2. 6C and 2.6 D).

We then determined whether H_2S and NO can interact to regulate angiogenesis. H_2S-induced EC proliferation was attenuated by eNOS knockdown (Figure 2. 7A and 2.7 B), whereas treatment with NO precursor L-arginine (1 mM) or NaHS (100 μM) alone significantly increased EC proliferation (Figure 2. 7B). Furthermore, NaHS (100 μM) treatment significantly increased the capillary-like tube formation of EC compared with the untreated cells (Figure 2. 7C). NaHS-induced increase in tube formation was significantly attenuated by co-treatment with L-NAME (200 μM), whereas L-NAME treatment alone had no significant effect on tube formation (Figure 2. 7C). The aortic tissues from CSE-KO mice showed a markedly decreased formation of new micro-vessels compared with WT mice. After treating the embedded aortic rings with NaHS, the sporting of vascular neogenesis was significantly increased in both CSE-KO and WT mice with markedly higher levels in CSE-KO mice (Figure 2. 7D). Similar to the effect of NaHS, L-arginine (a NO precursor)- stimulated vascular neogenesis in both CSE-KO and WT mice (Figure 2. 7D). Furthermore, the pro-angiogenic effects of H_2S on aortic rings from both CSE-

KO and WT mice were inhibited by L-NAME treatment (Figure. 2. 7D). L-NAME treatment inhibited new vessel formation from wild type aortic rings, but not that from CSE-KO aortic rings (Figure 2. 7D). Treatment of EC with NaHS (100 μM) increased the capillary-like tube formation, and co-treatment with a p38 or Akt inhibitor (SB202190 or LY294002) significantly reduced the H_2S effect (Figure 2. 7E). However, treatment of EC with LY294002 or SB202190 alone had no significant effect on tube formation (Figure 2. 7E). LY294002 or SB202190 blocked the proliferation induced by H_2S, and neither LY294002 nor SB202190 alone had any detectable effect (Figure 2. 7F), demonstrating that p38 MAPK and Akt are responsible for H_2S-induced EC proliferation and angiogenesis.

2. 5. Discussion

Gasotransmitters play important roles in angiogenesis [115, 119, 165, 257, 258]. Angiogenesis is important for the development of the cardiovascular system and sustaining blood supplies, wound healing, and fetus development [259-263]. In our present study, we found that H_2S can interact with NO to induce angiogenesis of both cloned EC line and freshly isolated primary mouse ECs. The mechanisms for H_2S action are mainly ascribed to the stimulation of the p38 MAPK /Akt and eNOS phosphorylation, which was followed by increased NO production.

Phosphorylation activates eNOS [115]. In our study, the phosphorylation of p38 MAPK precedes the phosphorylation of Akt in the H_2S signaling cascade, which was confirmed when inhibition of p38 MAPK abolished H_2S-induced phosphorylation of Akt. We also found that H_2S activated ERK phosphorylation with a time course similar to that for p38 MAPK activation. However, the inhibition of ERK did not affect H_2S-stimulated NO production. By altering the

phosphorylation of eNOS, H_2S regulated NO production in ECs. Our observation is consistent with another recent finding by Predmore *et al.* [264]who demonstrated that Na_2S (150 μM) treatment stimulated NO production in bovine arterial endothelial cells. While these authors illustrated the H_2S-dependent Akt mechanism that stimulates NO production, the involvement of other kinases, like p38 MAPK, or the synergistic partnership between H_2S and NO in angiogenesis were not addressed. Conversely, it has been reported that a high concentration of NaHS (300-3000 μM) significantly inhibited the activity of recombinant bovine eNOS [247]. It is worthy noted here that NaHS at this high concentration range unlikely bears physiological relevance.

We explored the possible interaction between H_2S and NO in angiogenesis regulation. *Ex vivo* aortic explants isolated from CSE-KO mice showed a remarkable decrease in vascular neogenesis when compared to WT mice. L-arginine treatment stimulated angiogenesis in the WT mice and to a lesser extent, in the CSE-KO mice. On the other hand, L-NAME treatment reduced new vessel formation in wild type mice, and this inhibitory effect was not significant in CSE-KO mice, suggesting that the angiogenic effect of NO might be mediated through H_2S biosynthesis. CSE overexpression stimulated EC proliferation, whereas CSE knockdown reversed this effect. Interestingly, we found that the pro-angiogenic effect of H_2S was partially attenuated in the presence of eNOS inhibitor L-NAME, or after eNOS knockdown using siRNA. Taken together, our results suggest that both gasotransmitters are required for optimal angiogenic activity, yet angiogenesis still proceeds in the presence of either H_2S or NO alone albeit to a reduced degree. Previous studies had reported that H_2S and NO can mediate angiogenesis without much knowledge about the H_2S-NO interaction on angiogenesis [191, 193]. Recently, one study reported that a mutually dependent relationship between H_2S and NO is important for

physiological control of different vascular function [165]. Our study used different angiogenesis model (CSE-KO mice *vs.* rat) and experimental conditions, and we found that H_2S and NO, alone or combined, can cause angiogenesis. H_2S-stimulated angiogenesis was partially but not completely inhibited by NO blockage, whereas in CSE-KO mice NO treatment stimulated angiogenesis but to a reduced level. The exact molecular mechanism underlying H_2S-mediated NO pro-angiogenic response is not clear.

In summary, our studies demonstrate that H_2S promotes NO production in ECs *via* the activation of a cascade of phosphorylation events, starting from p38 MAPK, Akt to eNOS. H_2S promotes EC tube formation, proliferation, and angiogenesis by NO-dependent and independent mechanisms as outlined in Figure 2. 8. Thus, H_2S may be a key regulator for angiogenic signaling pathways, whether they required NO or not. The elucidation of the H_2S-NO relationship in the vascular biology would improve our understanding of the pathogenic mechanisms for cardiovascular disease in general and angiogenic-related diseases in particular.

Acknowledgment

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Conflict of Interest

There is no conflict of interest of any kind with any of the authors.

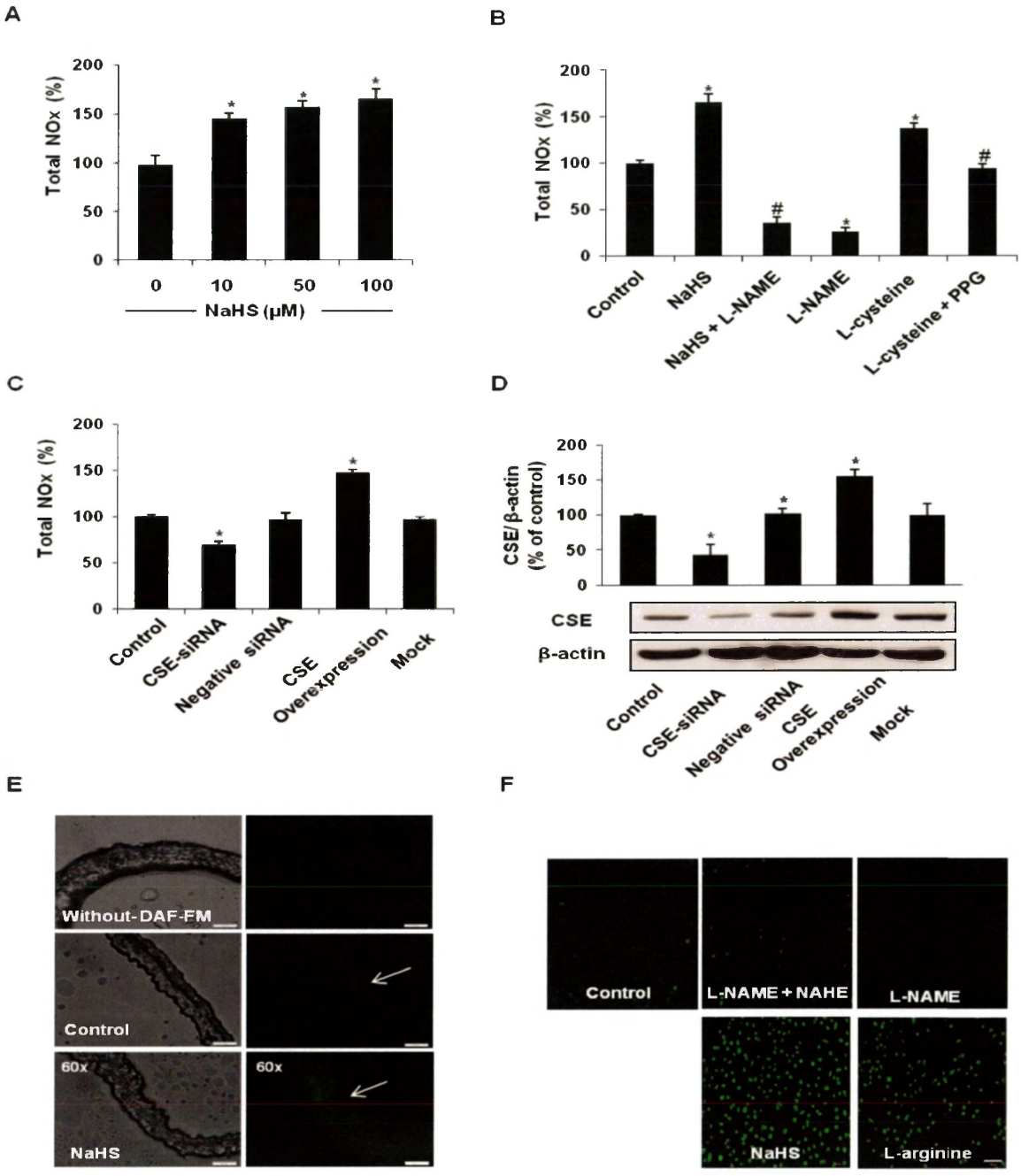

Figure 2. 1. H_2S stimulated NO production in ECs and aortic tissues. (**A**) The effect of NaHS on NO production in ECs detected by Griess assay, n=4, $^{*}P < 0.05$ *vs.* control. (**B**) The effects of NOS inhibitor L-NAME (200 μM, 1 hr), CSE inhibitor PPG (10 mM, 4 hrs), NaHS (100 μM, 30 min), and L-cysteine (6 mM, 30 min) on NO production detected by the Griess assay, n=3-4, $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* NaHS or L-cysteine treated groups. (**C**) The effects of CSE knockdown or overexpression on NO production assessed by the Griess assay. (**D**) The efficiency of CSE knockdown or overexpression, determined by western blot, n=3-4, $^{*}P < 0.05$ *vs.* control. The effect of NaHS (100 μM) and L-arginine (1 mM) treatment on NO production in isolated aortic tissues (Scale Bar: 50 μm) (**E**) and cultured ECs **(F)** using DAF-FM fluorescent probe (Scale Bar: 200 μm), n=3-4.

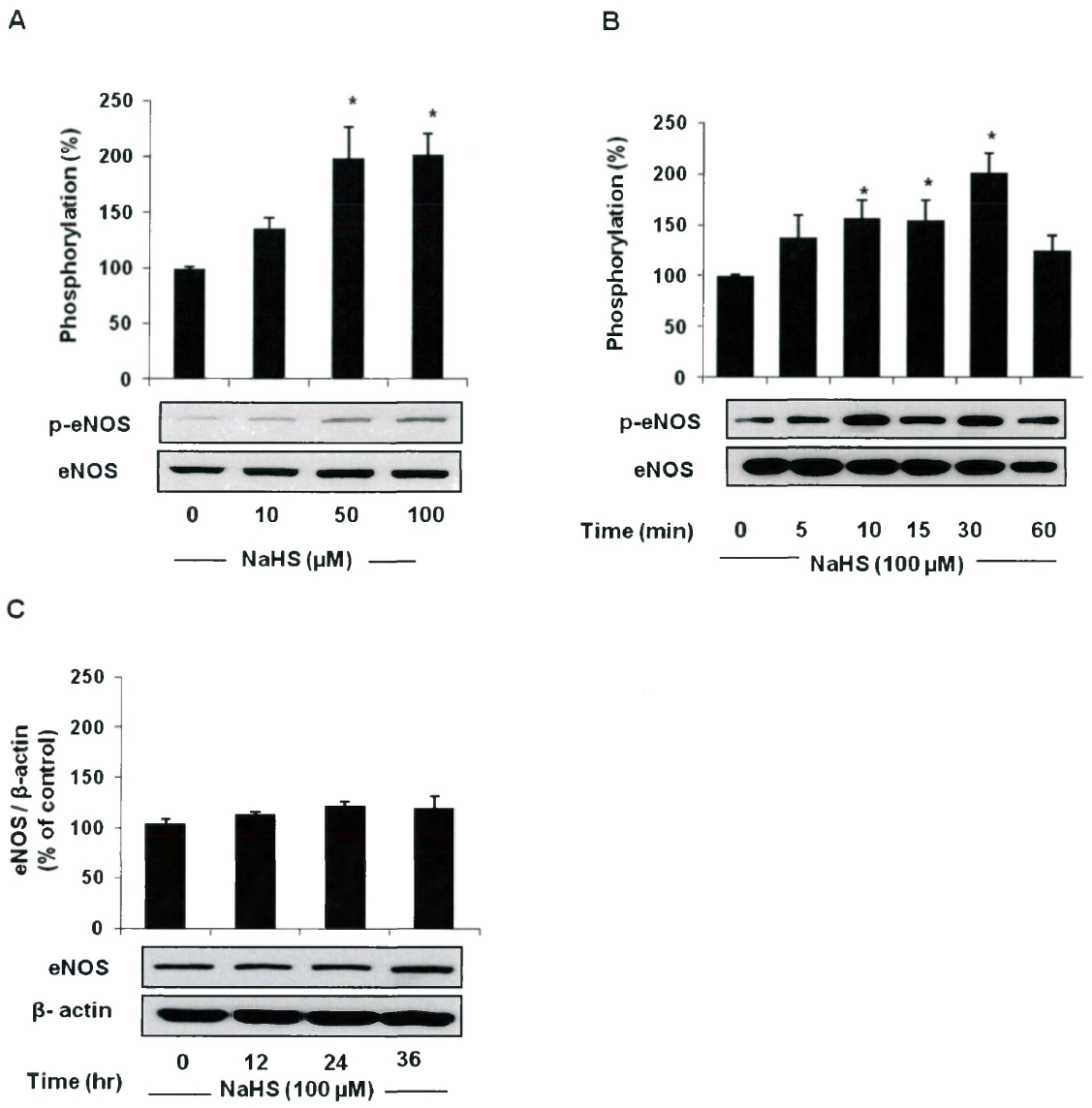

Figure 2. 2. H_2S stimulated the phosphorylation of eNOS in ECs. (**A**) The effect of NaHS treatment on eNOS phosphorylation. ECs were starved in DMEM medium free of serum for 24 hr and treated with different concentrations of NaHS for 30 min. Western blot analysis was conducted using anti-phospho-eNOS and anti-total eNOS antibody, n=3-4, $^{*}P < 0.05$ *vs.* control. (**B**) Time-dependent effect of NaHS treatment on the phosphorylation of eNOS. ECs were treated with NaHS (100 μM) for different periods (0 to 60 min). At the end of each time point, cells were collected and proteins lysates were analyzed by western blot, n=3-4, $^{*}P < 0.05$ *vs.* control. (**C**) The effect of NaHS treatment on eNOS expression level in ECs. The ECs were treated with NaHS (100 μM) for 12-36 hrs, and then cells were collected and proteins were subjected to western blot analysis. n=3-4, $^{*}P < 0.05$ *vs.* control.

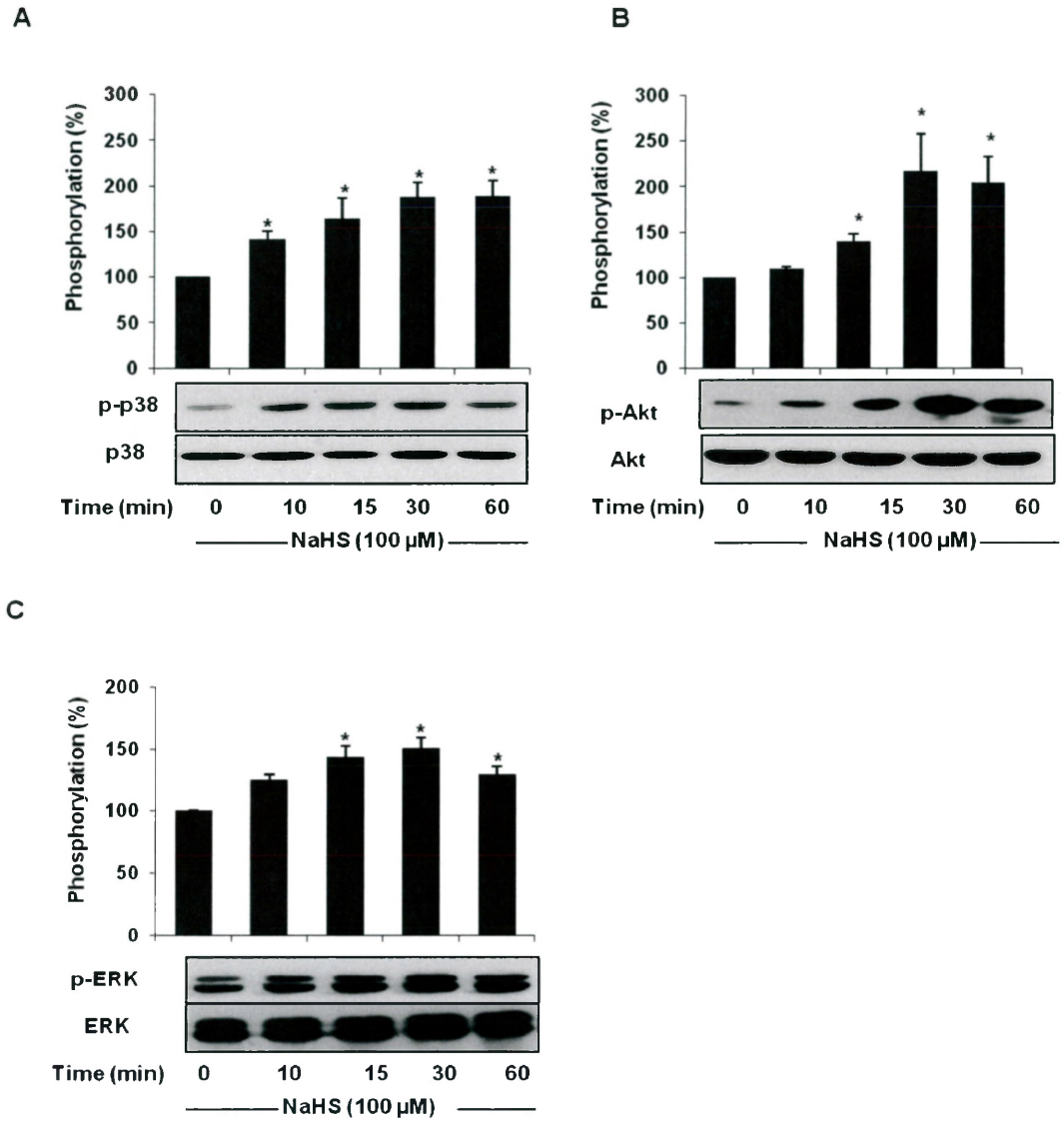

Figure 3. 3. H_2S-induced phosphorylation of p38 MAPK, Akt and ERK. ECs cells were treated with NaHS (100 μM) for different times (0-60 min). At the end of each time point, cells were collected and proteins lysates were analyzed by to western blot, using antibodies specific for the phosphorylated and total forms of (**A**) p38 MAPK, (**B**) Akt, and (**C**) ERK. Data were normalized to total protein level, n=3-4, $^{*}P < 0.05$ *vs.* control.

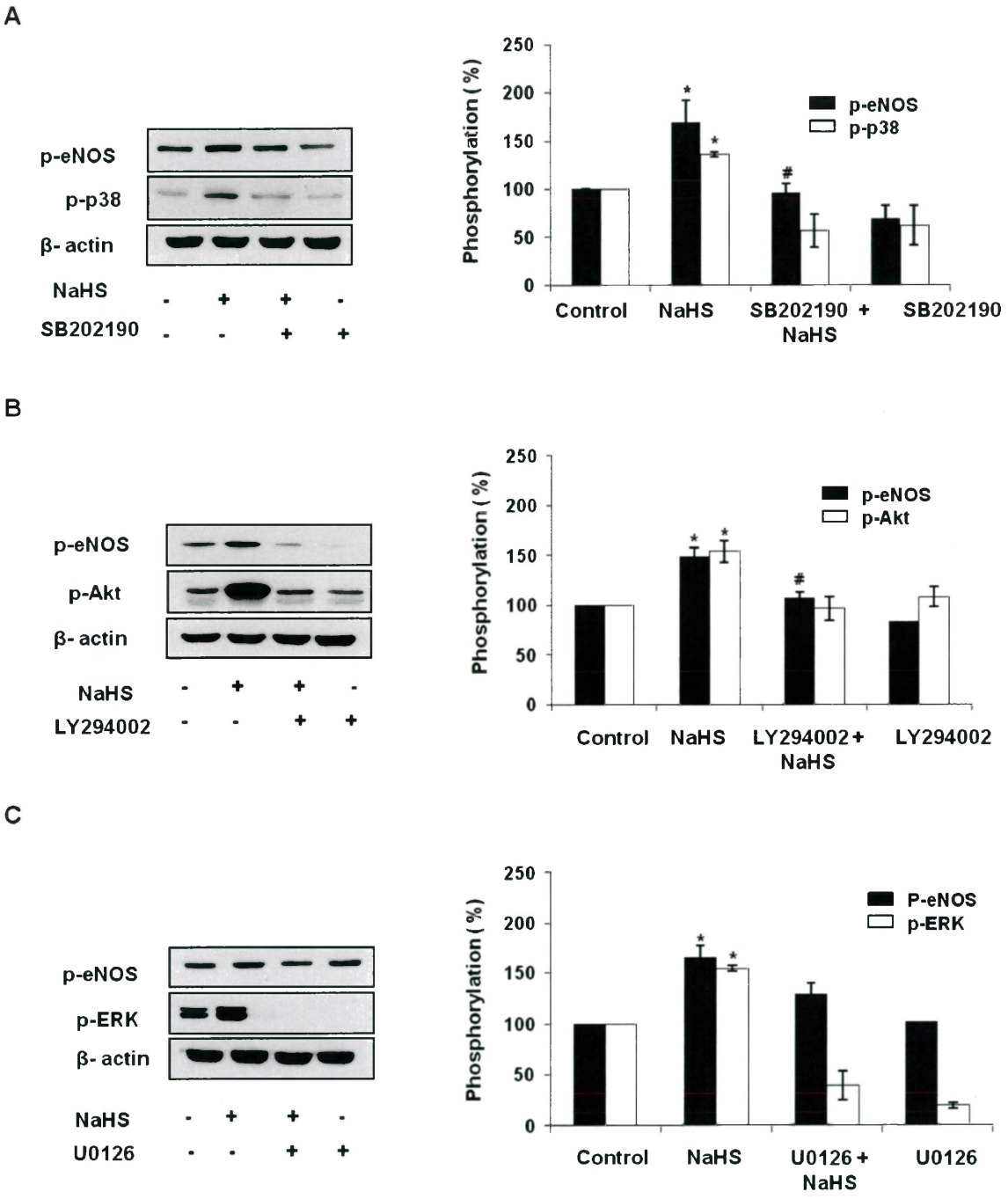

Figure 2. 4. H_2S-stimulated eNOS phosphorylation is dependent on p38 MAPK and Akt. ECs were pre-treated with (**A**) SB203580 (10 μM), (**B**) LY294002 (10 μM), and (**C**) U0126 (10 μM) for 1 hrs and then treated with NaHS (100 μM) for 30 min. Cell lysates were harvested and the level of phosphorylated forms of p38 MAPK, Akt, ERK, and eNOS were measured by western blot. n=3, $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* NaHS treated group.

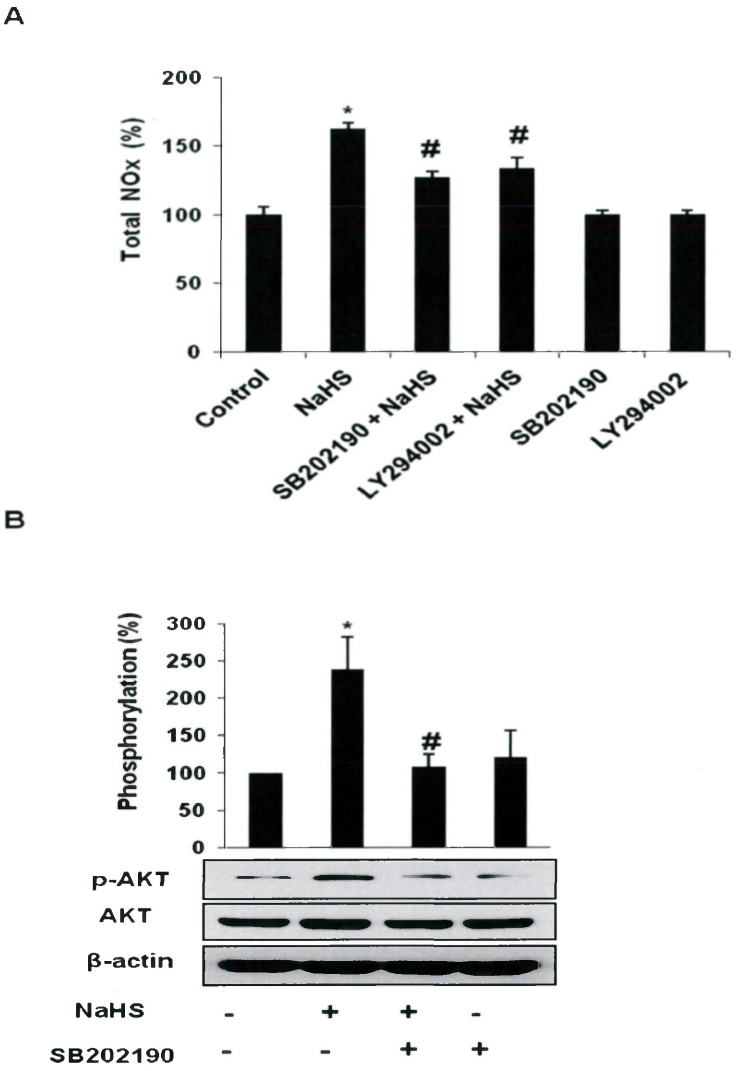

Figure 2. 5. Cross-talk between p38 MAPK and Akt in H_2S-induced NO production. The p38 MAPK inhibitor inhibited Akt and NO production induced by H_2S. (**A**) ECs were pre-treated with either SB203580 (10 μM) or LY294002 (10 μM) for 1 hrs, and then treated with NaHS (100 μM) for 30 min. At the indicated time point the NOx generation was assessed by Griess assay, n=3-4, $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* NaHS. (**B**) The phosphorylated Akt was measured by western blot after pre-treatment with SB203580 (10 μM) for 1 hr and NaHS (100 μM) treatment for 30 min, $^{*}P < 0.05$ *vs.* control, n=3-4.

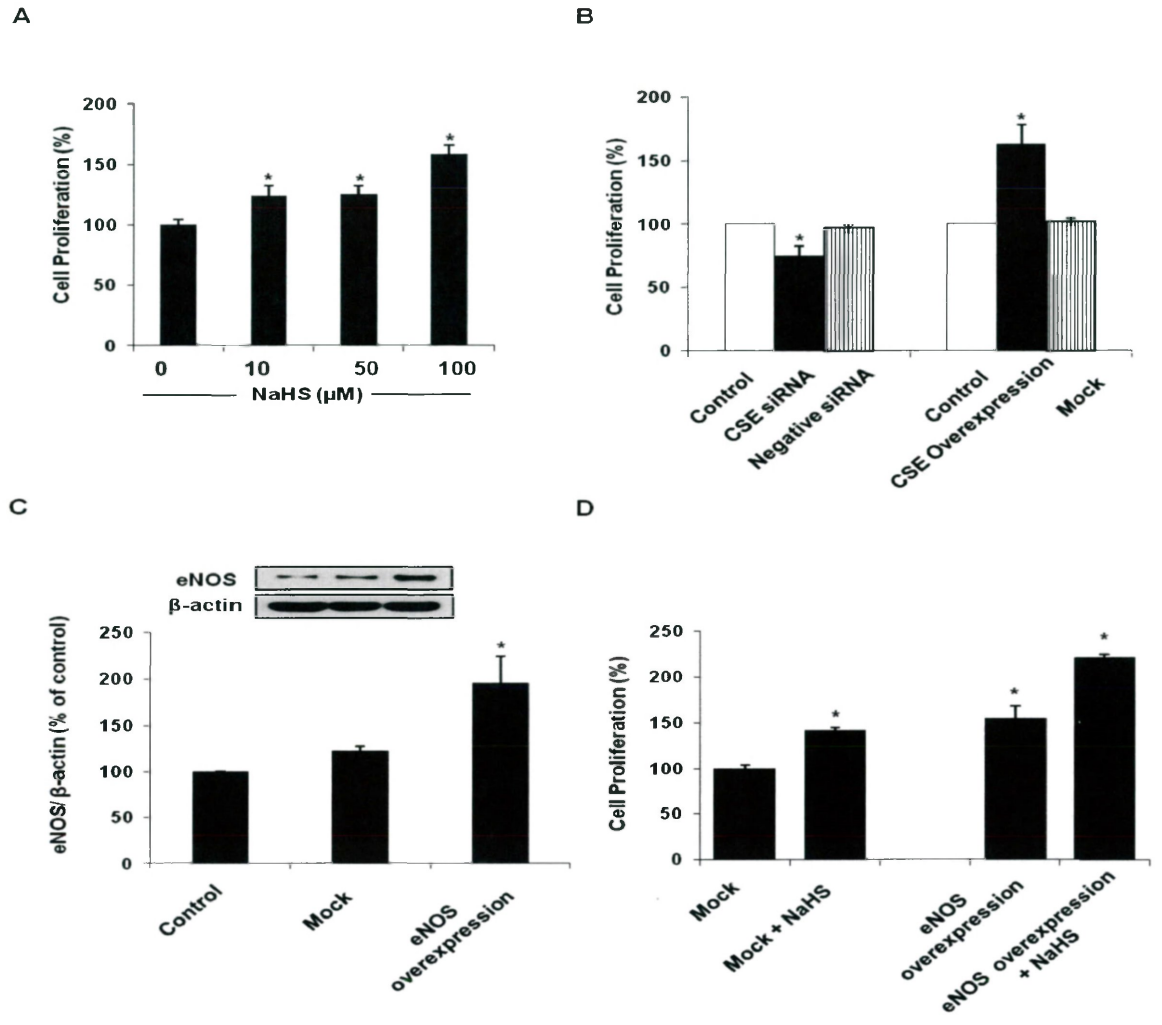

Figure 2. 6. H_2S-stimulated EC proliferation. (**A**) The effects of NaHS treatment on EC proliferation assessed using BrdU proliferation assay. n= 3, * $P < 0.05$ *vs.* control. (**B**) The effects of CSE knockdown or overexpression on EC proliferation. n=3, * $P < 0.05$ *vs.* control. (**C**) The efficiency of eNOS overexpression in ECs detected by western blot, n= 3, * $P < 0.05$ *vs.* Mock. (**D**) The effect of eNOS overexpression on EC proliferation. n= 3, * $P < 0.05$ *vs.* Mock.

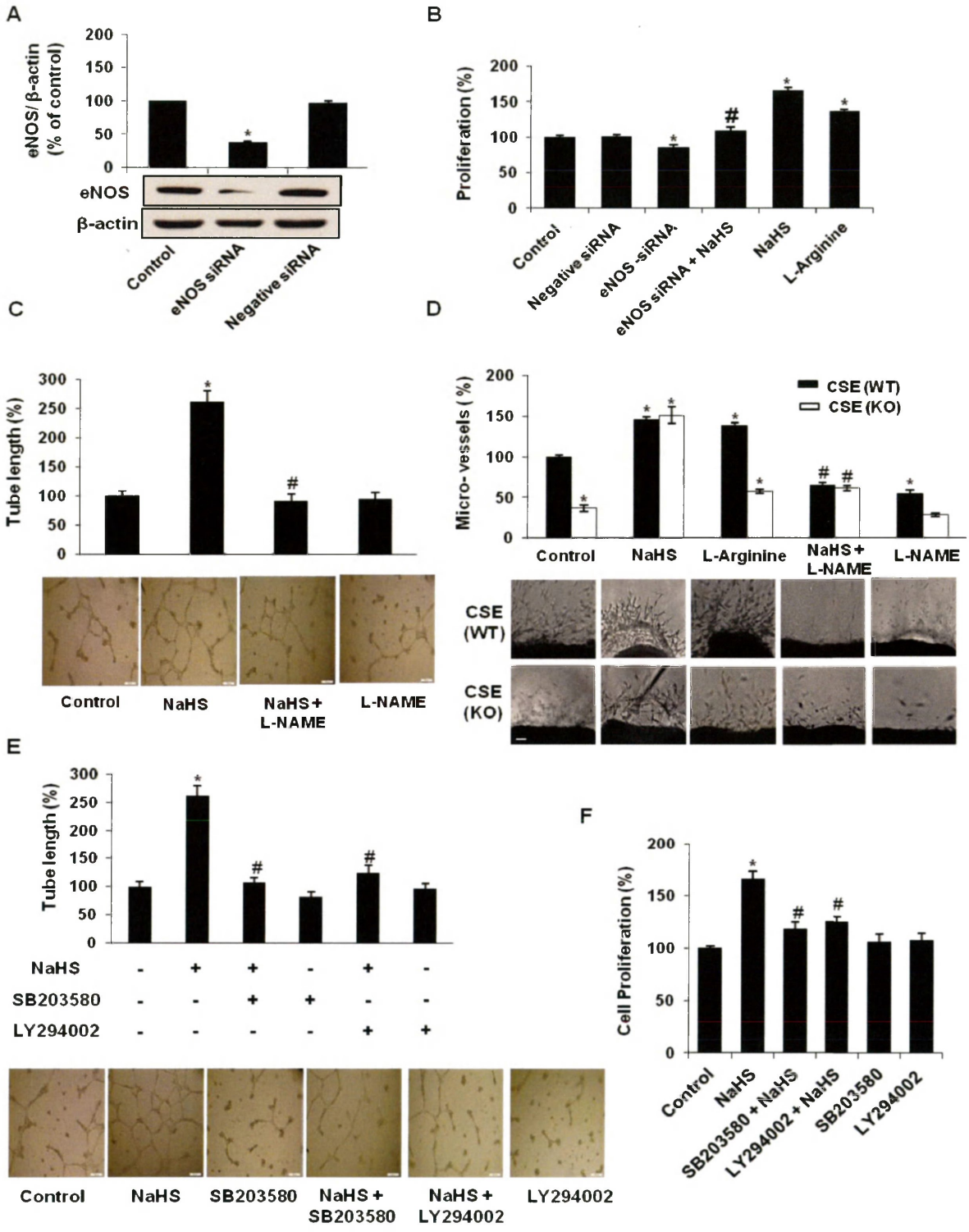

Figure 2. 7. H_2S interacts with NO to stimulate EC proliferation and angiogenesis. (**A**) The efficiency of eNOS knockdown transfection in EC detected by western blot. n= 3-4, $^{*}P < 0.05$ *vs.* control. (**B**) The effects of eNOS-knockdown (eNOS siRNA, 50 nM), NaHS (100 μM), and L-arginine (1 mM) treatments on EC proliferation evaluated by BrdU. assay. n = 3-4, $^{*}P < 0.05$

vs. control, $^{\#}P < 0.05$ *vs.* NaHS treated group. (**C**) H_2S-NO interaction on EC tube formation. The effects of NaHS (100 μM) and L-NAME (200 μM) on tube formation of ECs (Scale Bar: 500 μm). (**D**) The effects of L-NAME (200 μM), L-arginine (1 mM) and NaHS (100 μM) on angiogenesis (Scale Bar: 200 μm), n= 3-4 mice for each group, $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* NaHS treated group. (**E**) The involvements of p38 MAPK and Akt in EC proliferation and tube formation. ECs were pre-treated with p38 MAPK inhibitor SB202190 (10 μM) and Akt inhibitor LY294002 (10 μM) for 1hr, and treated with NaHS (100 μM) for 30 min. Cells (2×10^4 cells) were seeded on Martigel for 12 hrs to assist the formation of capillary-like structure (Scale Bar, 500 μm). (**F**) Cells were pre-treated with LY294002 or SB202190 and NaHS. The cells were cultured for 24 hrs for measurement of proliferation rate using BrdU. proliferation assay. n = 3-4, $^{*}P < 0.05$ *vs.* control.

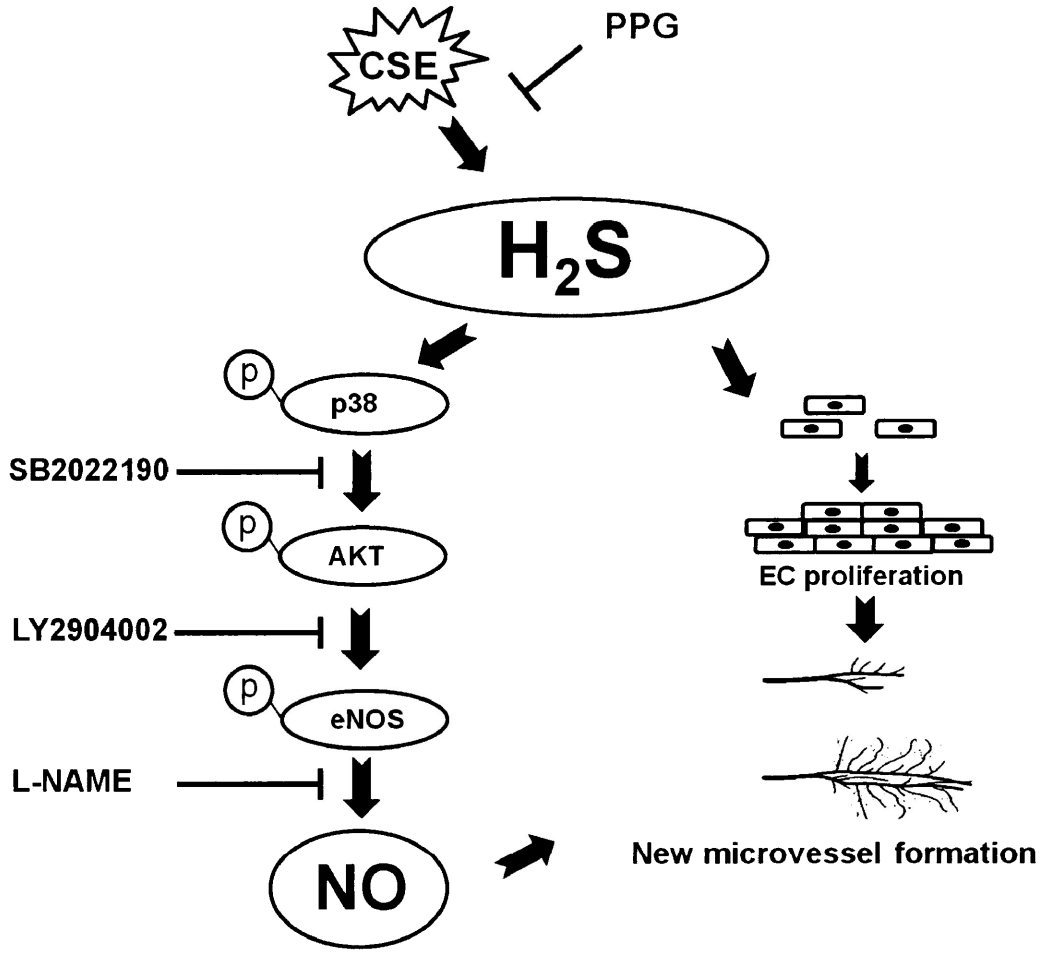

Figure 2. 8. Schematic representation of proposed pathways of H_2S-induced NO production and angiogenesis.

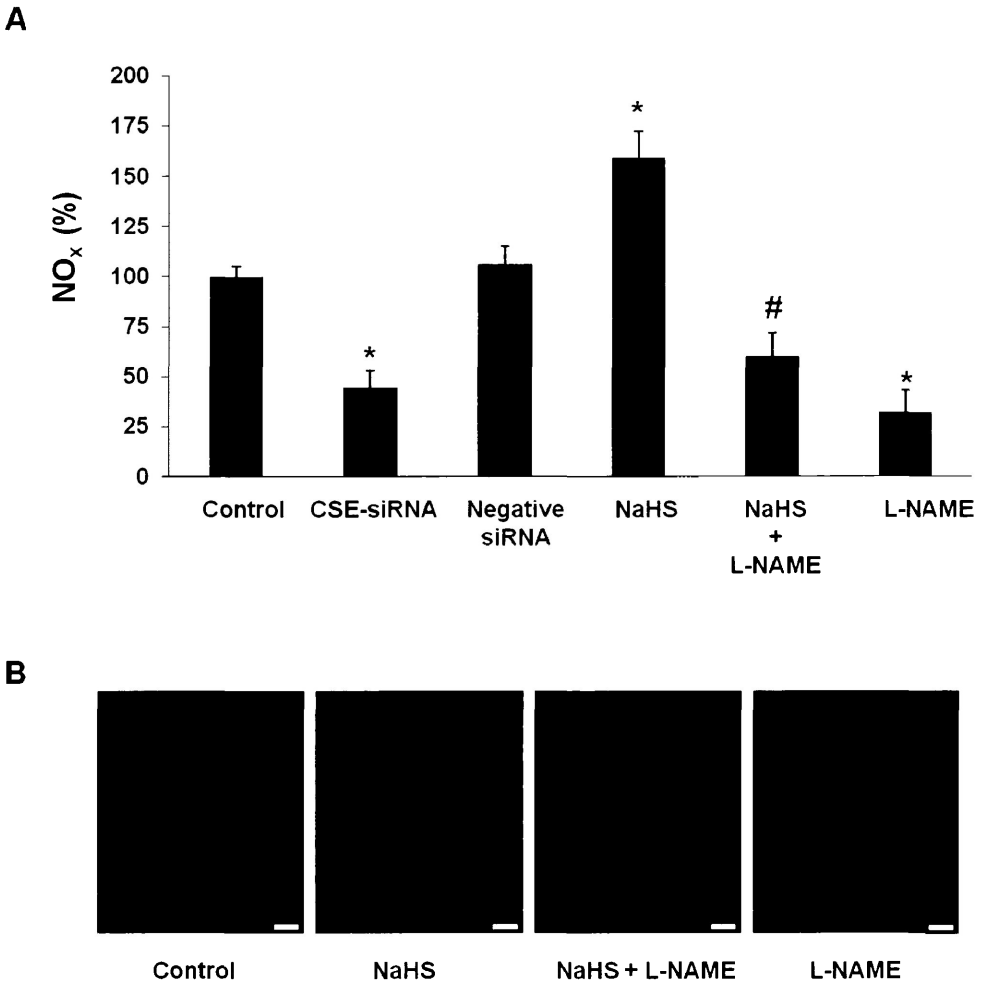

Figure 2. 9. (S1). The effect of H_2S on NO production in primarily cultured mouse aortic ECs. (**A**) The effects of CSE-siRNA knockdown and NaHS treatment on NO production detected by Griess assay, n=3, $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* NaHS treated group. (**B**) The effects of NaHS and L-NAME treatment on NO production detected by diaminofluorescein diacetate -based probes (DAF-FM), scale bars: 200 μm.

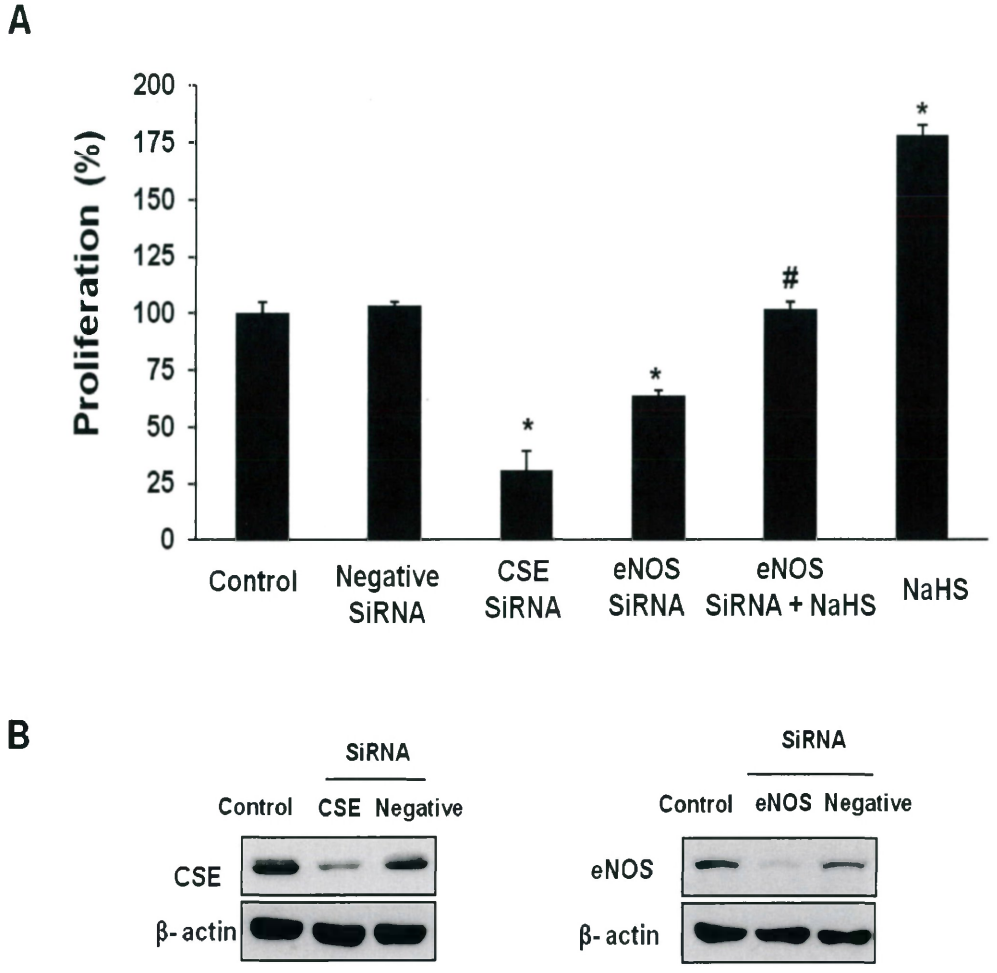

Figure 2. 10. (S2). The pro-proliferative effects of H_2S and NO on primarily cultured mouse aortic ECs. (**A**) The effects of CSE siRNA, eNOS siRNA, and NaHS treatments on EC proliferation assessed using BrdU proliferation assay, n= 3, $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* NaHS treated group (**B**) The efficiency of CSE siRNA and eNOS siRNA in ECs detected by Western blot.

CHAPTER 3

The coordination of *S*-sulfhydration, *S*-nitrosylation, and phosphorylation of endothelial nitric oxide synthase by hydrogen sulfide

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Running title: Interplay between *S*-sulfhydration and *S*-nitrosylation

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3. 1. Abstract

S-nitrosylation or *S*-sulfhydration of endothelial nitric oxide synthase (eNOS) alters the conformation of eNOS proteins and the production of NO. The goal of the present study was to investigate the interaction of *S*-nitrosylation and *S*-sulfhydration of eNOS and its structural and functional consequences. Our data indicate that eNOS was endogenously *S*-sulfhydrated and that NaHS (a H_2S donor) increased eNOS *S*-sulfhydration in a time-dependent manner. L-cysteine (a substrate of CSE) treatment increased *S*-sulfhydration of eNOS in aortic endothelial cells (ECs) isolated from wild type (WT) mice, but not that from cystathionine γ-lyase knockout (CSE-KO) mice. The level of eNOS *S*-sulfhydration in aortic tissue from CSE-KO mice was lower than that from WT mice. GSNO (a NO donor) induced, but NaHS reduced, eNOS *S*-nitrosylation. On the other hand, GSNO did not significantly alter eNOS *S*-sulfhydration. Mutation of one cysteine residue of eNOS (Cys-443-eNOS) completely eliminated eNOS *S*-sulfhydration and partially decreased its *S*-nitrosylation. NaHS or VEGF induced phosphorylation of WT-eNOS and Cys-443-eNOS. Mutation of Ser-1179 of eNOS did not affect its *S*-sulfhydration but abolished its phosphorylation. While the dominant configuration of eNOS proteins from WT mice was dimer, it was monomer of eNOS from CSE-KO mice or Cys-443- eNOS. In the presence of GSNO, more monomers were found with WT-eNOS, which was reversed by the subsequent treatment with NaHS. Cys-443-eNOS manifested itself as monomers, which was not changed by either GSNO or NaHS treatments. Compared with WT ECs, the production of NO was decreased, but superoxide increased, in CSE-KO ECs. NaHS increased NO production from both WT and CSE-KO ECs. NaHS decreased superoxide productions in CSE-KO ECs, but not in WT ECs. In summary, eNOS *S*-sulfhydration increases eNOS dimer coupling and NO bioavailability whereas eNOS *S*-nitrosylation generates opposite outcomes. *S*-sulfhydration and *S*-nitrosylation are

partially overlapping on the same cysteine residue. *S*-sulfhydration inhibits *S*-nitrosylation of eNOS, but not the other way around. As such, H_2S proves to be a critical gasotransmitter that coordinates multiple post-transcriptional modulations of given proteins.

Keywords: *S*-sulfhydration, *S*-nitrosylation, Phosphorylation, CSE, H_2S, eNOS, NO, eNOS uncoupling.

3. 2. Introduction

Hydrogen sulfide (H_2S) and nitric oxide (NO) are two gasotransmitters involved in the homeostatic regulation of vascular functions [2, 100]. In vascular walls, endothelial nitric oxide synthase (eNOS) is the predominant NOS isoform and cystathionine gamma-lyase (CSE) is mostly responsible for H_2S production [2, 100]. Both NO and H_2S function as endothelium-derived relaxing factors (EDRF) [19, 100, 265], and H_2S has been recently characterized as an endothelium-derived hyperpolarizing factor (EDHF) [160]. Like numerous proteins, eNOS is subjected to posttranslational modifications such as phosphorylation and *S*-nitrosylation [110]. Phosphorylation changes the structure of proteins due to transferring the negatively charged phosphates (PO_4^{3-}) by protein kinases onto hydroxyl groups (-OH) of the amino acid chains [266]. The phosphorylation cascades continue to function until protein phosphatases are activated and removed the phosphate group from the modified protein [266]. Protein *S*-nitrosylation occurs when NO covalently attaches to the thiol side chain (-SH) of cysteine residues to form *S*-nitrothiols (SNOs) [131, 132]. *S*-nitrosylation can be reversed by several de-nitrosylation enzymes like S-nitrosoglutathione reductase (GSNOR) and thioredoxin (Trx) [139]. GSNOR removes SNO through metabolism of GSNO to glutathione hydroxysulfenamide (GSNHOH) [138, 139]. The active site of Trx had two redox-active cysteine residues (Cys-Gly-Pro-Cys). Trx breaks disulfide bonds of its target protein and then binds with the protein [267]. One of the mechanisms by which H_2S mediates its effects is *via* protein *S*-sulfhydration, which is analogous to *S*-nitrosylation by NO. In this process H_2S covalently modifies cysteine residue in proteins to form hydropersulfides (-SSH) [140]. *S*-sulfhydration enhances cysteine activities, but *S*-nitrosylation most likely inhibits the cysteine reactivity. It has been reported that 1-2 % of proteins in liver total proteins were *S*-nitrosylated under physiological condition, while 10-25%

of proteins were *S*-sulfhydrated [140]. H_2S-induced *S*-sulfhydration of ATP-sensitive potassium (K_{ATP}) channels hyperpolarizes vascular smooth muscle cells and relaxes vascular tissues [141]. *S*-sulfhydration of intermediate (IK_{Ca}) and small conductance (SK_{Ca}) calcium-dependent potassium channels hyperpolarizes vascular ECs [142]. Moreover, *S*-sulfhydration of kelch-like ECH-associated protein 1 attenuated oxidative stress and delays cellular senescence in mouse embryonic fibroblasts cells [145]. It is now recognized that both H_2S and NO can modify protein cysteine residues. Yet, the interaction of *S*-nitrosylation and *S*-sulfhydration on the same cysteine residue under the same experimental conditions has not been elucidated adequately, let alone the structural and functional consequences of this interaction.

In the present study, we found that the same cysteine residue of eNOS was both *S*-sulfhydrated and *S*-nitrosylated. The interaction of these two thiol-binding mechanisms determined the conformation and function of eNOS. Whether eNOS phosphorylation was affected by *S*-sulfhydration and *S*-nitrosylation was also investigated.

3. 3. Material and Methods

Cell culture and transfection

In order to establish a cell line that stably expresses eNOS, control and mutant plasmids were transfected into HEK-293 cells (American Type Culture Collection, Manassas, VA) using Lipofectamine™ 2000 reagent as described by the manufacturer's protocol (Invitrogen, Burlington, ON, Canada). Transfected cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1.5 mg/mL geneticin (G418), which was supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (Sigma, Oakville, Canada). After 35-45 days of transfection, individual geneticin resistant-colonies were picked with verified stable

expression of eNOS. The cells were cultured in DMEM containing 0.5 mg/mL G148 for further passaging in an atmosphere of 95% O_2 and 5 % CO_2. Human umbilical vein endothelial cells (HUVECs-926) were kindly provided by Dr. Cora-Jean S. Edgell the (University of North Carolina, USA) [248], and cultured with DMEM containing 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin.

Isolation of primary ECs from the aorta in CSE-KO and WT mice

CSE-KO mice were homebred as previously described [19]. All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85 23, revised 1996) and approved by the Animal Care Committee of Lakehead University, Canada. CSE-KO mice and WT littermates of 10-12 weeks were anesthetized. Abdomen aortae were perfused with 1 ml phosphate buffered saline (PBS) containing 1000 U/ml heparin (Sigma). The aortae were then dissected out and immersed in DMEM containing 1000 U/ml heparin and 20% (v/v) FBS. Fat and connective tissues were rapidly cleaned off, and the aortae were rapidly tighten up with surgical ligation clip at one end and filled with collagenase type II (2 mg/ml) dissolved in DMEM and then the other end tightened. The aortae were incubated for 45 minutes at 37°C, and then the aortic ECs were released by flushing the aortae with 5 ml DMEM. The collected outflow was centrifuged at 1,200 × g for 5 minutes. The supernatant was discarded, and cell pellet obtained after centrifugation was resuspended in 2 mL DMEM with 10% (v/v) FBS and transferred to a 35 mm collagen type I coated-plates (Invitrogen). After 2 hours the medium was removed, cells were washed with PBS, and new DMEM was added containing 20% (v/v) FBS, 100 U/ml penicillin-G, 100 μg/ml streptomycin, 2 mM L-glutamine, 25 mM HEPES (pH 7.4),

100 μg/ml heparin, and 100 μg/ml EC growth supplement [249, 268]. The identity of aortic ECs was confirmed by the presence of endothelium-specific markers CD31 (Santa Cruz Biotechnology, Santa Cruz, CA) and eNOS (Cell Signaling Technologies, Beverly, MA, USA), and the absence of smooth muscle cell marker α-actin (Santa Cruz Biotechnology) by Western blotting (Figure 3. S1). Culture medium was changed every 2 days, and cultured cells of passages 3-7 were used.

Measurement of NO in ECs

Total nitrate/nitrite concentrations were measured by conversion of nitrate to nitrite. The cells were centrifuged, and supernatant were incubated with nitrate reductase (10 U/ml) and nicotinamide adenine dinucleotide 2′-phosphate (NADPH) (5 mM) for 1 hour at 37°C. After the incubation, 200 μL of the cell culture medium were tested by adding 100 μL of 1% sulfanilamide (solution A) for 10 minutes and then adding 100 μL of 0.3% N-1-naphthylethylenediamine dihydrochloride (solution B) for another 10 minutes at room temperature in darkness. Nitrite was quantified by spectrophotometer at 540 nm using sodium nitrite as standard (Promega, Madison, WI, USA).

The NO-specific fluorophore diaminofluorescein-DAF-FM probe (Invitrogen, Burlington, ON, Canada) was used to detect NO production. Equal number of cells was incubated with 5 μM DAF-FM for 30 minutes at 37°C in darkness. After the incubation, cells were washed to remove excess probe, replaced with fresh medium, and incubated for additional 10 minutes before the observation under a fluorescent microscope (Olympus IX71, Olympus America, PA, USA). The DAF-FM loaded cells were also assayed using a fluorescence

microplate reader (FLUOstar OPTIMA, BMC Lab tech, Ortenberg, Germany) with excitation and emission maxima at 495 and 515 nm, respectively [269].

Measurements of superoxide in ECs

Dihydroethidium (DHE) is membrane permeable and reacts with superoxide (O_2^-) to form ethidium, which in turn intercalates with DNA to produce nuclear fluorescence. Aortic ECs from WT and CSE-KO mice (passage 3-5) were used in these experiments. The tested cells were treated with DHE (10 μmol/L) (Sigma) for 30 minutes at 37°C in darkness. The production of superoxide was detected using a fluorescence microplate reader (FLUOstar OPTIMA) with an excitation wavelength of 485 nm and emission of 620 nm [223].

Site-directed mutagenesis

Plasmids pcDNA3.1 containing wild-type eNOS gene and Ser-1179-eNOS mutant (serine replaced with alanine) were purchased from Addgene (Cambridge, MA) [109]. The eNOS mutant of cysteine replacement with glutamine (Cys-443-eNOS) was generated using QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) [145]. The primer sequences of eNOS gene (GenBank # M89952.1) were the following (Forward) 5'-AGGCCAGGGGGGGCCAGCCCGCCGACTGGG-3' and (Reverse) 5'-GCCCAGTCGGCGGGCTGGCCCCCCCTGGCCT-3'. Briefly, the PCR reactions contained 10 ng templates of DNA, 125 ng of each of the primers, 200 μM of each dNTP, 1 × Pfu DNA polymerase reaction buffer and 2.5 U of DNA polymerase (Pfu). The PCR cycling parameters used for the reaction were one cycle at 95 °C for 1 minute, further 18 cycles of 95 °C for 50

seconds each, one cycle at 60°C for 50 seconds, one cycle at 68°C for 10 minutes, and extension at 68°C for 7 minutes. Positive clones of eNOS were identified by electrophoresis on 1% (w/v) agarose gel. The plasmid DNA templates from positive clones of eNOS were prepared using the QIAprep spin miniprep kit (Qiagen, Toronto, Canada). DNA sequencing was performed to screen the correct mutation of eNOS at the Paleo-DNA Laboratory at the Lakehead University, ON, Canada.

_S_-sulfhydration and _S_-nitrosylation assays

The cells or tissues were collected and washed twice with ice-cold PBS and then suspended in 250 µl HEN buffer (250 mM HEPES-NaOH, 1 mM EDTA, and 0.1 mM neocuproine). For *S*-sulfhydration detection, 100 µM deferoxamine (DFO) was added. Cell suspensions were sonicated for three times (5-10 second/each) on ice using a cell sonicator (Sonic Dismemrator Model 100, Fisher Scientific). The samples were centrifuged at 14,000 × g for 15 minutes at 4°C, and then the supernatants were collected. Four volumes of the blocking buffer (HEN buffer, 2.5% SDS and 20 mM *S*-methyl methanethiosulfonate (MMTS)) were added for 20 minutes at 50°C with shaking in the darkness to block the free thiol (-SH). The pre-chilled acetone was added for 20 minutes at -20°C to stop the MMTS reduction and precipitate the proteins. The proteins were centrifuged at 2,000 × *g* at 4°C for 10 minutes, and protein pellets were collected and resuspended in HEN buffer containing 1% SDS, 40 mM biotin-HPDP, 1 mM ascorbic acid, and then incubated for 3 hours at 25°C in darkness. For *S*-sulfhydration detection, no ascorbic acid was added at this step [140, 145]. The streptavidin biotin-binding protein beads were washed 3 times with PBS and incubated with the proteins for 1 hour at 25°C. After the incubation, the beads were washed with PBS for 5 times and spun down at 5,000 × *g* for 15

second. The biotinylated proteins were eluted from the beads by re-suspending the beads into loading sample buffer (3% SDS, 1% β-mercaptoethanol, 62.5 mM Tris-base and 0.005% bromophenol blue) at 37°C for 20 minutes with shaking. The protein samples were finally heated at 95°C for 5 minutes and subjected to Western blotting analysis to detect the *S*-nitrosylated or *S*-sulfhydrated proteins [140, 145, 270]. Densitometric quantification was performed using Alpha Digi Doctor Software (Richardson, TX, USA) and NIH image software- Image J. *S*-nitrosylation and *S*-sulfhydration levels were defined as the ratio between the *S*-sulfhydrated or *S*-nitrosylated eNOS and their total forms and expressed as the percentage of the controls.

Western blotting

Tested cells or aortic tissues were collected using cold PBS and incubated in a lysis buffer containing 0.5 M EDTA, 1 M Tris-Cl (pH 7.4), 0.3 M sucrose, and a mixture of protease inhibitors. The cell extracts were sonicated three times (5-10 second/each) on ice using a cell sonicator (Sonic Dismemrator Model 100, Fisher Scientific). Cellular extracts were separated by centrifugation at 14,000 × *g* for 15 minutes at 4°C. The supernatants were collected, and protein concentrations were measured using Bradford assay. Equal amounts of proteins were loaded on 7.5 % sodium dodecyl sulfate-polyacrylamide gels and run at 120 v for 1.5 hours. Gels were transferred to nitrocellulose membranes (Pall Corporation, Pensacola, FL, USA) at constant current of 200 mA for 3 hours using BioRad transfer system (Bio-Rad Laboratories, Inc. CA, USA). The membranes were incubated with primary antibodies at 4°C overnight. The dilution ratios for the antibodies were 1:1000 for anti-phospho-eNOS (Ser-1179) (Cell Signaling Technologies), anti-eNOS (Cell Signaling Technologies), anti-CSE (Proteintech Group, Chicago, IL, USA) and anti-PECAM (CD31) (Santa Cruz Biotechnology); and 1:10000 for anti-α-actin

(Santa Cruz Biotechnology) and anti-β-actin antibodies (Sigma). Membranes were then incubated with appropriate conjugated secondary antibody for 2 hours at room temperature. After the incubation, the membranes were washed 3 times with 1 × PBS and 1% Tween for 30 minutes. The membranes were visualized using enhanced chemiluminescence western blotting system (GE Healthcare, Piscataway, NJ, USA).

Determination of eNOS uncoupling

The monomer and dimer forms of eNOS (M-eNOS and D-eNOS) were detected by using low-temperature polyacrylamide gel electrophoresis (LT-PAGE) and Western blotting [223]. Briefly, eNOS protein extracts were prepared from aortae or cultured cells on ice. Equal amounts of the isolated proteins were mixed with sample buffer (62.5 mM Tris-HCl (pH 6.8), 40% glycerol, 0.01% bromophenol blue) and kept on ice. The electrophoresis was performed at constant current of 35 mA at 4°C for 4 hours. To preserve dimer, all gels and buffers were prepared without sodium dodecyl sulfate-polyacrylamide and preequilibrated to 4°C prior to electrophoresis, and the buffer tank placed in an ice bath during electrophoresis to maintain gel temperature below 15°C. The gels were transferred to the nitrocellulose membrane, and eNOS protein expression analyzed by Western blotting using anti-eNOS antibody (Cell Signaling Technologies) for both dimer and monomer at 1:1000 dilutions.

Statistical analysis

Statistical comparisons were made using Student's *t* or two-way ANOVA followed by Tukey post hoc tests as applicable using Origin 8 software (OriginLab Corporation, MA, USA). Significant level was set at $P< 0.05$.

3. 4. Results

S-sulfhydration of eNOS

S-sulfhydration and *S*-nitrosylation eNOS proteins in aortic tissues of WT mice were detected under basal conditions (Figure 3. 1A). Treatment of WT aortic tissue lysate with 100 μM NaHS increased eNOS *S*-sulfhydration, started from 15 minutes and lasted up to 2.5 hours (Figure 3. 1A). GSNO at 100 μM (a NO donor) maximally increased eNOS *S*-nitrosylation during the first 15 min and then this increase declined to the basal level during the following 150 minutes (Figure 3. 1A). The level of eNOS *S*-sulfhydration was significantly lower in aortic tissues from CSE-KO mice than that from WT mice (Figure 3. 1B). Treatment with L-cysteine (a CSE substrate) (0.1 – 10 mM) for 1 hour markedly increased *S*-sulfhydration of eNOS in aortic ECs isolated from WT mice in a concentration dependent manner (Figure 3. 1C). In contrast, L-cysteine failed to elicit *S*-sulfhydration of eNOS in aortic ECs isolated from CSE-KO mice (Figure 3. 1C).

The reciprocal effects of H_2S and NO on eNOS *S*-nitrosylation and *S*-sulfhydration

Treatment of aortic lysates with GSNO (200 μM) for 30 minutes increased eNOS *S*-nitrosylation (Figure 3. 2A). Treatment with NaHS (100 μM) for 30 minutes had no effect on eNOS *S*-nitrosylation (Figure 3. 2A). NaHS (100 μM) co-treatment for 30 minutes abolished the effect of the proceeding GSNO treatment on eNOS *S*-nitrosylation (Figure 3. 2A). NaHS (100 μM) treatment, but not GSNO (200 μM), of aortic lysates for 30 minutes increased *S*-sulfhydration (Figure 3. 2B). GSNO (200 μM) co-treatment for 30 minutes of aortic lysates did not change the effect of the proceeding NaHS treatment on eNOS *S*-sulfhydration (Figure 3. 2B).

The roles of Cys-443 and Ser-1179 in eNOS *S*-sulfhydration and *S*-nitrosylation

Treatment of HEK-293 cell lysates with NaHS (100 μM) increased *S*-sulfhydration of the heterologously expressed WT-eNOS (Figure 3. 3A). This NaHS treatment, however, failed completely to induce *S*-sulfhydration of Cys-443-eNOS (Figure 3. 3A). The phosphorylation of eNOS was abolished after Ser-1179 mutation to alanine (Figure 3. 3B). Treatment of cell lysates with GSNO (200 μM) increased *S*-nitrosylation of WT-eNOS and Cys-443-eNOS (Figure 3. 3C). Treatment of cell lysates with NaHS (100 μM) induced *S*-sulfhydration of Ser-1179-eNOS (Figure 3. 3D). Basal phosphorylation level was the same for Cys-443-eNOS and WT-eNOS (Figure 3. 3E). NaHS (100 μM) or VEGF (20 ng/ml) treatments increased the phosphorylation of both WT-eNOS and Cys-443-eNOS (Figure 3. 3E). GSNO (200 μM) and NaHS treatments had no effect on the phosphorylation of Ser-1179-eNOS (Figure 3. 3E).

The effect of H_2S on eNOS coupling in vascular tissues

Next, we determined the effects of NaHS and GSNO co-treatment on the quaternary structure (monomer and dimer) of eNOS proteins. Dimeric and monomeric forms of eNOS were separated by low temperature-PAGE and visualized by immunoblotting with anti-eNOS antibody. The amounts of eNOS dimers presented in aortic tissues from WT mice was around 16 time of the monomers, but eNOS dimers in CSE-KO aortae tissue was only around 0.2 of its monomer level (Figure 3. 4A). L-NAME (200 μM) treatment of the isolated mouse aorta ECs for 1 hour did not alter the dimer/monomer ratio of eNOS (Figure 3. 4B). Treatment of ECs with GSNO (200 μM) alone or with L-NAME decrease the eNOS dimers/monomer ratio (Figure 3. 4B). GSNO (200 μM) treatment of the isolated ECs for 30 minutes destabilized, but NaHS (100 μM) treatment for 30 minutes stabilized, eNOS dimers (Figure 3. 4B). Importantly, NaHS (100

μM) co-treatment for 30 minutes reversed the proceeding effect of GSNO treatment on eNOS dimer (Figure 3. 4B). The expression of total eNOS was not changed by GSNO, NaHS or L-NAME treatments when compared with the controls with no treatments (Figure 3. 4B).

To determine whether the sulfhydrated cysteine was required for the eNOS dimer assembly we examined the dimer/monomer ratio of Cys-443-eNOS in HEK cells. Cys-443-eNOS migrated as monomers in LT-PAGE, whereas the heterologously expressed WT-eNOS manifested itself as both monomers and dimers. NaHS (100 μM) treatment of HEK-293 cells stabilized eNOS dimers, but GSNO (200 μM) induced more eNOS monomers, in WT-eNOS (Figure 3. 5A). NaHS (100 μM) treatment for 30 minutes reversed the effect of the proceeding 30 minutes- GSNO treatment. Neither NaHS (100 μM) nor GSNO (200 μM) caused dimer formation of Cys-443-eNOS (Figure 3. 5A).

NaHS (100 μM) treatment increased NO production from WT-eNOS-expressed HEK-293 cells but not from Cys-443-eNOS expressed cells. GSNO (200 μM) increased, but L-NAME (200 μM) decreased NO production from either WT-eNOS or Cys-443-eNOS (Figure 3. 5B). NaHS co-treatment enhanced the effect of the proceeding GSNO treatment on NO production from either WT-eNOS or Cys-443-eNOS. L-NAME pre-treatment for 1 hr did not alter GNSO-induced NO production in these HEK cells (Figure 4. 5B).

The effect of H_2S on superoxide and NO in aortic ECs

Aortic ECs isolated from CSE-KO mice (CSE-KO-ECs) showed higher levels of superoxide and lower levels of NO compared to those from WT mice (Figure 3. 6). NaHS (100 μM) treatment for 1 hour decreased the levels of superoxide in CSE-KO-ECs but not in WT-

ECs. GSNO (200 μM) treatment for 1 hour increased the formation of superoxide in WT-ECs and CSE-KO-ECs (Figure 3. 6A). NaHS (100 μM) treatment for 1 hour did not change the effect of the preceding GSNO treatment on superoxide levels (Figure 3. 6A). Both NaHS (100 μM, 30 minutes) and GSNO (200 μM, 1 hour) treatments increased the levels of NO in aortic ECs from CSE-KO and WT mice. NaHS (100 μM) treatment for 30 minutes increased the effect of the preceding GSNO treatment on NO levels in CSE-KO-ECs (Figure 3. 6B).

3. 5. Discussion

S-nitrosylation and *S*-sulfhydration are two posttranslational modification of cysteines, playing important roles in cellular regulation and signaling in many organisms [136]. Two previous and important studies have shown that the same cysteine at GAPDH and NF-kappaB proteins can be *S*-nitrosylated and *S*-sulfhydrated [13, 36]. However, *S*-sulfhydration and *S*-nitrosylation of these same proteins were conducted in separate experiments, not at the same time under the same conditions. How these two posttranslational modifications interact with each other was not addressed either. An initial study showed that *S*-nitrosylation of GAPDH at Cys-150 abolished its catalytic activity [38]. A study 5 years later showed that *S*-sulfhydration of GAPDH at Cys-150 augmented its activity [140]. *S*-nitrosylation of p65 at Cys-38 residue inhibited NF-kappaB-dependent gene transcription [271] but *S*-sulfhydration of the same decrease cell apoptosis [143]. Under the same experimental conditions and at the same time, we found that *S*-sulfhydration and *S*-nitrosylation interact with each other by competitively modifying the same cysteine residue of eNOS. More importantly, we for the first time demonstrated that *S*-sulfhydration reverses *S*-nitrosylation of eNOS but *S*-nitrosylation has no effect on *S*-sulfhydration. *S*-nitrosylation occurs faster but is less stable than *S*-sulfhydration of eNOS as evidenced in the following. *i*) The significant increase in the *S*-nitrosylation signal

started from 15 minutes whereas *S*-sulfhydration signal started from 30 minutes. *ii*) The *S*-nitrosylation signal reduce to the basal level after 1 hour treatment whereas *S*-sulfhydration signal stays until 2.5 hours. *iii*) The co-treatment with NaHS to induced *S*-sulfhydration, reduced *S*-nitrosylation. *iv*) Co-treatment with GSNO to induced *S*-nitrosylation, did not reduced *S*-sulfhydration. The differences between *S*-sulfhydration and *S*-nitrosylation can be explained by the nature of the chemical reaction between H_2S and NO and their target thiols [272]. The chemical reaction for *S*-nitrosylation (-S-N=O) appears to be kinetically favored reaction whereas *S*-sulfhydration (S-S-H) appears to be thermodynamically favored. The kinetically favored reaction usually happens faster, but the final product is not stable. Whereas, thermodynamically favored reaction happens slower and more stable. Likewise, we have found that *S*-sulfhydration reverses *S*-nitrosylation in eNOS protein, but *S*-nitrosylation has no effect on *S*-sulfhydration. The chemical strength of the *S*-nitrosylated bond (S-NO) is weaker (12-20 kcal/mol) compared to *S*-sulfhydrated bond (S-SH) (60 kcal/mole), this clearly explains why *S*-nitrosylated bond in eNOS can break easier compared to *S*-sulfhydrated one. In addition, the possible explanation why *S*-sulfhydration reverses *S*-nitrosylation, is that H_2S produces the HS^- and this can turn NO-thiol into good leaving group to produce thiosulfide bond as shown in equation: $HS^- + RS\text{-}NO \rightarrow NO + RS\text{-}SH$. However, *S*-nitrosylation cannot do the same (reveres) for *S*-sulfhydration because NO cannot break the strong S-S bond. Therefore, our results suggest that H_2S maintain the extent of eNOS *S*-nitrosylation by a reversible modification *via* *S*-sulfhydration, associated with enzyme activation.

The interaction between *S*-sulfhydration and other posttranslational modification in eNOS protein has not been explored previously. In our study, we found that H_2S increased the phosphorylation of WT-eNOS and Cys-443-eNOS, while H_2S failed to *S*-sulfhydrate Cys-443-

eNOS, implying that eNOS *S*-sulfhydration is not directly linked to eNOS phosphorylation. The phosphorylation of eNOS at Ser-1179, which is located within the reductase domain, increases eNOS activity by enhancing reductase activity and calcium sensitivity [53, 100]. In addition to inhibition of calmodulin dissociation from eNOS and enhancement of the internal rate of eNOS electron transfer [115]. The eNOS phosphorylation of Ser-1179 results in a negative charge inducing a conformational change that shifts the entire FMN domain to allow enhanced electron transfer through the reductase domain, activating eNOS [95]. Here we found that eNOS mutation at Ser-1179 can still be *S*-sulfhydrated (Figure. 3. 3D). Although, Ser-1179-eNOS can no longer be phosphorylated (Figure. 3. 3B). Thus our data indicate that phosphorylation of eNOS at Ser-1179 would not be necessary linked to *S*-sulfhydration.

The functional quaternary structure of eNOS proteins is a dimer which can produce NO [53]. The uncoupling of eNOS into monomers has been found in many forms of endothelial dysfunction such as hypertension [273], type II diabetes [274], and age-related erectile dysfunction [275]. Uncoupling of eNOS results in a higher superoxide and lower NO bioavailability [276]. A previous study showed that *S*-nitrosylation decreased dimer levels of eNOS from bovine ECs [135]. Our present study for the first time demonstrated the impact of H_2S and *S*-sulfhydration on eNOS dimer stability. The aortic tissue isolated from CSE-KO mice has less dimer/monomer ratio of eNOS than those from WT mice. This tells that the lack of formation of eNOS dimer formation possibly due to CSE-defiaency, which is further substantiated by the effect of exogenous H_2S treatment to induced *S*-sulfhydration. GSNO induced eNOS monomer and H_2S co-treatment restored the eNOS dimer. GSNO induced more eNOS monomer which is consistent with previous reports [135]. We speculate that H_2S-induced *S*-sulfhydration and reverses *S*-nitrosylation to stabilize the eNOS dimer. One of the functional

consequences of H_2S-enhanced eNOS dimer stability is the decreased NO level in CSE-KO-ECs (Fig. 6) where both endogenous H_2S level and eNOS dimer level are low (Fig. 4A). NaHS treatment of WT-ECs increased the levels of eNOS dimer (Fig. 4B) and NO (Fig. 6C), providing additional evidence to link *S*-sulfhydration and dimer coupling of eNOS to NO production. The bioavailability of NO is dependent on both of its production and destruction [53]. We found that the levels of superoxide were higher in CSE-KO-ECs than in WT-ECs, which were suppressed by NaHS treatment. The underlying mechanisms for these effects of H_2S could be related to *S*-sulfhydration and stabilization of eNOS. For NO to be produced, the electrons donated by NADPH must move to the reductase domain of one monomer of eNOS and proceed one by one *via* FAD and FMN redox carriers to the heme group in the oxygenase domain of another monomer of eNOS [100]. In the oxygenase domain, electrons interact with the heme iron and BH_4 at the active site to catalyse the reaction of oxygen with L-arginine to generate citrulline and NO [100]. However, when the dimer of NOS is uncoupled ferrous-dioxygen complex is dissociated. As a result superoxide is generated from the oxygenase domain instead of NO [100]. The mechanisms by which *S*-nitrosylation or *S*-sulfhydration modulate eNOS dimer are not clear. However, one study has found that NO can destroy the zinc tetrathiolate bond at the dimer interface of eNOS upon *S*-nitrosylation [135]. These authors found that there was an increase in release of zinc when eNOS was exposed to NO [135]. Whereas, after exposure of eNOS to TPEN, which is a membrane-permeable zinc chelator and decreased the intracellular level of zinc, the eNOS dimers converted into monomers [101]. However, the effect of TPEN was partially blocked by exogenous zinc exposure, but not by Mg^{2+} or Cu^{2+} [101]. The inactivation of uncoupling or monomeric form of eNOS happens because the prerequisite electron transfer between two monomers of eNOS cannot occur [135]. Here we propose that

H_2S-induces *S*-sulfhydration stabilizes eNOS dimers by preventing *S*-nitrosylation leading to dimerization (Figure 3. 7).

The competitive interaction between *S*-sulfhydration and *S*-nitrosylation of eNOS was confirmed by the use of Cys-443-eNOS in our study. Not all cysteine residues in proteins are accessible to posttranslational modification [277, 278]. The presence of metal ions (Mg^{2+} or Ca^{2+}), local pH, and the acid base motifs affect thiol reactivity to *S*-nitrosylation [131, 279-281] and would equally affect *S*-sulfhydration. There are 28 cysteine residues in eNOS protein, some of these cysteine residues located in the reductase domain and others located in the oxygenase domain. The location of Cys-443 at eNOS is in between acid (glutamine) and base (arginine) amino acids and is the last residue close to the C terminus of oxygenase domain in the dimer interface of eNOS, this might serve as good target for *S*-sulfhydration. In our study, a detectable increase in *S*-nitrosylation was seen after GSNO treatment in WT-eNOS, however, this level were decreased after Cys-443 mutated to glutamine , but not completely abolished, implying that Cys-443 is only one of multiple cysteine residues that can be *S*-nitrosylated. In contrast, mutation at Cys-443 completely eliminated *S*-sulfhydration of eNOS, indicating that *S*-sulfhydration of eNOS occurs only at Cys-443.

In summary, *S*-sulfhydration and *S*-nitrosylation interact with each other by competitively modifying the same cysteine residue of eNOS. H_2S increases eNOS *S*-sulfhydration and decreases *S*-nitrosylation, facilitates eNOS dimer coupling, and increases NO bioavailability. *S*-sulfhydration reverses *S*-nitrosylation of eNOS, but *S*-nitrosylation and phosphorylation of eNOS has no effect on *S*-sulfhydration. Therefore, curbing *S*-nitrosylation and stabling eNOS dimer may represent an important regulatory mechanism for the molecular and cellular effects of H_2S in the cardiovascular system as well as other systems.

Acknowledgment:

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Conflict of interest:

The authors state no conflict of interest.

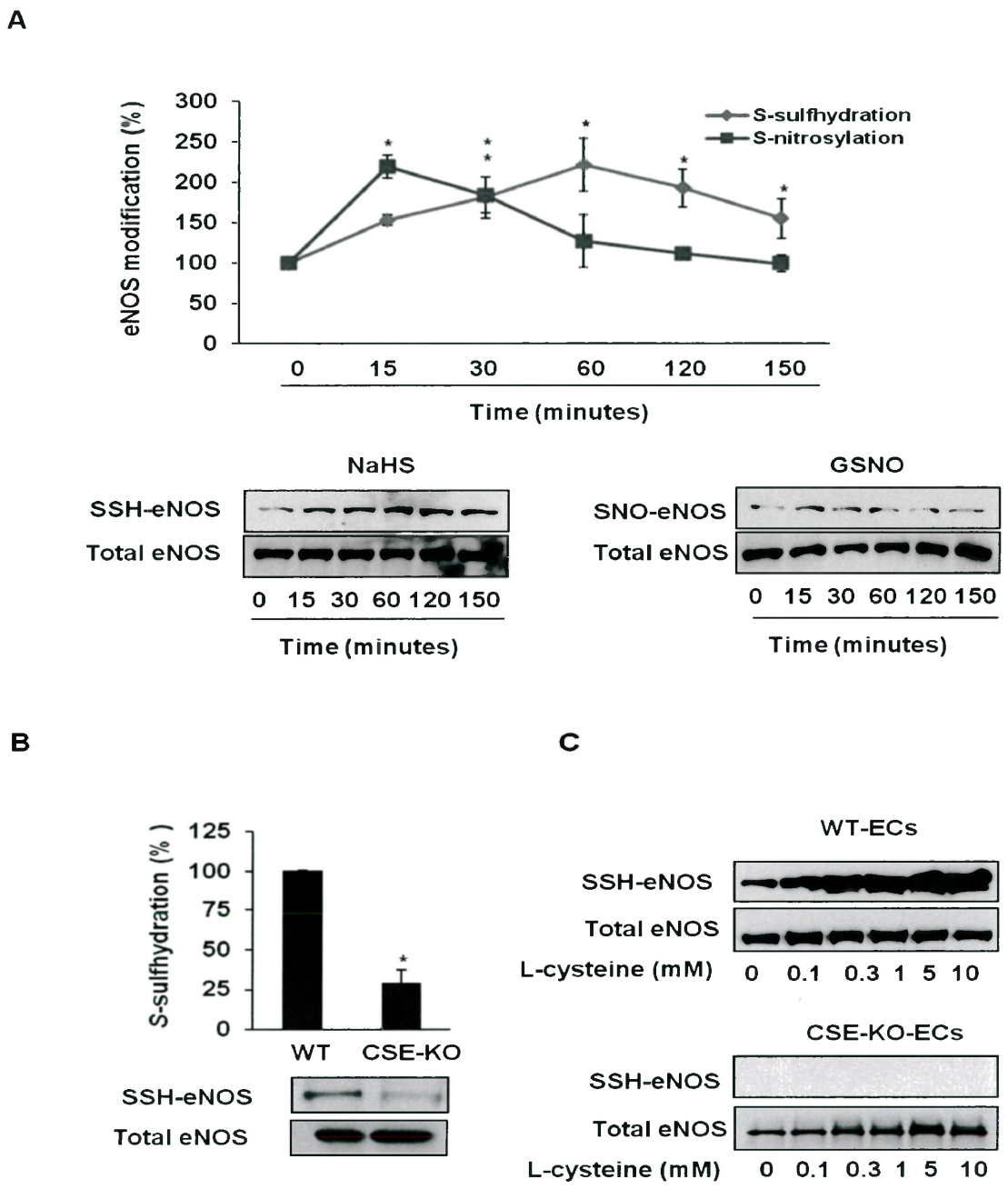

Figure 3. 1. *S*-sulfhydration and *S*-nitrosylation of eNOS from isolated aortic ECs or aortic tissues. (A) EC lysates were treated with NaHS (100 μM), or GSNO (200 μM) for 15 to 150 minutes and subjected to *S*-sulfhydration or *S*-nitrosylation assay. n=4, $^{*}P< 0.05$ *vs.* control. **(B)** *S*-sulfhydration of aortic eNOS from CSE-KO and WT mice. n=3. Each individual experiment was from 20 CSE-KO and WT mice, $^{*}P< 0.05$ *vs.* WT mice. **(C)** The effect of L-cysteine (0.1 - 10 mM) treatment on eNOS *S*-sulfhydration in primarily cultured aortic ECs isolated from WT and CSE-KO mice. The representative Western blotting was taken from 3 independent experiments. SSH-eNOS represents *S*-sulfhydrated eNOS. SNO-eNOS represent *S*-nitrosylated eNOS. eNOS modification before the addition of NaHS or GSNO (time 0) is defined as 100%.

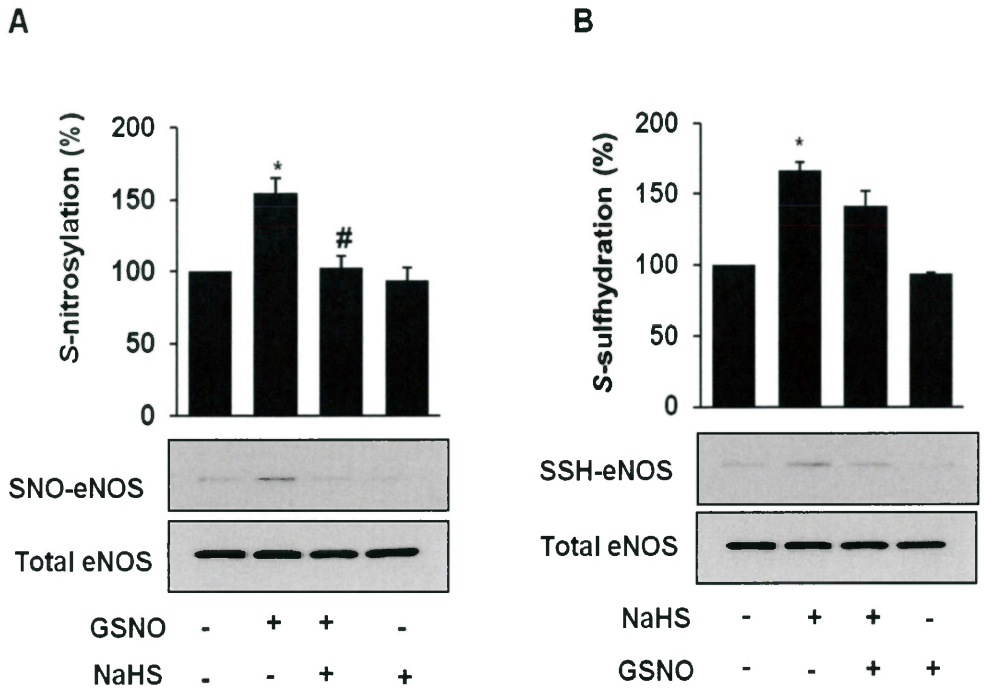

Figure 3. 2. The effects of NO and H_2S on *S*-sulfhydration and *S*-nitrosylation. Aortic ECs lysates were treated with GSNO (200 μM) alone or followed by NaHS (100 μM) for 30 minutes at 37°C and then subjected to *S*-nitrosylation assay (**A**), or *S*-sulfhydration assay (**B**). n=4 for each experiment, $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* GSNO treated group. SNO-eNOS represent *S*-nitrosylated eNOS. SSH-eNOS represents *S*-sulfhydrated eNOS. The *S*-nitrosylation or *S*-sulfhydration of controls without the addition of GSNO or NaHS is defined as 100%.

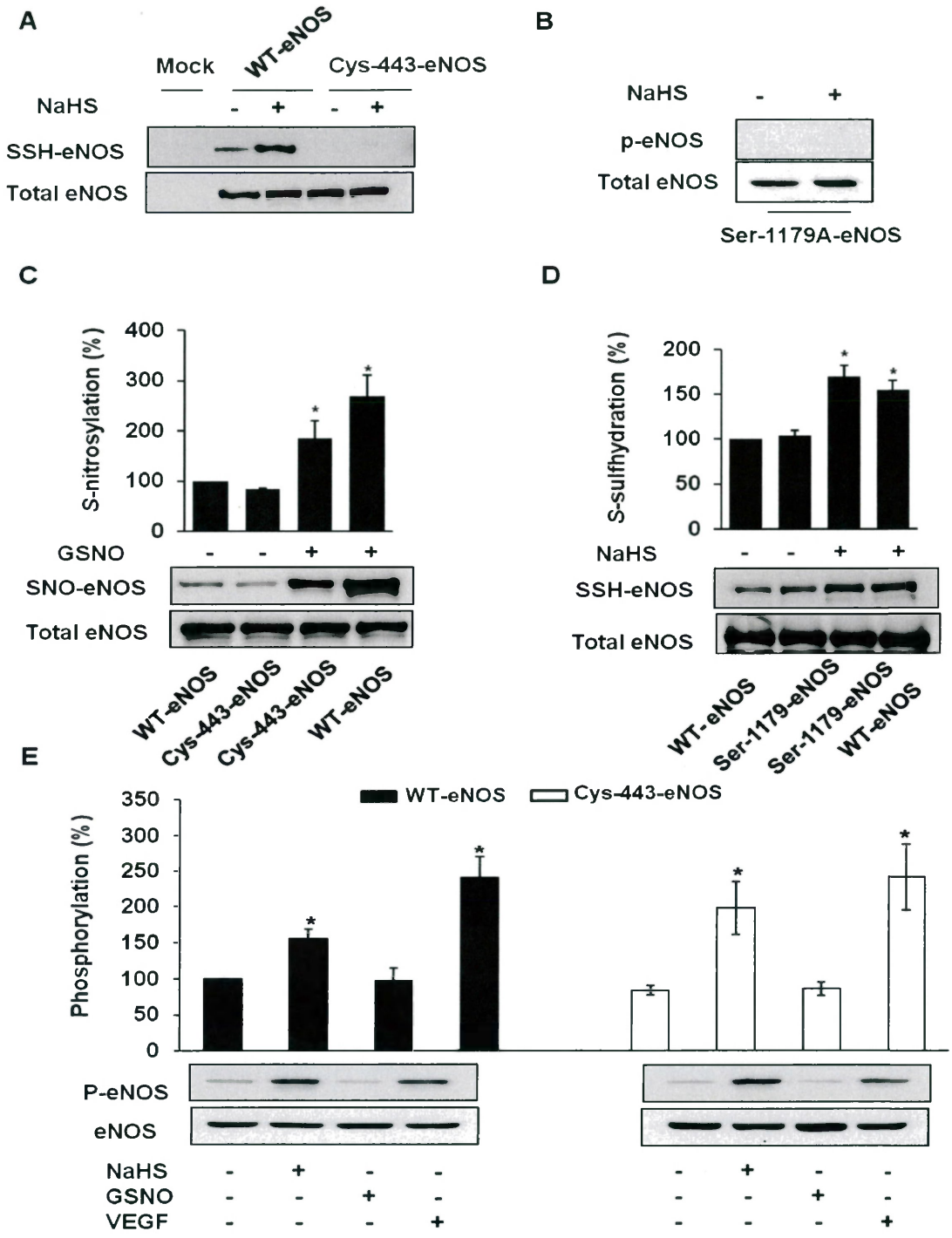

Figure 3. 3. The effects of Cys-443 and Ser-1179 mutations on eNOS *S*-sulfhydration and *S*-nitrosylation. HEK-293 cells were transfected with plasmids encoding wild-type eNOS (WT-eNOS), Cys-443-eNOS, or Ser-1179-eNOS. The cell lysates were collected and treated for 30 minutes at 37°C. (**A**) The effects of NaHS (100 μM) treatment on *S*-sulfhydration of WT-eNOS and Cys-443-eNOS. (**B**) The effect of NaHS (100 μM) treatment on eNOS phosphorylation in Ser-1179-eNOS mutant. (**C**) The effects of GSNO (200 μM) treatment on *S*-nitrosylation of WT-eNOS and Cys-443-eNOS, n=4 for each experiment, $^{*}P < 0.05$ *vs.* control. (**D**) The effects of

NaHS (100 μM) treatment on *S*-sulfhydration of WT-eNOS and Ser-1179-eNOS. n=3 for each experiment, $^{*}P < 0.05$ *vs.* control. (**E**) The effects of NaHS (100 μM), GSNO (200 μM) and VEGF (20 ng/ml) on phosphorylation of WT-eNOS and Cys-443-eNOS. Phospho-specific eNOS (P-eNOS) was detected using Western blot analysis against the level of total eNOS n=3-4 for each experiment, $^{*}P< 0.05$ *vs.* controls (HEK-293 transfection with pcDNA3.1 empty plasmid (Mock)). SNO-eNOS represent *S*-nitrosylated eNOS. SSH-eNOS represents *S*-sulfhydrated eNOS. The *S*-sulfhydration or *S*-nitrosylation before the addition of NaHS or GSNO is defined as 100%.

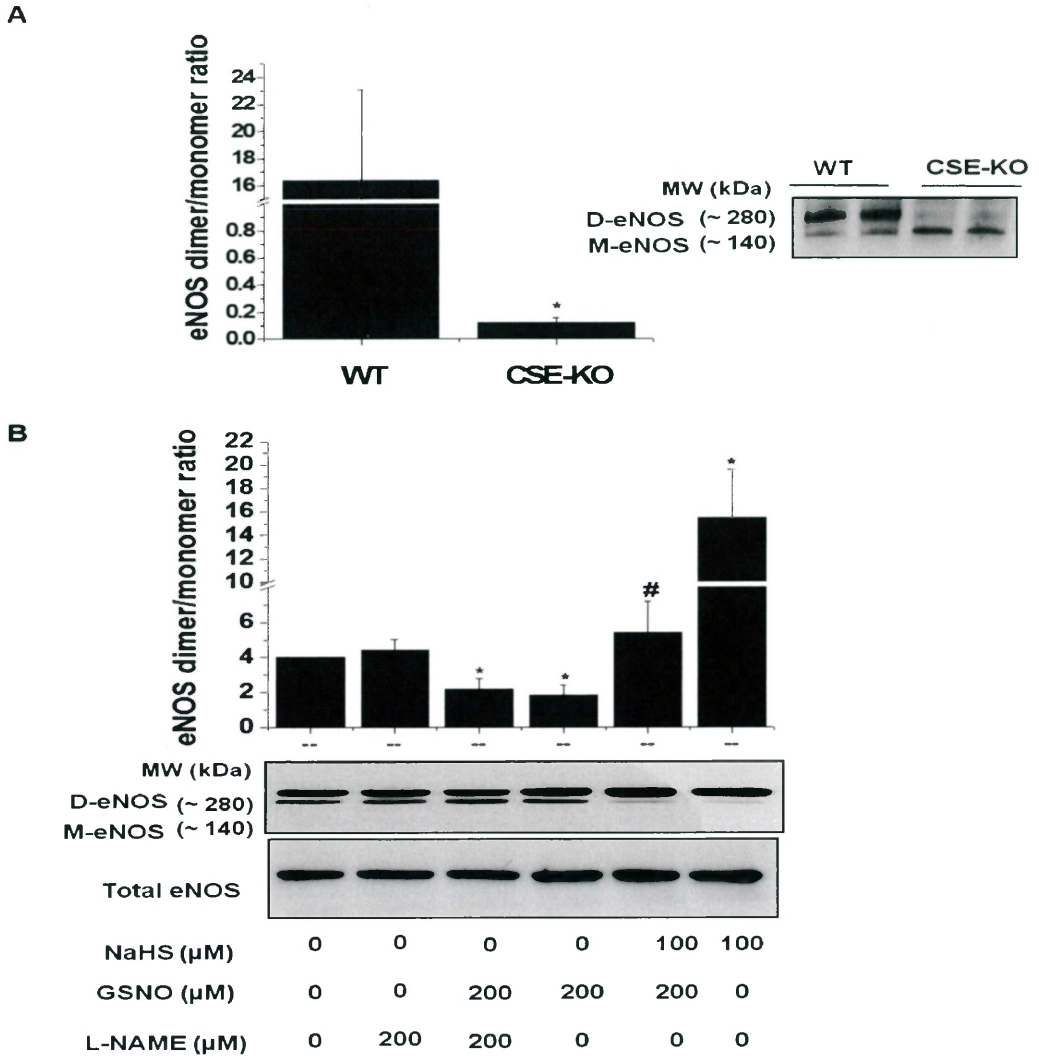

Figure 3. 4. The effect of H_2S on the quaternary structure (monomer and dimer) of eNOS proteins. (**A**) The relative abundance levels of eNOS monomers and dimers in aortic tissues from WT and CSE-KO mice. n=4, but each individual experiment was from 4 WT or CSE-KO mice. $^{*}P< 0.05$ *vs.* WT mice. (**B**) The effects of NaHS (100 μM), GSNO (200 μM) and L-NAME (200 μM) treatments on eNOS dimer stability in primarily cultured aortic ECs isolated from WT mice. n=3, $^{*}P < 0.05$ *vs.* control. $^{\#}P < 0.05$ *vs.* NaHS treated group. D-eNOS represent the dimeric form of eNOS. M-eNOS represent the monomeric form of eNOS.

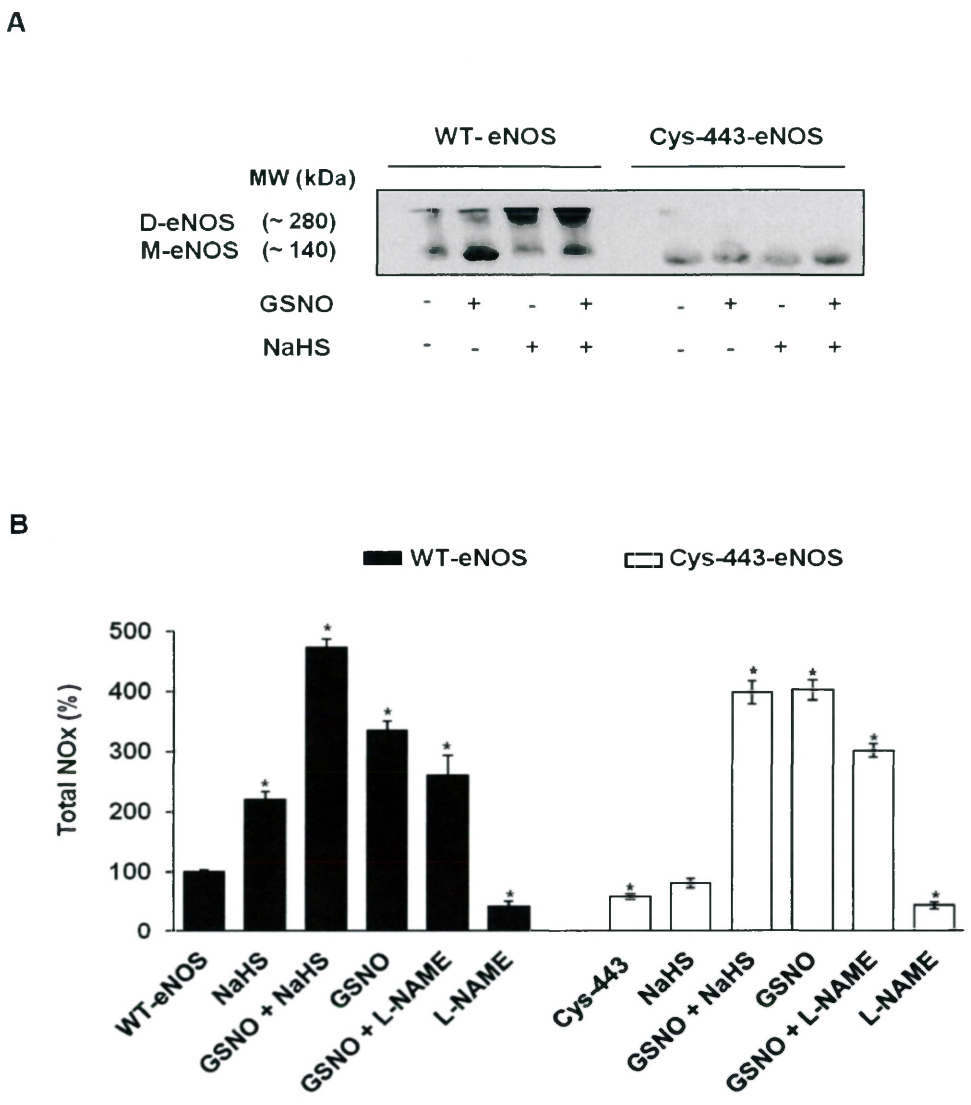

Figure 3. 5. Dimer stability of, and NO production from, wide-type (WT) and Cys-443-eNOS heterologously expressed in HEK-293 cells. The cells were treated under different conditions for 30 minutes at 37°C. (**A**) Dimer stability of WT-eNOS and Cys-443-eNOS, shown as the representative results from a total of 3 experiments, detected using LT-PAGE. D-eNOS represent the dimeric form of eNOS. M-eNOS represents the monomeric form of eNOS. (**B**) NO productions of WT-eNOS and Cys-443-eNOS detected with Griess assay. n=3-4, $^{*}P < 0.05$ *vs.* WT-eNOS control.

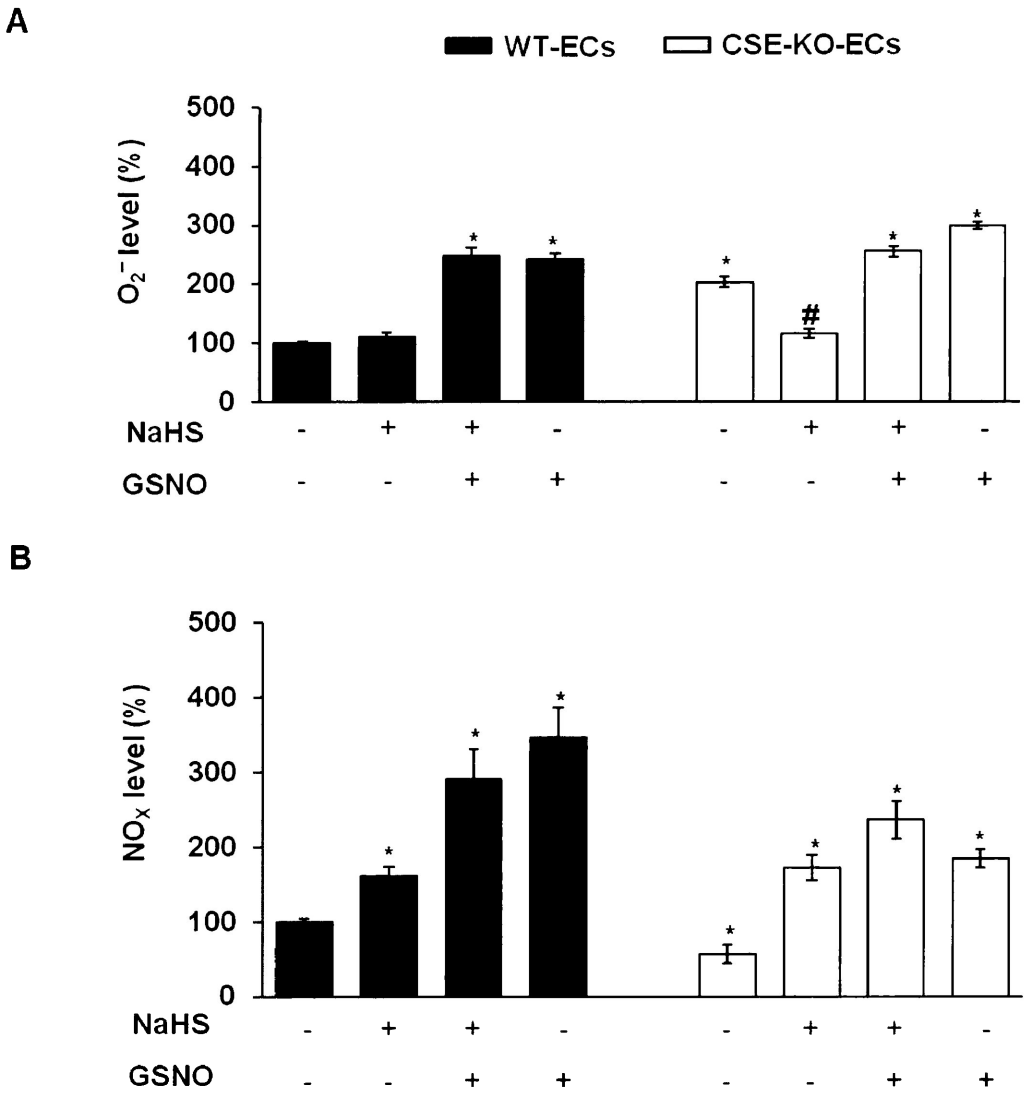

Figure 3. 6. The effects of H_2S and NO on superoxide and NO levels in aortic ECs isolated from WT and CSE-KO mice. WT-EC and CSE-KO-ECs were treated with NaHS (100 μM), GSNO (200 μM) or combined for 1 hour at 37°C, and then the levels of superoxide (**A**) and NO (**B**) were detected. n=4-5, $^{*}P < 0.05$ *vs.* WT-EC without any treatment. $^{\#}P < 0.05$ *vs.* NaHS and GSNO treated group.

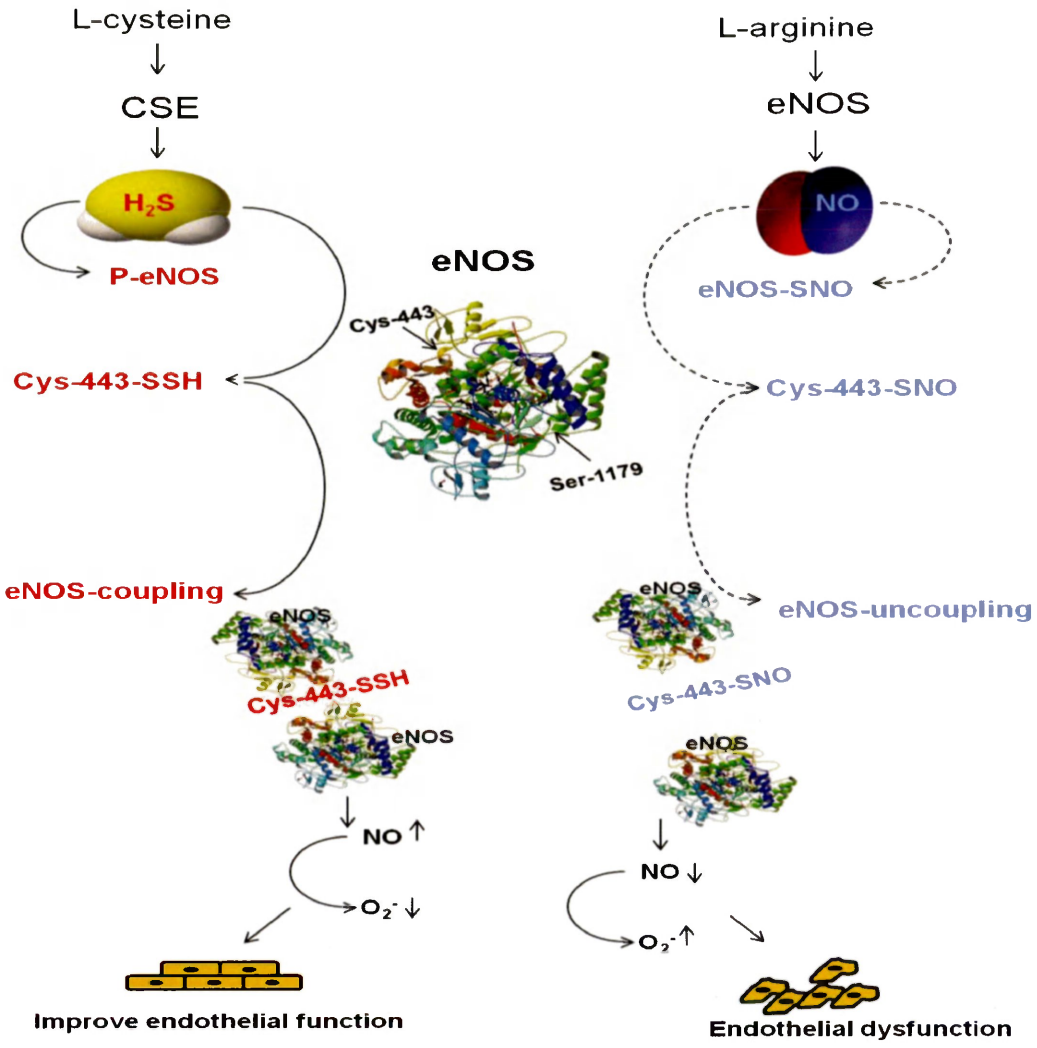

Figure 3. 7. Schematic model for the proposed H_2S - NO interactions on the cysteine modification. NO induces *S*-nitrosylation of eNOS by modifying several cysteine residues, and uncouples eNOS dimer. The uncoupling of eNOS dimers will produce less NO and more superoxide in ECs. Whereas H_2S *S*-sulfhydrates eNOS at Cys-443 and stabilizes eNOS dimers. This will lead to more NO production and lower superoxide level, which may improve endothelial function.

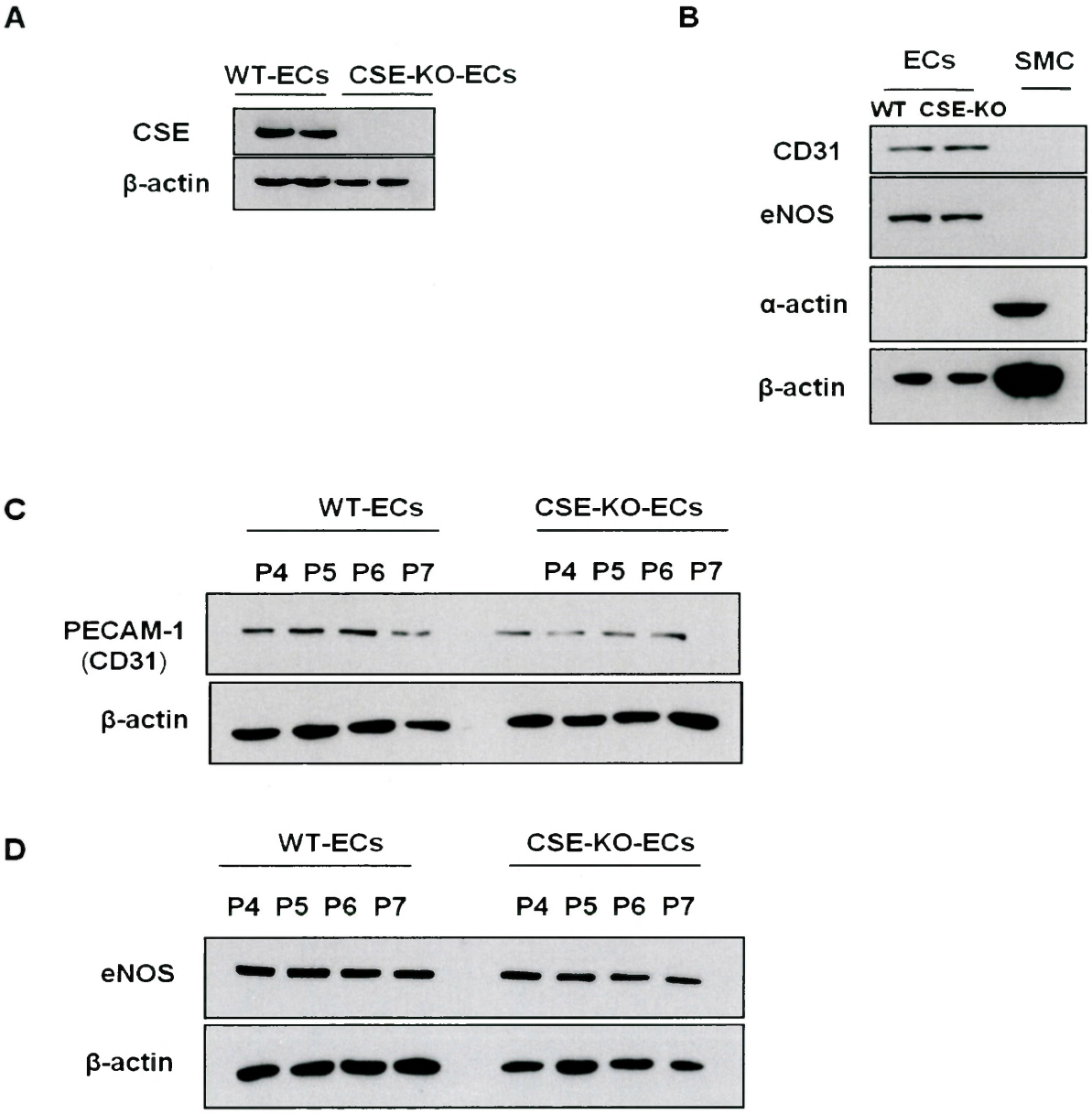

Figure 3. 8. (S1). The identification of primarily cultured aortic endothelial cells (ECs) isolated from WT mice and CSE-KO mice. (A) CSE protein expression detected using Western blot in WT-ECs and CSE-KO ECs. n=5. (**B**) The expression of EC markers (CD31 and eNOS) and SMC marker (α-actin), n=4. (**C**) The expression of EC markers (CD31 and eNOS) in different passages (P4 - P7) of primary isolated aortic ECs. n=3. β-actin was used as a loading control in all studies.

CHAPTER 4

This chapter summarizes my contribution in a paper which has been published under the title:

Hydrogen sulfide is an endogenous stimulator of angiogenesis

Papapetropoulos A, Pyriochou A, Altaany Z, Yang G, Marazioti A, Zhou Z, Jeschke MG, Branski LK, Herndon DN, Wang R, Szabó C.

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4. 1. Introduction

Angiogenesis, the development of new capillaries form pre-existing vessels, requires the coordinate activation of ECs, which migrate and proliferate in response to growth factors to form functional vessels [282]. The endothelium-derived NO is a well known mediator of angiogenesis. Vascular endothelial growth factor (VEGF) is a signal protein produced by cells that stimulates angiogenesis. VEGF stimulates the release of NO from ECs and upregulates the expression of eNOS [283, 284]. In contrast, when the NO bioactivity is reduced the angiogenesis is attenuated as well. Dysregulated angiogenesis contributes to tumor growth, psoriasis, arthritis, neuro-degeneration, wound healing defects and hair loss [282]. In the vascular wall, ECs is both targets and sources of H_2S. However, the role of endogenous H_2S in angiogenesis is not known. The goal of the current study was to investigate the role of endogenous H_2S in angiogenesis and wound healing using CSE- KO mice model.

4. 2. Materials and methods

In vitro angiogenesis assay

CSE-KO was generated as previously described [19]. Aorta were isolated from young (7-8 week old) CSE-KO and WT mice. To prepare aortic segments, mice were euthanized by CO_2 and the aorta were removed and rinsed in DMEM media (100 U/ml penicillin streptomycin and 0.25 ug/ml amphotericin B). The isolated aortae were cleaned of adipose tissue, rinsed three times and cut into segments around 3mm in length, care being taken while cleaning and cutting to avoid damage of endothelial lining segments. Using forceps one aortic segment was placed into the center of 48-multiwell plates containing 400 ul of bovine fibrinogen solution (3 mg/ml in M199 medium; Sigma). Gelation of the fibrinogen was induced using bovine thrombin (1.5

U/ml; Sigma). After 20 min, 400 ul of DMEM was added with antibiotics (100 U/ml penicillin, 100 ug/ml streptomycin, 0.25 ug/ml amphotericin B and 10% FCS) in each well. After 24 hours, the medium was removed, the gels were washed and VEGF (20 ng/ml) was added for another 2 days. The number of new microvessels was quantified by visual count under inverted microscope with bright field on the third day [285].

Wound healing assay

The second generation of 7-8 week old male CSE-KO offspring and WT were anaesthetized by intraperitoneal injection of ketamine HCl and xylazine. The dorsum of each mouse was shaved and sterilized by alcohol swabs. A ~100-mm^2 scald wound (approximately 5% total body-surface area) was created on the dorsal surface. In brief, a heated metal stick with 100-mm^2 surface immersed in constant temperature 90°C water bath was layed on the dorsum of mouse for 10 second. This procedure created a second-degree burn [286, 287]. In this model, only the epidermis and a superficial portion of the skin appendages are injured, and the interwoven pattern of collagen fibers in most of the dermis are retained [287]. Once mice recovered from anesthesia, they were placed back to the cage and maintained under standard conditions in the animal facility. Every three day after wounding, a ruler was aligned next to the wound to allow direct macroscopic measurement to the wound area and digital photographs were taken. All wound pictures were standardized according to a measurement ruler included in the images using Adobe Photoshop software 6.0 (Adobe Systems Inc, San Jose, CA). The wound surface area quantification analysis was performed by using AlphaEase FC (version 5.0.1). Wounds are considered healed when the wound area is completely closed (usually in 3-weeks), the epithelial covering is restored, and the surface of the wound is smooth, homogenous in color, without residual defects [288]. All animal experiments were conducted in accordance with

approved protocols by the Animal Health Care Committees of Lakehead University, Canada. All animals were maintained on standard rodent chow, and had free access to food and water.

H_2S measurement

H_2S production was measured *via* the methylene blue assay [166]. Briefly, 200µl of culture media from each treatment were collected, and added to microcentrifuge tubes containing zinc acetate (1% w/v, 600 µl) to trap H_2S. After 5 minutes, the reaction was terminated by adding 400 µl of *N, N*-dimethyl-*p*-phenylenediamine sulphate (20 µM in 7.2 M HCl) and 400 µl of FeCl3 (30 mM in 1.2 M HCl). After the mixture was kept in dark for 20 minutes, 300 µl of trichloroacetic acid (10% w/v) was added to precipitate any protein that might be present in the culture media. Subsequently, the mixture was centrifuged at 10,000 × g for 10 mins. H_2S in the sampled culture media interacts with *N,N*-dimethyl-*p*-phenylenediamine sulphate to form methylene blue, and the absorbance of the resulting solution was determined at 670 nm [289]. H_2S concentration in the culture media was calculated against the calibration curve of standard H_2S solutions.

Detection of CSE expression in skin tissue using immunoblot analysis

Back skin were cleaned from the fair and cleaned with ethanol. Skin samples were collected and incubated in a lysis buffer containing 0.5 M EDTA, 1 M Tris-Cl (pH 7.4), 0.3 M sucrose, and a protease inhibitors mixture (Sigma). The skin extracts were homogenized three times (5-10 seconds/each) on ice using a cell homogenizer. Skin extracts were separated by centrifugation at 14,000 × g for 15 min at 4°C. Supernatants were collected, and the same amounts of proteins were separated on 10% SDS-polyacrylamide gels and blotted onto

nitrocellulose membranes (Pall Corporation, Pensacola, FL, USA). Anti-CSE antibody was used at 1:5000 (Proteintech Group, Chicago, IL, USA). CSE primary antibody incubation was performed at 4 °C overnight, and anti-β-actin antibody was at 1:10000 (Sigma) as a loading control. Membranes were visualized using enhanced chemiluminescence western blotting system (GE Healthcare, Piscataway, NJ, USA).

Data analysis

Data are expressed as means ± SEM. Statistical comparisons between groups were performed using ANOVA followed by a post-hoc or Student's *t* test.

4. 3. Results and conclusion

VEGF is an important factor for angiogenesis control. It is well known that VEGF increase the production of NO in ECs. We have found that VEGF (20 ng/mL) treatment increased H_2S production in the cultured ECs (Figure 4. 1). Next, we isolated the aortic artery from WT and CSE-KO mice. The isolated rings were cultured for 72 hours to study the effect of CSE knockout on the formation of new-microvessels. We have found that aortic rings isolated from CSE-KO mice exhibited markedly reduced micro-vessel formation in response to VEGF treatment, when compared to wild-type littermates (Figure 4. 2). In addition, we examined the role of endogenous H_2S in wound recovery by a wound healing assay. This assay allows us to observe the healing process *in vitro* in which the EC on the edges of the artificial wound migrate toward the wound area. The influence of endogenous H_2S was measured by observing the difference in the size of the wound areas between WT and CSE-KO mice. After 6 days of post-

injury we noticed that the wound areas in WT mice were consistently smaller than in CSE-KO mice. After 9 days the wound healed even faster by almost 50% recovered compared wild type mice (Figure 4. 3). This effect is due to the absence of CSE, as we confirmed that CSE expression was completely abolished and not detected in CSE-KO mice skin tissue (Figure 4. 3A). We conclude that endogenous H_2S stimulates angiogenesis and wound healing.

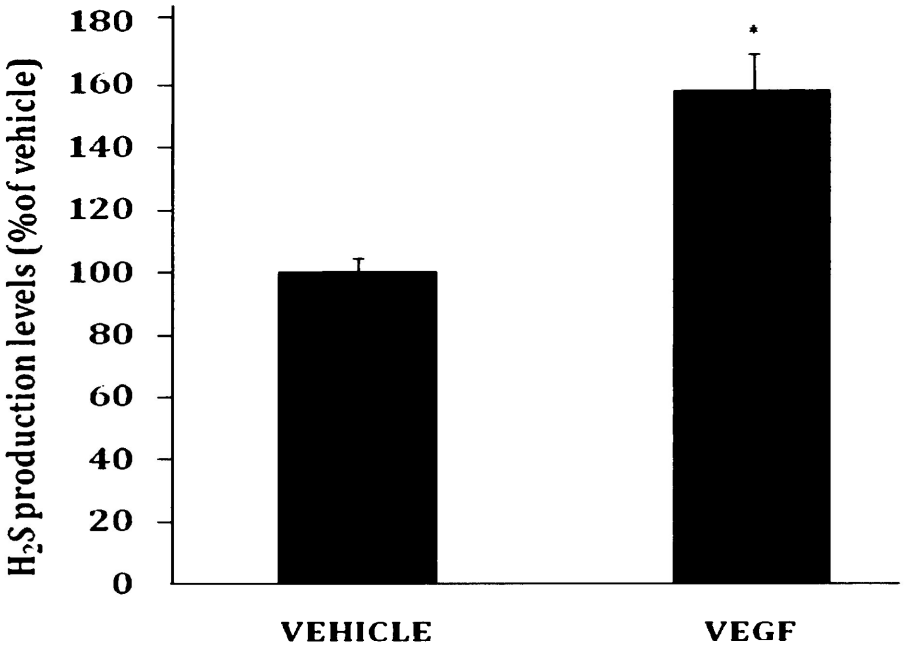

Figure 4. 1. Production of H_2S in HUVECs. H_2S levels were determined by methylene blue assay in control HUVECs, and in response to VEGF (20 ng/mL) stimulation for 10 min. $n = 5$; $^{*}P < 0.05$ *vs.* vehicle.

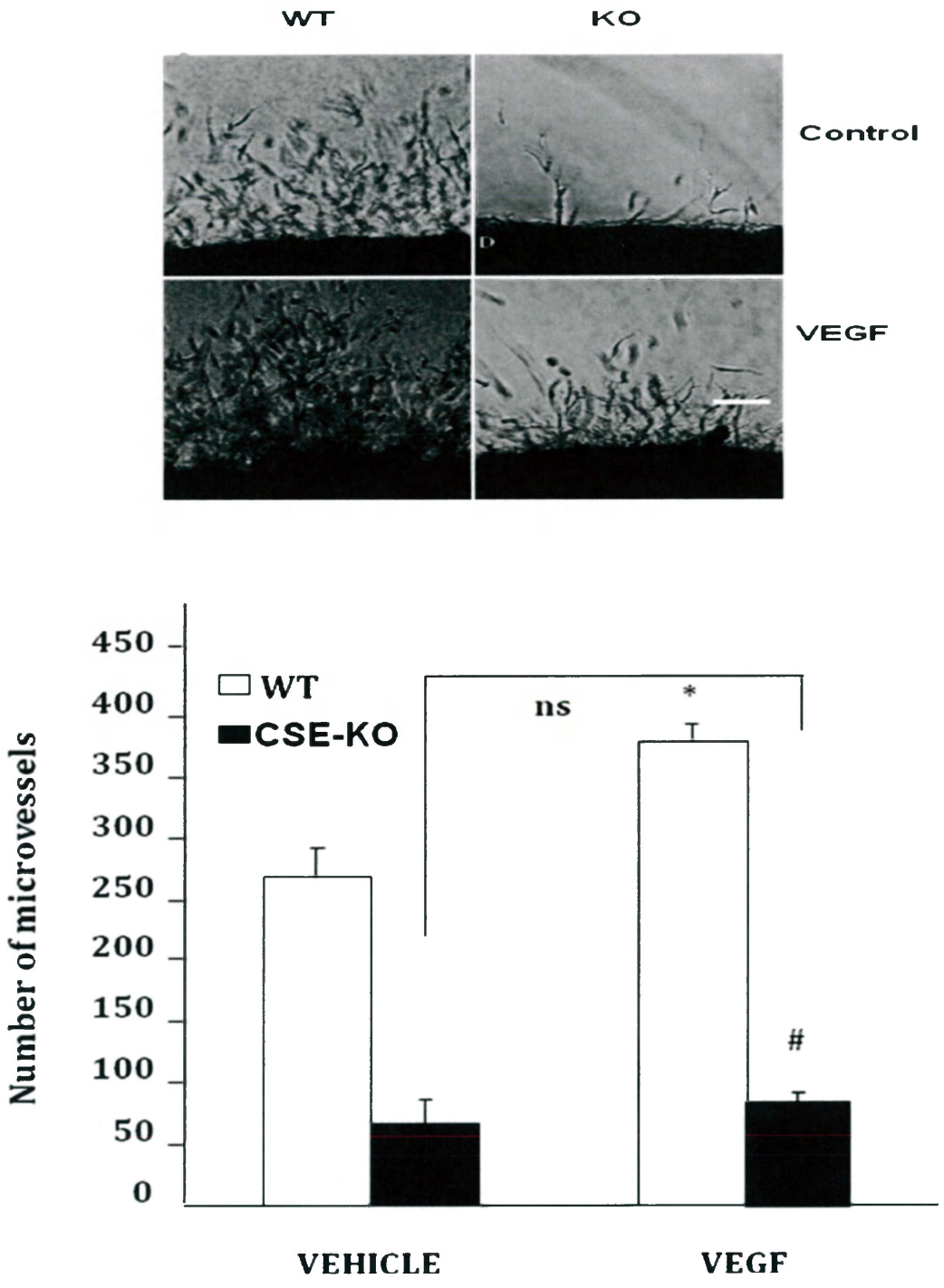

Figure 4. 2. *In vitro* angiogenesis assay in CSE knockout mice. A CSE knockout mouse shows a reduction in formation of new microvessels in the present or absent of VEGF treatment than treated VEGF and control wild type mice. n =5, * $P<0.05$. *Scale Bar* = 100 μm.

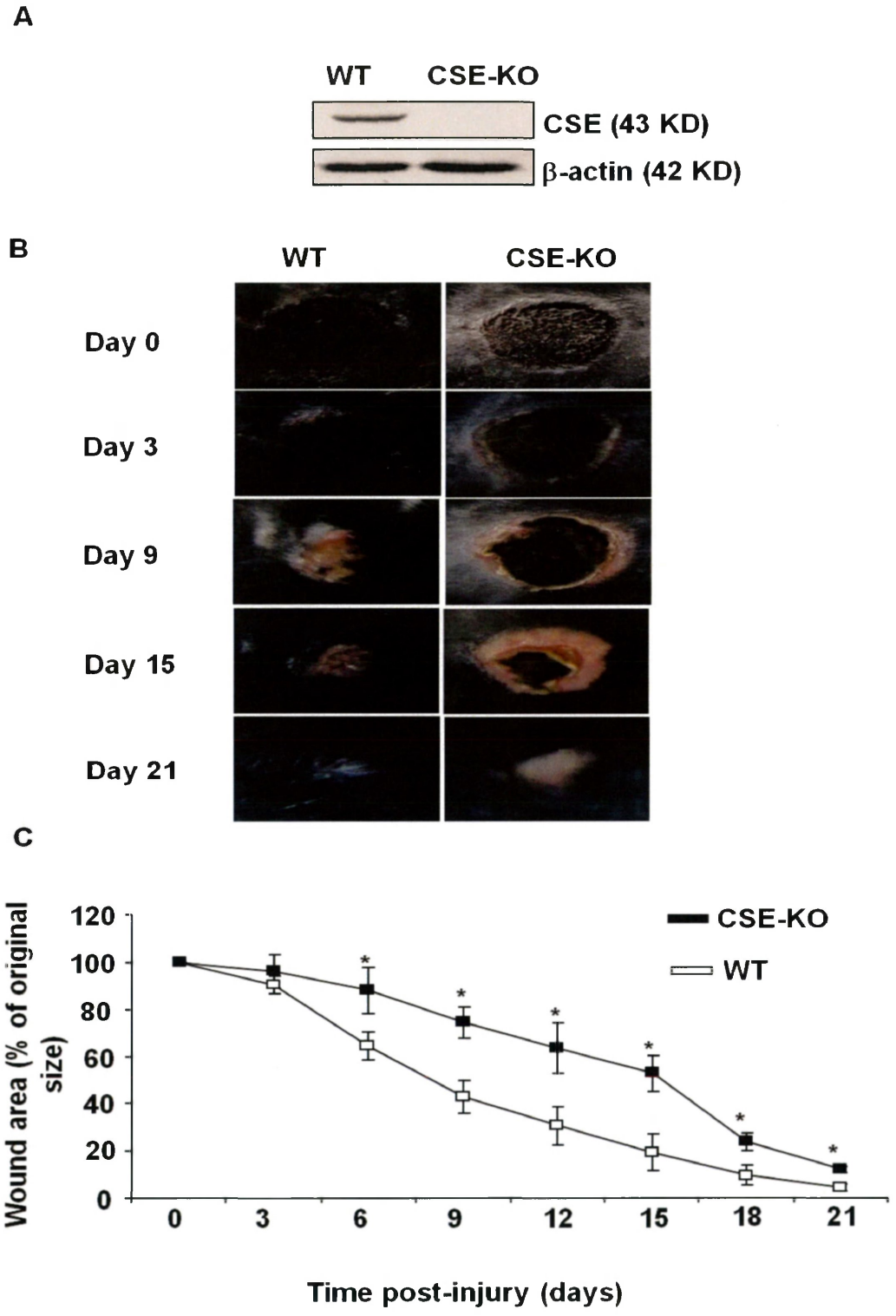

Figure 4. 3. Evaluation of wound healing in WT and CSE-KO mice. (**A**), Lacked CSE protein expression in skin tissue of CSE-KO mice. (**B**) Representative photographs of the wound sites at the indicated time post-injury. (***C***) Changes in total wound area during the evaluation interval. Four animals for CSE wild-type group and 5 for CSE knockout mice. Results represent mean + SEM. * $P<0.05$.

CHAPTER 5

DISCUSSION AND CONCLUSION

GENERAL DISCUSSION

There are similarities among gasotransmitters in terms of their production and effects, but also some differences in their mechanism of action [3]. For the similarities, H_2S and NO can be produced enzymatically, and reduce blood pressure [19, 79]. In terms of different mechanisms of action, H_2S-induced *S*-sulfhydration and usually increase the function of the modified proteins [140] whereas the same proteins can have decreased activities once *S*-nitrosylated by NO [290]. To date, the molecular mechanisms for the interaction of H_2S and NO on the same protein remain unclear and the functional outcomes of this interaction are largely unknown. To clarify these unsettled matters was the motivation of my current PhD thesis study.

In this thesis, as shown in Chapters 2 and 3, I have found that both endogenous and exogenous H_2S were involved in eNOS regulation and NO release. H_2S did not significantly change the expression levels of eNOS. However, H_2S affects the posttranslational modification of eNOS. For example, H_2S increased eNOS phosphorylation and *S*-sulfhydration, thus increasing NO production. However, phosphorylation mainly depends on enzymes or kinases to transfer phosphate groups from high-energy donor molecules such as ATP on to specific amino acid [291]. On the other hand, *S*-sulfhydration is a direct chemical interaction of H_2S with the protein, and achieved non-enzymatically. The functional outcomes of these two modes of post-translational modifications are the same, *i.e.* increased eNOS activity and NO generation. Phosphorylation of eNOS did not affect its *S*-sulfhydration since mutating the eNOS phosphorylation site Ser-1179 did not prevent H_2S to S-sulfhydrate eNOS. *S*-sulfhydration of eNOS also did not affect its phosphorylation since the mutation of the *S*-sulfhydration residue (Cys-443) of eNOS did not affect VEGF- or H_2S-induced phosphorylation. The lack of interaction between phosphorylation and *S*-sulfhydration on the same protein may be explained

by the involvement of different amino acid residues. Furthermore, *S*-sulfhydration of eNOS inhibits its *S*-nitrosylation because NaHS inhibits GSNO-induced *S*-nitrosylation. Conversely, *S*-nitrosylation of eNOS did not significantly inhibit its *S*-sulfhydration as GSNO did not inhibit NaHS-induced eNOS *S*-sulfhydration. To our knowledge, this is the first report to demonstrate the *S*-sulfhydration of eNOS and reveal the novel competitive interplay between *S*-nitrosylation and *S*-sulfhydration of eNOS.

In Chapters 2 and 4 we report that H_2S plays a key role in angiogenesis along with NO. The formation of microvessels from cultured aortic rings isolated from CSE-KO mice was reduced compared to that from WT-mice, showing the effect of endogenous H_2S. The supplementation of NaHS to the culture medium increased the formation of microvessels, showing the effect of exogenous H_2S. Increased angiogenesis in the presence of H_2S may also explain, at least in part, our observation of the beneficial effects of H_2S on wound repair. Delayed wound healing is seen in CSE-KO mice, and exogenous H_2S accelerates this process.

The effects of endogenous H_2S on eNOS regulation have been described in detail in Chapters 2 and 3. Whether these two gasotransmitters H_2S and NO interact in the regulation of crucial endothelial functions is addressed. Interestingly, we found that NO levels were decreased by CSE inhibitor (PPG) or CSE-siRNA knockdown, and so did angiogenesis. In contrast, NO levels were increased after CSE gene overexpression, exogenous H_2S or L-cysteine treatments, and so did angiogenesis. Additionally, treatment with L-cysteine increases the *S*-sulfhydration of eNOS only in WT-ECs, but not in CSE-KO-ECs. These results show that CSE-yielded endogenous H_2S acts as an endogenous modulator of eNOS protein in ECs. *S*-sulfhydration of eNOS might explain the dependence of NO production on H_2S level.

The angiogenic effect of H_2S is affected by NO levels. For example, eNOS-knockdown by siRNA partially blocks the proliferative effect of NaHS. L-NAME treatment did not completely block new microvessels formation induced by NaHS in both WT and CSE-KO aortic rings. Likewise, the NO angiogenic response is also affected by H_2S levels. Cultured aortic rings isolated from CSE-KO mice exhibited a reduced new microvessels outgrowth in response to L-arginine, compared with WT controls. We have found that eNOS activation and angiogenesis is mediated by H_2S-induced phosphorylation of p38 and Akt signaling pathways. A recent study showed a cooperative action of H_2S and NO in angiogenesis due to increased intracellular cGMP and PKG activation [165]. These authors clam that "H_2S and NO mutually relies on each other and it is almost absolute in the case of angiogenesis (because blocking eNOS completely abrogates the angiogenic effects of H_2S, whereas silencing of H_2S markedly reduces the angiogenic effect of NO)" [165]. However, their results in Figure 1A [165] clearly showed that L-NAME treatment did NOT completely block the proliferative effect of NaHS. Indeed, NaHS effect was decreased due to the lack of NO but it still proceeded to induce angiogenesis. This interpretation is consistent with our own observation that L-NAME or eNOS-siRNA partially reduces H_2S angiogenic effect.

In Chapter 3, we isolated ECs from WT and CSE-KO mice. We further investigate the functional differences between these cells under different conditions, including their production of superoxide and NO, their eNOS dimer status and eNOS *S*-sulfhydration. We found that superoxide production levels in CSE-KO-ECs were increased, whereas the NO levels were decreased compared to WT-ECs. In contrast, H_2S treatment decreases superoxide levels and increases NO levels in both WT and CSE-KO-ECs. The mechanism behind these effects is still unclear, but it seems that H_2S produced by CSE act as endogenous modulator for eNOS dimer

stability. In this regard, we show that eNOS proteins from both CSE-KO-ECs and CSE-KO aortic tissues were predominantly monomers and have less *S*-sulfhydration compared to WT-ECs or WT-aortic tissues, which were predominantly dimers. In addition, exogenous H_2S treatment increases *S*-sulfhydration and eNOS dimer in WT-ECs. Mutation at Cys-443 residue completely eliminated *S*-sulfhydration of eNOS, and generated more eNOS monomers, indicating that *S*-sulfhydration of eNOS occurs only at Cys-443 and it may play a role in stabilizing eNOS dimer.

CONCLUSION

Our studies provide evidence that H_2S is a critical regulator for eNOS, the main NO producing enzyme in ECs. H_2S stimulates NO production, promotes angiogenesis, accelerates wound healing, and decreases superoxide levels in ECs.

Mechanistically, H_2S stimulates the phosphorylation of p38 and Akt protein kinases, which leads to eNOS phosphorylation at Ser-1179 and enhances NO production. H_2S also directly modulates eNOS through *S*-sulfhydration (Figure 5. 1). *S*-sulfhydration decreases *S*-nitrosylation of eNOS and increases eNOS dimer stabilization, thus increasing NO bio-availability and decreasing superoxide levels in ECs.

The mechanisms by which protein is post-translationally regulated have been investigated for nearly three decades. *S*-sulfhydration is a novel post-translational modification mechanism that coordinates many other post-translational modification mechanisms to exert profound impact on the net outcomes of the modified proteins.

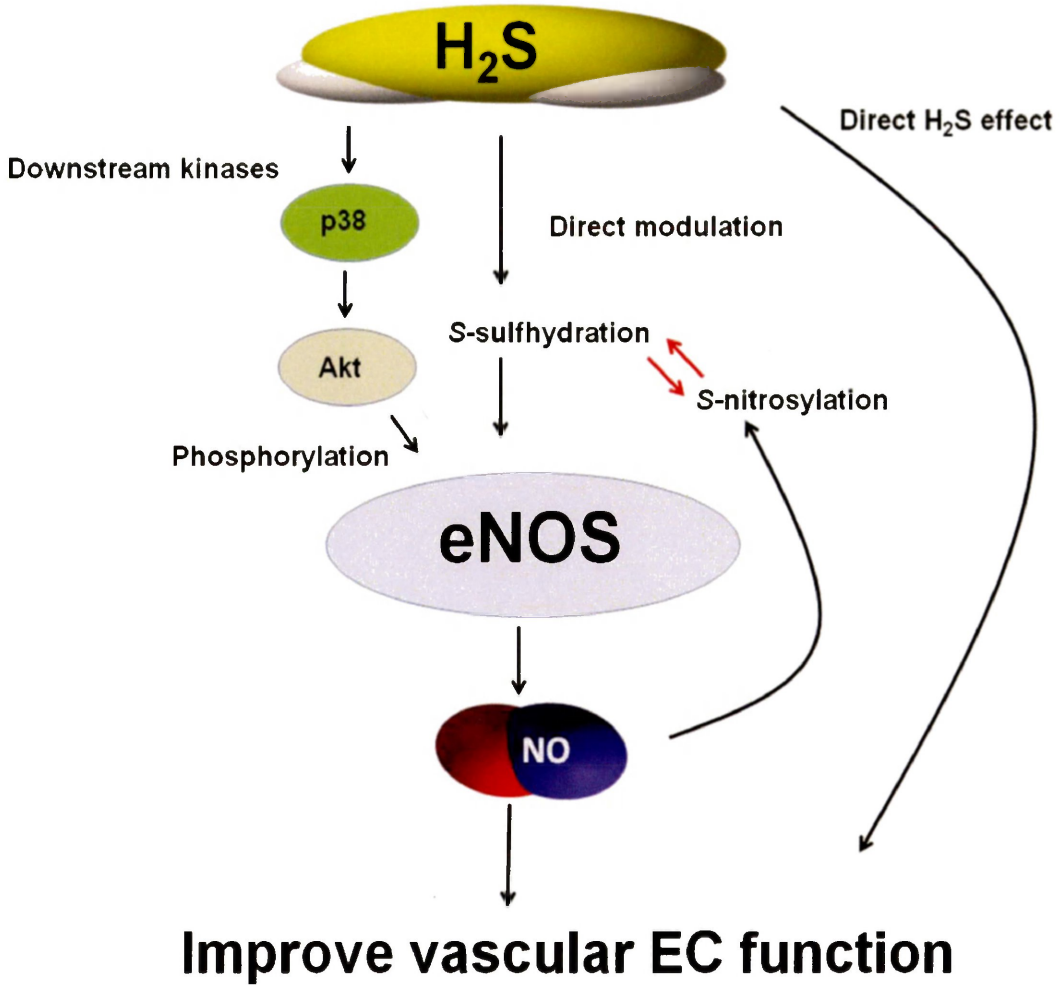

Figure 5. 1. H_2S and NO interaction in vascular endothelial cells

SIGNIFICANCE OF THE STUDY

Vascular disease represents a global medical problem with high morbidity and mortality. World Health Organization (WHO) has listed cardiovascular diseases as the number one cause of death worldwide in 2011. In 2007, 1.3 million Canadians (4.8% of Canadians population) reported having heart disease [292]. Every 7 minutes in Canada, someone dies from heart disease or stroke [293]. Restoring the blood supply to the injured or diseased tissues is a critical goal for successful treatment of many cardiovascular diseases. Pro-angiogenic therapy is a novel treatment option that can help restore circulation in people with blocked arteries and cardiovascular diseases. Angiogenesis is a complex multistep process, but mostly depends on EC-response to an angiogenic stimulus. Scientists have been trying different strategies to stimulate new blood vessel formation in areas where current blood vessels are blocked in order to supply the affected area with oxygen and nutrients. Our studies have discovered novel mechanisms for the molecular and cellular effects of H_2S on controlling and induction of angiogenesis, which can be useful as a possible therapeutic strategy to stimulate angiogenesis. Furthermore, eNOS stabling or biosynthesis of NO in vascular tissues is impaired in most of the patients with vascular diseases and at advanced age. Our data demonstrated that administration of exogenous NO in a high concentration increases eNOS uncoupling and eNOS *S*-nitrosylation, thus increasing superoxide and decreasing NO level, which is related to the pathophysiology of EC dysfunction diseases. In contrast, H_2S and NO combined treatment or H_2S alone induces eNOS *S*-sulfhydration, decreases *S*-nitrosylation, prevent eNOS uncoupling, thereby decreasing superoxide and increasing NO production in vascular ECs. Thus, the supplementation of H_2S and NO to individual's cells and tissues will stimulate eNOS activity and prevent their uncoupling consequence, which offers much more beneficial protection than NO administration alone.

LIMITATIONS OF THE STUDY

A. Research Limitations

Phosphorylation of eNOS at serine 1179 activates eNOS and NO production [116], but phosphorylation of eNOS at theronine 495 inhibits eNOS and NO production [294]. In Chapter 2, we only investigated the phosphorylation of eNOS-Ser-1179. Based on this result, we concluded that phosphorylation of eNOS would not affect the *S*-sulfhydration of the same. Clearly, investigating other phosphorylation sites such as eNOS inhibitory site theronine 495 could have generated a more comprehensive understanding for the correlation of eNOS phosphorylation and *S*-sulfhydration.

In Chapter 2, the interaction between H_2S and NO on EC proliferation was investigated using siRNA for eNOS and L-NAME to block NO in ECs. L-NAME or siRNA-based gene silencing is often not as precise or thorough as a traditional gene deletion. Therefore, using eNOS-knockout mouse model or cells will further confirm our finding.

B. Methodological Limitations

The biotin switch assay for the detection of *S*-nitrosylated proteins was developed by Jaffrey and Snyder [295]. The modified assay for biotin switch for detection of *S*-sulfhydrated protein was developed by Snyder group [140]. Biotin switch assay is considered as the most reliable method that is more easily adapted to the study of previously mentioned protein modification. However, biotin switch assays have two major technical drawbacks. *1)* Due to the high liability and redox sensitivity of S-NO bond, SNO can be lost easily or gained artificially

during sample preparation [296]. *2)* The sensitivity of the assay depends on the effective block of the free thiol by MMTS and the effectiveness of ascorbic acid as a reducing agent [297, 298]. With the biotin switch assay, free thiols are blocked by MMTS, and then SNO modifications are specifically reduced with ascorbic acid. After blocking, the modified thiol will be simultaneously labeled with a thiol-reactive biotin (which forms a mixed disulfide with the modified thiol). Finally, thiol-biotinylated proteins were pulled down with streptavidin-agarose and analyzed by Western blot. The effectiveness of each chemical in every step will affect the detection efficiency of biotin switch assay. In Chapter 3, we only used biotin switch assay to detect *S*-sulfhydration and *S*-nitrosylation of protein samples. Using LC-MS/MS will add a further confirmation to our data because it will directly measure the *S*-sulfhydrated or *S*-nitrosylated proteins, without applying MMTS, biotin or ascorbic acid to protein samples. In addition, LC-MS/MS can distinguish between *S*-sulfhydration and *S*-nitrosylation based on protein mass shift, whereas biotin switch assay depends on thiol-biotin-streptavidin-agarose binding, and analyzed by western blot.

FUTURE DIRECTION

As a follow-up on this study, the suggested future directions are:

1. To further investigate the molecular mechanism of *S*-sulfhydration. The following questions are still burning and yet to be answered: *a)* what factors determine the occurrence of eNOS *S*-sulfhydration? *b)* what are the de-*S*-sulfhydration mechanisms for eNOS? *c)* how does *S*-sulfhydration modulate the activity of eNOS?
2. To gain more insights into the precise molecular mechanism of H_2S-induced NOS dimer stability. This also may include exploring the influences of H_2S on eNOS co-factors: BH_4, heme, and zinc, which are all proposed to be involved in eNOS dimer stability.
3. To further investigate how the same H_2S signal in vascular tissue stimulates EC proliferation but inhibits SMC proliferation.

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