

Interplay of Iron and Hydrogen sulfide in pathophysiology of vascular damage

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A DISSERTATION SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL

FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN BIOLOGY

YORK UNIVERSITY

TORONTO, ONTARIO

January 2026

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Abstract

Hydrogen sulfide (H₂S) and iron are fundamental regulators of redox biology, exerting opposing influences on oxidative stress and vascular homeostasis, yet their mechanistic interplay remains unresolved. This dissertation addresses this gap through a series of studies spanning molecular, mitochondrial, cellular, and whole-animal levels. The molecular basis of H₂S–iron interactions in vascular smooth muscle cells was defined, demonstrating that endogenous H₂S upregulates ferritin expression, balances apoptosis–autophagy signaling, and protects against iron overload–induced cellular dysfunction. These findings were then extended to mitochondrial physiology, showing that CSE-derived H₂S safeguards mitochondrial respiration, spare respiratory capacity, and membrane potential under iron stress, thereby enhancing cellular resilience. To enable accurate translational assessment, a high-performance liquid chromatography method using a methylene blue/methylene green (MBMG) system was developed, establishing a robust, sensitive, and stable platform for plasma H₂S quantification with broad research applicability. Applying this method in murine models of acute iron overload demonstrated that CSE deficiency led to impaired ferritin upregulation, increased vascular iron deposition, elastin degradation, inflammatory remodeling, and severe vasomotor dysfunction—effects that were mitigated in wild-type mice via compensatory CSE/H₂S induction. Collectively, these studies establish a continuum of evidence linking H₂S to iron metabolism and vascular adaptation, from cellular and mitochondrial mechanisms to systemic physiology. By integrating conceptual review, mechanistic cell studies, methodological innovation, and translational animal models, this thesis identifies endogenous H₂S as both a biomarker and a therapeutic target for iron-related vascular disease.

Acknowledgements

I would like to express my deepest gratitude to my supervisor, **Dr. Rui Wang**, for his invaluable guidance, encouragement, and unwavering support throughout the course of my research and the completion of this thesis. His mentorship has been instrumental in shaping my scientific perspective and academic growth.

I am sincerely thankful to **Dr. Ming Fu**, our lab manager, for his continuous technical support, insightful suggestions, and dedication throughout my research. His assistance has been crucial to the success of my experimental work.

I would also like to extend my heartfelt appreciation to my thesis committee members, **Dr. Gary Sweeney** and **Dr. John C. McDermott**, for their constructive feedback, valuable advice, and encouragement, which have greatly enhanced the quality of this work.

My deepest gratitude goes to my beloved wife, **Madiha**, whose love, patience, and steadfast support have been my greatest source of strength. This thesis is lovingly dedicated to my children—**Daniya, Eshmal, and Zaviyar**—who bring endless joy and purpose to my life.

I wish to acknowledge my late grandfather, whose dream was to see me earn a Ph.D. Although he passed away in 2023, his belief in me continues to inspire every achievement I make.

Special thanks are due to my mother-in-law, **Mrs. Nazir** whose generosity and care—especially in coming to Canada to help us with our children—allowed me to focus on my research. I am equally grateful to my neighbor, **Mr. Zeeshan**, for his kindness and commitment in helping our family by

picking up my children from school over the past four years. Your support will always be remembered with deep appreciation.

Finally, I would like to express my sincere thanks to my lab colleagues—**Dr. Quihui Cao**, **Dr. Tayebbah Amanpour**, and **Dr. Richa Verma**—for their collaboration, helpful discussions, and friendship. Working alongside such dedicated and talented individuals has been a privilege.

To all who have supported and believed in me throughout this journey, I offer my heartfelt and sincere thanks.

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List of Abbreviations

3-MP: 3-mercaptopyruvate

AD: Alzheimer's disease

ALK: activin receptor like kinase

AMPK: Adenosine monophosphate-activated protein kinase

ANOVA: Analysis of variance

ATF4: Activating transcription factor 4

Atg5: autophagy related 5 protein

BBB: blood brain barrier

BrdU: Bromodeoxyuridine

CAT: cysteine aminotransferase

CBS: cystathionine β -synthase

CP: ceruloplasmin

CSE: cystathionine γ -lyase

Dcytb: duodenal cytochrome B

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DMT1: divalent metal transporter 1

EDHF: endothelium-derived hyperpolarizing factor

eIF2 α : Eukaryotic translation initiation factor 2

EPO: erythropoietin

ER- α : estrogen receptor- α

ERFE: erythroferrone

ERK: extracellular signal-regulated kinase

FAC: Ferric ammonium citrate

FLVCR1a: feline leukemia virus subgroup C receptor-related protein 1a

FPN: ferroportin

GSH: glutathione reduced

GSSG: glutathione oxidized

HCP1: heme carrier protein 1

HH: hereditary hemochromatosis

HIF: hypoxia induced factor

HRG1: heme responsive gene 1

ICAM1: intracellular adhesion molecule 1

IGF-1: insulin like growth factor 1

IL-6: interleukin 6

IRE: iron response elements

IRE-BP: iron response element binding proteins

IRP1: Iron regulatory proteins 1

ISR: Integrated stress response

ISRIB: Integrated stress response inhibitor

JAK/STAT: Janus kinase/ signal transducer and activator of transcription

KEAP1: Kelch-like ECH-associated protein 1

L-NAME: L-NG-Nitro arginine methyl ester

LC3-II: microtubule-associated protein 1A/1B-light chain 3-II

LIC: liver iron concentration

MCP1: monocyte chemoattractant protein 1

MST: 3-mercaptopyruvate sulfurtransferase

NCOA4: Nuclear Receptor Coactivator 4

NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells
NFT: neurofibrillary tangles
NLRP3: NLR family pyrin domain containing 3
NOX: NADPH oxidase
NRAMP1: natural resistance-associated macrophage protein
NRF2: nuclear factor erythroid 2-related factor 2
NTBI: non-transferrin bound iron
ODQ: 1H-[1=2=4]oxadiazolo-[4=3-a]quinoxalin-1-one
P38/MAPK: P38/mitogen activated protein kinase
PBS: Phosphate-buffered saline
PD: Parkinson's disease
RNS: reactive nitrogen species
ROS: reactive oxygen species
RSS: reactive sulfur species
SAH: S-adenosyl-homocysteine
SAM: S-adenosylmethionine
SDR-2: stromal cell derived receptor 2
SOD: superoxide dismutase
STEAP2: six-transmembrane epithelial antigen of prostate 2
TBS: Tris-buffered saline
TfR1: transferrin receptor 1
TNF- α : tumor necrosis factor- α
VCAM1: vascular cell adhesion molecule 1
VEGF: vascular endothelial growth factor

VSMCs: Vascular smooth muscle cells

ZIP14: ZRT/IRT-like protein 14

Chapter 1: Introduction

Signaling integration of hydrogen sulfide and iron on cellular functions

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Note: Parts of this chapter has been published as: Arif, H. M., Qian, Z., & Wang, R.

(2022). Signaling integration of hydrogen sulfide and iron on cellular functions. *Antioxidants & Redox Signaling*, 36(4-6), 275-293.

Contributions: H.M.A. conducted the literature review and wrote the first draft. Z.Q. provided feedback on clarity and interpretation. R.W. guided revisions and offered supervisory input. H.M.A. carried out all writing and revisions at each stage.

The sections and headings in this chapter are structured to align with the formatting guidelines of

Antioxidants & Redox Signaling journal.

1.1. Abstract

Significance: Hydrogen sulfide (H₂S) is an endogenous signaling molecule, regulating numerous physiological functions from vasorelaxation to neuromodulation. Iron is a well-known bioactive metal ion, being the central component of hemoglobin for oxygen transportation and participating in biomolecule degradation, redox balance, and enzymatic actions. The interplay between H₂S and iron metabolism and functions impact significantly on the fate and wellness of different types of cells. *Recent advances:* Iron level *in vivo* affects the production of H₂S via non-enzymatic reactions. On the other hand, H₂S quenches excessive iron inside the cells and regulates the redox status of iron. *Critical issues:* Abnormal metabolism of both iron and H₂S are associated with various conditions and diseases like iron overload, anemia, oxidative stress, cardiovascular and neurodegenerative diseases. The molecular mechanisms for the interactions of H₂S and iron are unsettled yet. Here we review signaling links of the production, metabolism and their respective and integrative functions of H₂S and iron in normalcy and diseases. *Future direction:* Physiological and pathophysiological importance of H₂S and iron as well as their therapeutic applications should be evaluated jointly, not separately. Future investigation should expand from iron-rich cells and tissues to the others in which H₂S and iron interaction has not received due attention.

Keywords: H₂S biology, Iron metabolism, iron-H₂S interaction

1.2. Introduction

Iron and H₂S are important chemical species which lie on the opposite ends of redox spectrum. Where excess of iron creates oxidative stress, H₂S offers antioxidant protection. The metabolism of H₂S and iron in mammalian cells are also intertwined. Through a non-enzymatic process, iron participates in H₂S production. On the other hand, H₂S regulates iron uptake, transport, and accumulation. The interaction between H₂S and iron affects cellular homeostasis and constitutes important signaling crosstalk. Albeit with great efforts, our understanding of the intertwined metabolism of H₂S and iron in mammalian cells and their respective and integrated roles in redox signaling has been limited.

This review article aims at identifying the relevant knowledge gaps on iron and H₂S biology. Multiple mechanisms are presented to interpret the reported metabolism of H₂S and iron, respectively, and their impacts to each other under different physiological and pathophysiological conditions. We further reviewed differential impacts of iron and H₂S on cellular redox balance, considering one being a prooxidant and the other being antioxidant. Finally, the functional consequences of the interaction of H₂S and iron are discussed, focusing on the cardiovascular system, the liver, and the brain.

1.3. H₂S metabolism

H₂S is produced in eukaryotes, enzymatically from three main enzymes: cystathionine γ -lyase (CSE) (EC 4.2.1.22), cystathionine β -synthase (CBS) (EC 4.4.1.1), and 3-mercaptopyruvate sulfurtransferase (MST) (EC 2.8.1.2). Where CSE and CBS can use both L-cysteine and

homocysteine as the substrates, MST only uses 3-mercaptopyruvate (3-MP) as a substrate, which is a derivative of L-cysteine (176). Methionine is converted to homocysteine in one carbon metabolism with S-adenosylmethionine (SAM) and S-adenosyl-homocysteine (SAH) as intermediate products (72, 128). Entering the reverse-transsulfuration pathway, homocysteine converts to cystathionine catalyzed by CBS (154). Cystathionine is converted to L-cysteine using CSE. H₂S is released from L-cysteine in the presence of CSE or CBS (176). Similarly, cysteine can be converted to 3-MP using cysteine aminotransferase (CAT). MST catalyzes the conversion of 3-MP to H₂S (73). CBS is the main enzyme for H₂S production in the brain, whereas CSE is the main enzyme in cardiovascular and liver systems (21, 196). MST is mainly found in heart and endothelium in both cytoplasm and mitochondria (124, 146). Our lab has recently shown that MST is the main enzyme for H₂S production in chicken red blood cells (RBCs) (63). Figure 1 summarizes enzymatic and non-enzymatic H₂S production pathways in eukaryotes.

Non-enzymatic sources of H₂S in eukaryotes include cysteine reaction with iron and vitamin B6 (200) or reduction of elemental sulfur in which protein bound sulfur or sulfur containing compounds release H₂S (74, 98, 142). Vitamin B6 initiates a nucleophilic attack on cysteine, forming a cysteine-alimine. Further, free or heme-bound iron reacts with the cysteine-alimine to form a cysteine-quinonoid. Ferric ions react with cysteine-quinonoid to eliminate the thiol group as H₂S and the resulting de-sulfurated alimine undergoes hydrolysis to yield vitamin B6, ammonia and pyruvate (Figure 2A). A classic example of non-enzymatic H₂S production is the reduction of elemental sulfur using NADPH (98). Searcy and Lee showed that RBC cell lysates can react with GSH, NADH and NADPH to form H₂S non-enzymatically (142). They further showed that H₂S may be formed through inorganic sulfur using reducing equivalents from glucose oxidation in phospho-gluconate pathway

in RBCs (Fig. 2B-1). Phospho-gluconate pathway represents 5 to 10% of glucose metabolism in RBCs but becomes more active in oxidative stress (142). Searcy and Lee predicted that 2 glucose molecules reacting with 6 elemental sulfur and 3 water molecules yield 3 lactate, 6 hydrogen sulfide and 3 carbon dioxide molecules (Fig. 2B-1). However, their empirical data showed a ratio of 6 hydrogen sulfide to 2 carbon dioxide. They theorized this with the release of pyruvate instead of lactate but did not give a balanced reaction. A balanced equation would be achieved with 4 molecules of sedoheptulose (Fig. 2B-2), a metabolite in phospho-gluconate pathway which coincidentally is also increased in oxidative stress (29, 192). This phenomenon also hints an antioxidative role of H_2S . Similarly, thiosulfates can undergo reduction by interacting with reduced glutathione (GSH) to release H_2S (Fig. 2C). Leskova and coworkers showed this phenomenon in human umbilical vein endothelial cells (86).

Where enzymatic production of H_2S is part of complex series of reactions in cysteine metabolism and the expression of H_2S -producing enzymes can be induced or inhibited, non-enzymatic production of H_2S is less regulated via non-specific reactions. There would be no difference in the biological functions of H_2S whether it is produced enzymatically or non-enzymatically as it is the same molecule. The functional differences, if any, may be accounted by the differences in the concentrations of H_2S produced and the co-products from different pathways. Non-enzymatically produced H_2S may contribute to basal levels of H_2S in blood stream (200) where H_2S -producing enzymes are less expressed. Moreover, iron-dependent non-enzymatic production of H_2S may offer a protection mechanism against iron induced oxidative stress. This mechanism will be discussed in detail later in this article.

H₂S can be exhaled or pass through urine or feces as sulfate or thiosulfate (56, 177). H₂S can also be oxidized in mitochondria to thiosulfate and further to sulfite or sulfate catalyzed by sulfate detoxifying enzymes, such as rhodanase (20, 126). Methylation of H₂S to a non-toxic compound, dimethylsulfide, by thiol S-methyltransferase is another possible fate of H₂S (181). Lastly, H₂S can be scavenged by different proteins containing metallic groups or disulfide molecules, such as methemoglobin which is a hemoglobin derivative with oxidized ferric irons (Fe³⁺) (165). The neutral H₂S molecule rapidly binds with Fe³⁺ in methemoglobin in vitro and in vivo with an association constant of $3.5 \times 10^3 \text{ M}^{-1} \text{ S}^{-1}$. This association is pH independent, but its dissociation is pH dependent. The dissociation of H₂S increases with an increased acidity and the dissociation curve shifts to right with an increase in pH (26). This is similar to Bohr's effect for oxygen which governs loading and unloading of oxygen from hemoglobin depending on partial pressure of CO₂ and local pH. RBCs may unload oxygen and H₂S to tissues with increased metabolic activity to enhance local oxygen and increase vasodilation (59). Figure 3 summarizes transport of H₂S via methemoglobin. Other molecules which may bind H₂S include oxidized glutathione, catalase, and horseradish peroxidase (21, 27).

H₂S moves intracellularly by free diffusion. It can also diffuse out of the cell for paracrine signaling. Bearden and coworkers found presence of extracellular H₂S, produced by CSE and CBS in the blood as part of plasma proteome (9). Moreover, H₂S can bind to oxidized, ferric hemoglobin (methemoglobin) and be transported in the circulation as a potential endocrine signaling molecule (59).

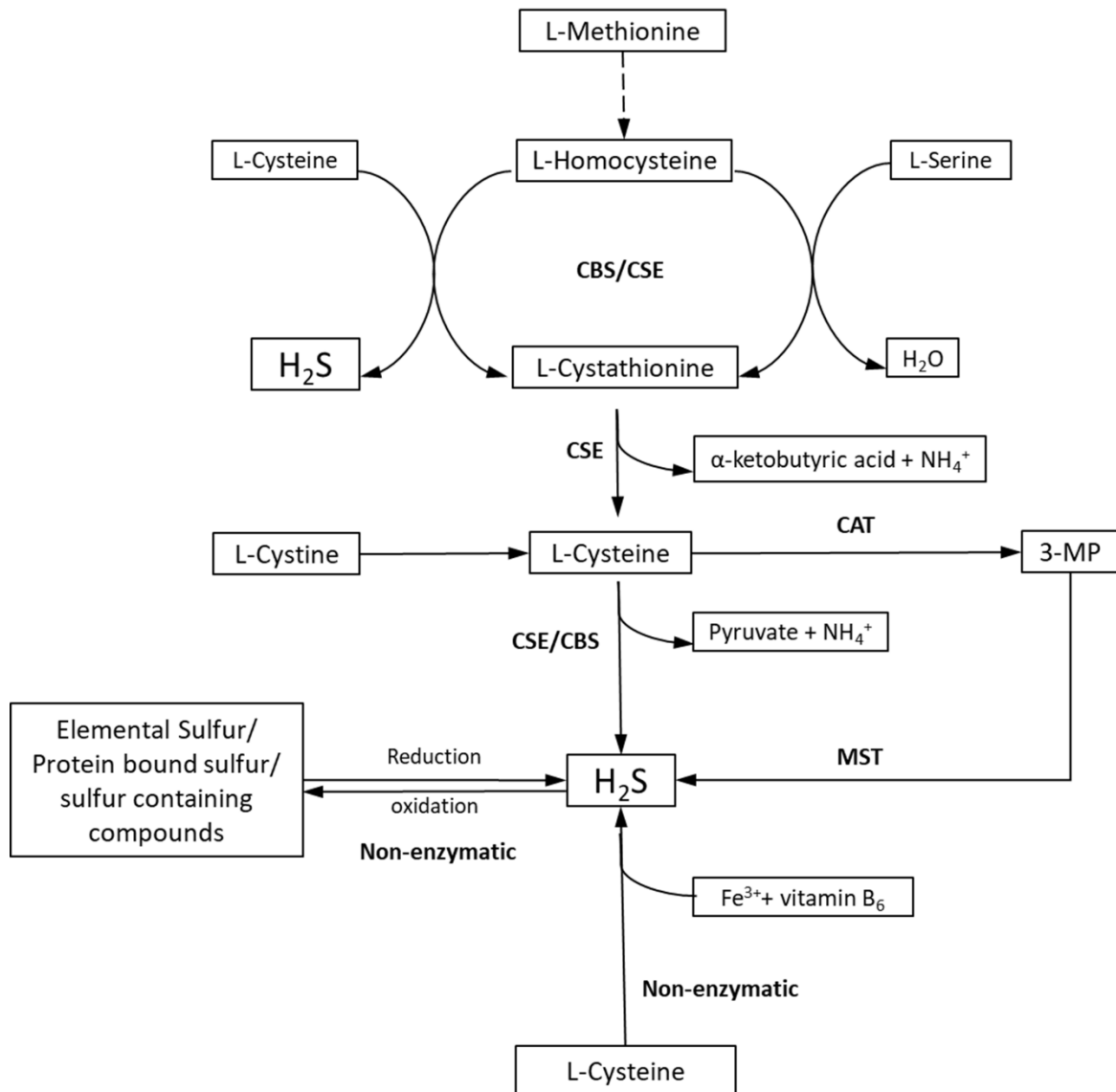


Figure 1. Summary of important enzymatic and non-enzymatic H₂S generation pathways. CBS and CSE can act on L-homocysteine to convert it to L-cystathionine releasing H₂S. CSE further converts L-cystathionine to L-cysteine and subsequently pyruvate and ammonia, along with H₂S. Cysteine can be converted to 3-MP by CAT which can be further broken down to release H₂S by MST. Non-enzymatic H₂S production is conducted by reduction of elemental or bound sulfur, or reaction of ferric ions with L-cysteine in the presence of vitamin B₆.

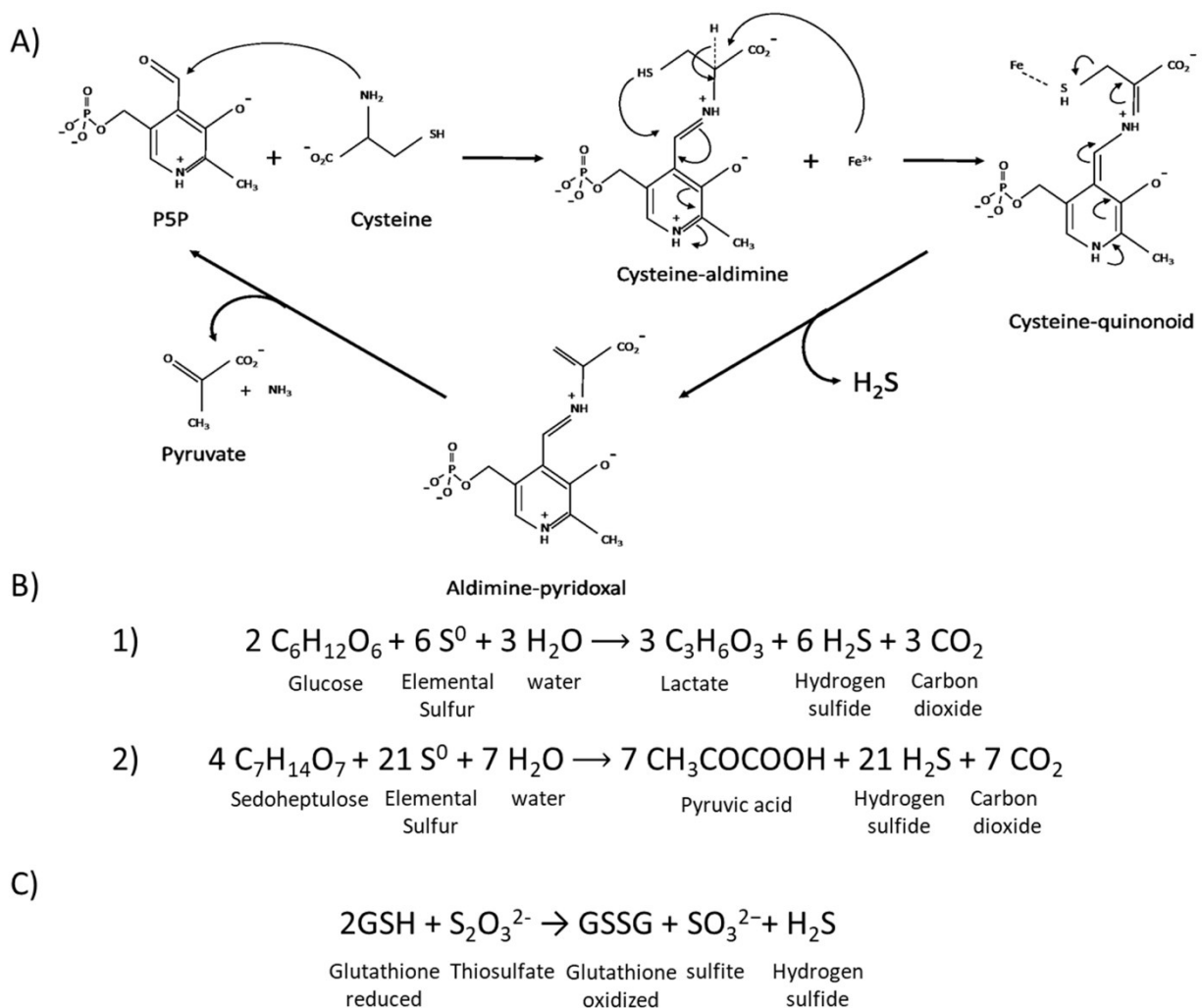


Figure 2. Reactions leading to non-enzymatic H₂S formation in cells. Cysteine reacts with pyridoxal 5 phosphate (P5P) to form a cysteine-aldimine complex which reacts with Fe³⁺ to form cysteine-quinonoid. H₂S is released from cysteine-quinonoid via elimination, resulting in aldimine-pyridoxal that further dissociates back into P5P, pyruvate and ammonia (A). Glucose or its reduced equivalent sedoheptulose reacts with elemental sulfur to form lactate or pyruvate, H₂S and CO₂ (B). Equation B-2) is speculative based on literature review. Thiosulfate reacts with glutathione (reduced) to form glutathione (oxidized), sulfite and H₂S (C).

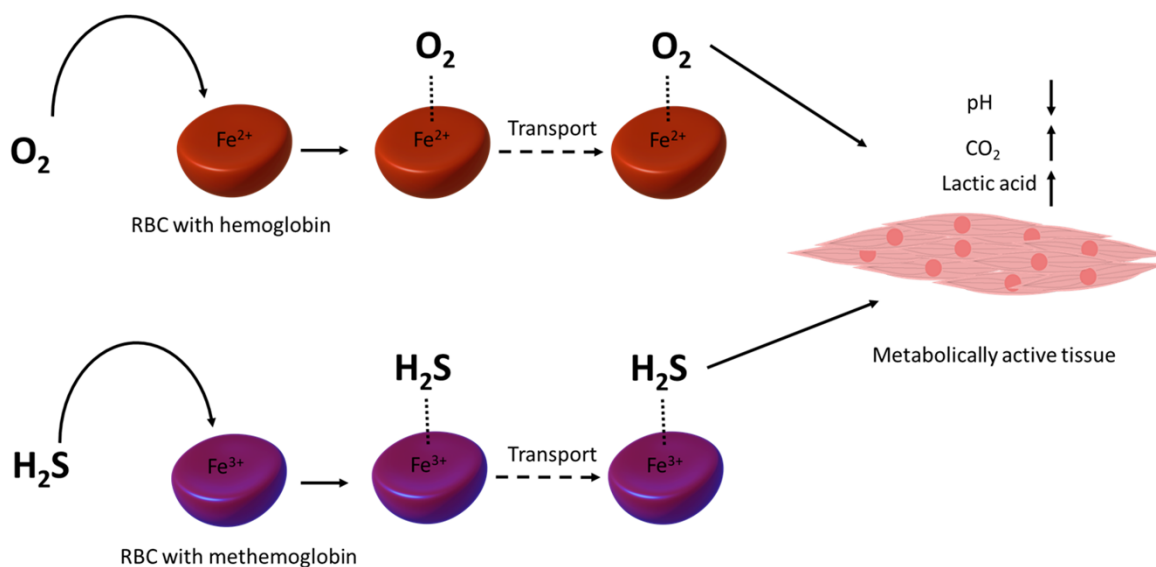


Figure 3. Transport of oxygen via hemoglobin (Fe²⁺) and H₂S via methemoglobin (Fe³⁺) in RBCs to metabolically active tissues.

1.4. Iron metabolism

Human body contains 2.5 to 4.0 grams of iron, predominantly present in hemoglobin and in lesser quantities in myoglobin, and in various enzymes and transport proteins (16). Iron is involved in a wide array of physiological functions, such as erythropoiesis, oxygen transport, degradation of lipids, proteins and nucleic acids. It is also used in myocardial and muscle metabolism, function of thyroid gland, CNS and immune system (57). Iron exists in multiple states and forms in vivo, including Fe³⁺ (ferric), Fe²⁺ (ferrous), bound iron in heme group or in iron-sulfur (Fe-S) clusters such as 2Fe₂S or 4Fe₄S (83, 172). Each form is tightly regulated by complex mechanisms. Most abundant form of iron is the one bound to heme, followed by transferrin and ferritin (1). Free ionic forms of iron are kept at a very low concentration to avoid toxicity by free radicals (32). Transferrin levels in plasma are sufficient to bind more than 99% of iron which leaves rare possibility of free

iron. Any free iron is usually loosely complexed with molecules such as citrate or serum albumin. However, in diseases such as atransferrinanaemia, hemochromatosis and thalassemia, plasma free iron levels overwhelm transferrin binding capacity (131).

Free iron enters the cell as Fe^{2+} through ion channels or transporters like divalent metal transporter 1 (DMT1), ZRT/IRT-like protein 14 (ZIP14). Whereas DMT1 chiefly prefers and transports iron in the cell, it can also transport copper, zinc and manganese. DMT1 deficient mice showed decreased iron concentration in blood, liver, spleen and heart but not other metal ions (144). ZIP14 is a zinc transporter but it transports iron as well (71). Moreover, iron may enter through L-type Ca^{2+} channels as well (45, 96, 119). Ionic Fe^{3+} gets reduced to Fe^{2+} by ferrireductases like six-transmembrane epithelial antigen of prostate 2 (STEAP2), stromal cell derived receptor 2 (SDR-2) and duodenal cytochrome B (Dcytb) outside the cell (15). Dietary heme enters the enterocyte by heme carrier protein 1 (HCP1) transporter protein (145). Transferrin-bound iron binds to a transferrin receptor (TfR1) and is internalized as an endosome. Further, the iron is released from transferrin as Fe^{3+} and reduced to Fe^{2+} by neighboring STEAP2 ferrireductase in the endosome before being transported into the cytoplasm through DMT1 (6). Free iron as Fe^{2+} constitutes the labile iron pool (LIP) which may increase through breakdown of heme by heme oxygenase (HO) (188). Further, Fe^{2+} can bind to apoferritin to become ferritin and be stored as Fe^{3+} (6). Free iron inside the cell may be incorporated as a cofactor for iron-containing proteins involved in processes such as ROS generation and degradation of biomolecules (57, 81). Free iron can be exported out of the cell via ferroportin transporter along with ferroxidase enzymes, hephaestin and ceruloplasmin. Ceruloplasmin converts Fe^{2+} to Fe^{3+} outside the cell (15). Most of the iron requirements for cellular metabolism and function are fulfilled by recycling existing iron. An example of this process is heme

recycling by macrophages which engulf senescent RBCs and break them down into free iron or heme which is moved into cytoplasm via natural resistance-associated macrophage protein (NRAMP1) or heme responsive gene 1 (HRG1) transporter (76, 201). The heme can be broken down in cytoplasm by heme oxygenase or exported out of the cell via Feline leukemia virus subgroup C receptor-related protein 1a (FLVCR1a) (201).

H₂S can interact with a wide variety of ion channels (177). For example, H₂S is a known K_{ATP} channel opener through which it mediates vasorelaxation (61). One possible interaction between H₂S and iron is that H₂S can inhibit L-type Ca²⁺ channels by S-Sulfhydrylation as observed in mouse pancreatic beta cells (159). Iron can enter the cell through L-type Ca²⁺ channels as observed in cardiomyocytes (118). Inhibition by H₂S may decrease the amount of iron entering in the cell.

Total iron concentration inside body is largely controlled through the protein hepcidin. It is secreted by the liver into circulation when plasma iron concentration is high (Figure 4). Hepcidin blocks ferroportin by binding to it, causing its ubiquitination followed by internalization and degradation (134). This results in the blockade of iron movement into circulation from enterocytes, macrophages or hepatocytes (43, 113). If there is an abnormal increase in hepcidin in circulation, such as in the case of inflammation, higher level of hepcidin may lead to iron deficiency (113). Conversely, abnormally low hepcidin, as in the case of certain hereditary hemochromatosis (HH), leads to iron overload (91, 114). Human body loses 1-2 mg of iron daily by skin and enteric desquamation or through blood loss (171).

Iron status in vivo can be determined by different parameters, such as hemoglobin concentration, serum iron, serum ferritin, tissue ferritin, liver iron concentration (LIC), and non-transferrin bound iron (NTBI) levels in the blood or tissues. Each parameter changes with respect to different physiological conditions and can be used as an indicator for disease. However, careful considerations need to be made before making conclusion based on iron values as they tend to be different between different age groups, gender and disease models (18, 38, 44, 125, 162). Table 1 summarizes total iron concentration (free and bound irons) in different biological samples.

Whereas hepcidin controls overall iron in the body, intracellular regulation of iron level depends on iron response elements (IRE) and iron response element binding proteins (IRE-BP). IREs are short sequences on untranslated regions (UTR) of mRNA which can bind to IRE-BP (83). For example, mRNA of ferritin contains an IRE near 5' UTR and mRNA of TfR1 has multiple IREs near 3' UTR. If cellular iron content is low, IRE-BP becomes active and binds to IRE of ferritin mRNA to destabilize it, resulting in decreased storage and more free form of iron. Conversely, IRE-BP binding to IRE of TfR1 mRNA stabilizes it, resulting in increased expression of TfR1 and increased intake of iron. With high cellular iron concentrations, IRE-BP becomes inactive and results in the exact opposite effect on ferritin and TfR1 (83, 108).

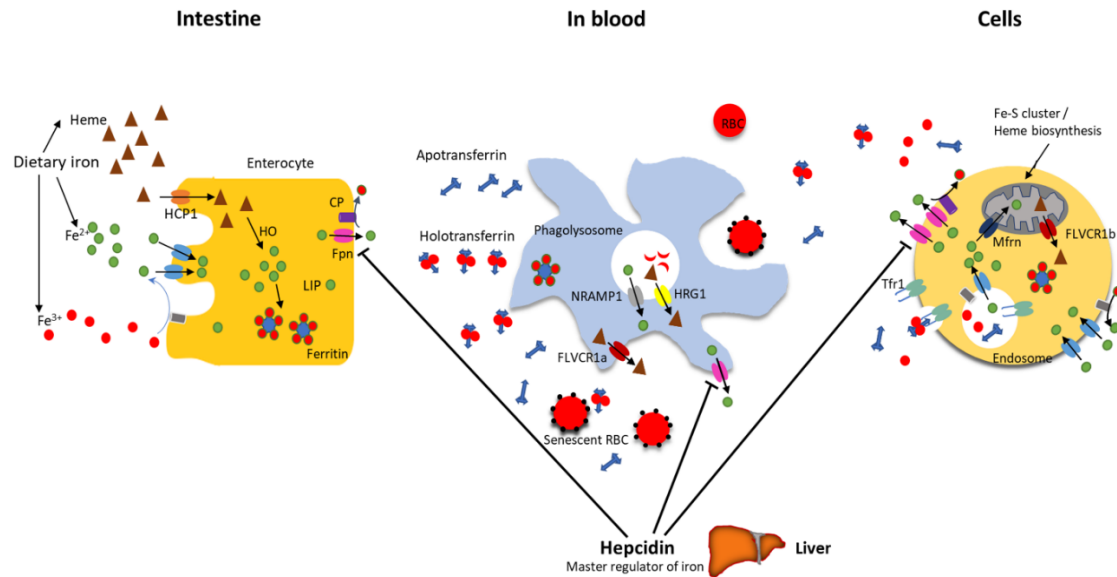


Figure 4. Uptake, transportation, and recycling of iron. Heme enters enterocytes after binding with HCP1 receptor and release iron via heme oxygenase (HO). DMT1, ZIP14 or L-type Ca^{2+} channels provide pathways for Fe^{2+} entering enterocytes. Fe^{3+} first gets reduced to Fe^{2+} by ferrireductase like STEAP2/SDR2/Dcytb or ceruloplasmin (CP). Transferrin-bound iron is internalized via binding to transferrin receptor (Tfr1) and endocytosis after which the iron in Fe^{3+} form is released and converted to Fe^{2+} by ferrireductase like STEAP2 and transported to cytoplasm via Fe^{2+} channels, like DMT1 or ZIP14. Free iron inside the cell is in Fe^{2+} form which constitutes labile iron pool (LIP). Heme can be degraded to release Fe^{2+} via heme oxygenase. Heme is shuttled among intracellular organelles via 1) transportation to cytoplasm from mitochondria via FLVCR1b receptors; 2) moving to cytoplasm via HRG1 transporter. Iron in the form of Fe^{2+} can be moved from intracellular organelles to cytoplasm through NRAMP1 transporter and moved out of the cell via ferroportin (Fpn) which is regulated by hepcidin secreted by the liver. Hepcidin blocks Fpn, leading to reduced release of iron from enterocytes or other types of cells.

Table 1. The concentrations of total iron and selective iron proteins in different organs.

Aortic tissue iron concentration	Human	<300 mg iron/g dry tissue weight (140)
	Mouse (C57BL/6J)	<200 mg iron/g dry tissue weight (140)
Brain iron concentration	Human	28–122 mg iron/kg dry tissue weight (78)
	Mouse (C57BL/6J)	18.3-19.8 mg iron/kg dry tissue weight (52)
Liver iron concentration	Human	0.2-2 mg iron/g dry tissue weight (153)
	Mouse (C57BL/6J)	0.181 mg iron/g dry tissue weight (189)
Heart iron concentration	Human	0.09-78.64 mg iron/g dry tissue weight (24)
	Mouse (C57BL/6J)	0.513 mg iron/g dry tissue weight (189)
Plasma iron concentration	Human	10-30 μ M (42)
	Mouse (C57BL/6J)	17-23 μ M* (27)
Serum ferritin concentration	Human	65-670 pM (111)
	Mouse (C57BL/6J)	241 pM** (106)
	Human	9-32 μ M (111)

Serum iron concentration	Mouse (C57BL/6J)	19-21 μM^* (101)
Serum transferrin concentration	Human	2-3.6 g/L (111)
	Mouse (C57BL/6J)	2.6-3.2 g/L (88)

*SI Units were converted.

** 1 mg of ferritin is equal to 8 mg of stored iron (111)

1.5. Iron and H₂S in redox signaling

Oxidative stress is the accumulation of pro-oxidants which overwhelms the body's natural antioxidant systems [50]. The word "oxidative stress" has a frightening connotation, but events of oxidative stress also occur at a homeostatic level which is essential for redox signaling, known as oxidative eustress (149). Redox signaling encompass cellular processes involving reactions where electron transfer occurs through free radicals, redox active metals or related species. Overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in oxidative stress leads to widespread damage of biomolecules like proteins, lipids and nucleic acids (104). The reactive species of note include the superoxide ($\text{O}_2^\bullet$), hydroxyl ($^\bullet\text{OH}$), alkoxyl ($\text{RO}^\bullet$), peroxy ($\text{ROO}^\bullet$), and nitroxyl radical ($\text{NO}^\bullet$), along with hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH) and hypochlorous acid (HOCl) (152). The most notable source of ROS includes

mitochondria (mitoROS) where electron transport chain may generate $O_2^\bullet$, $^\bullet OH$, and H_2O_2 (39). Complex I release $O_2^\bullet$ when molecular O_2 reacts with reduced FMN (flavin mononucleotide) when there is a high NADH/NAD⁺ ratio in matrix. Complex III produces $O_2^\bullet$ in the presence of respiratory inhibitors like antimycin; and complex II produces $O_2^\bullet$ when complex I and II are inhibited (39). Other sources of mitoROS include enzymes like α -ketoglutarate dehydrogenase complex and pyruvate dehydrogenase complex (97, 155). Cytoplasmic production of ROS (cytoROS) includes ROS generation through NADPH oxidase (NOX) family and nitric oxide synthases (eNOS, iNOS and nNOS) (39).

Superoxide and hydrogen peroxide generated either in cytoplasm or mitochondria are freely membrane permeable and can react with iron to form the highly reactive and toxic hydroxyl radical through fenton reaction (110). Under normal metabolic conditions superoxide leads to the more favorable oxidation of Fe^{2+} to Fe^{3+} along with ROS generation. If superoxide levels are high, however, the less favorable reduction of Fe^{3+} to Fe^{2+} along with ROS generation takes place (25, 104). The source of the free Fe^{2+} or Fe^{3+} is the LIP in cytoplasm (cLIP) and mitochondria (mLIP) (17). Iron metabolism and formation of LIP is discussed in the previous section and summarized in Figure 4. There are several conditions which can lead to iron overload. Hereditary hemochromatosis is an autosomal recessive disorder which leads to increased absorption of dietary iron. There are four types of HH: type 1 due to a fault in *HFE* gene required for iron sensing; type 2 due to a fault in hemojuvelin, a regulator of hepcidin or hepcidin itself; type 3 due to a fault in transferrin receptor 2; and type 4 due to a fault in ferroportin (127). Down regulation of hepcidin is common in HH which leads to iron overload (182). The increased iron is accumulated in parenchyma of organs such as liver and heart and is responsible for widespread organ damage due to oxidative stress (182).

Similar to HH, iron overload is also observed in diseases where repeated blood transfusion is required, such as thalassemia (49), sickle cell anemia (49), chronic kidney disease (133), liver disease (107) and certain cancer types like colon-rectal (164) and liver cancer (54). Iron overload catalyzed ROS can induce generalized damage to biomolecules such as carbohydrates, lipids, proteins and membranous structures. A secondary effect of iron catalyzed ROS is the induction of stress and inflammation signaling pathways like Janus kinase/signal transducer and activator of transcription (JAK/STAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and p38/mitogen activated protein kinase (p38/MAPK) (19, 26, 33, 151).

Oxidative stress can be countered through enzymatic and non-enzymatic antioxidant mechanisms (14, 132). The enzymatic antioxidants include the enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase, heme oxygenase and redox proteins like glutaredoxins, thioredoxins and peroxiredoxins. The non-enzymatic antioxidants include low molecular weight compounds like GSH, NADPH, , vitamin C and E along with trace metals like selenium (14, 132). Any $O_2^\bullet$ that is converted to H_2O_2 by SOD can be reduced to water and oxygen by catalase or water only by glutathione peroxidase utilizing GSH molecules (183). Collectively, SOD, catalase and glutathione peroxidase constitute first line antioxidant defense, whereas other enzymes and molecules like glutathione, ascorbic acid and vitamins are considered second line of defense (55).

Another species critical to redox signaling are reactive sulfur species (RSS). Although sulfur is part of antioxidant mechanisms, as explained in subsequent paragraphs, its role in “reductive stress” has been recently brought forward (116). Sulfur has lower electronegativity than oxygen, which is why it can exist in more oxidation states. RSS can exist as hydrogen persulfide or polysulfide (H_2S_2 or H_2S_n); protein sulfide or polysulfide (PSH, $PS(S)_nH$, $PS(S)_nP$); small weight polysulfides ($RS(S)_nH$

or $\text{RS(S)}_n\text{R}$); sulfenic acids or nitrosothiols (RSOH or RSNO) (75). RSS generation involves a loss of electron from 2 H_2S molecules to form 2 $\text{HS}^\bullet$ which can recombine to form persulfides or polysulfides (116). Whether the formation of persulfide can be categorized as a redox process remains to be determined. Recently, Akaike and coworkers have shown strong potency of cysteinyl-tRNA synthetases in forming cysteine polysulfides in mitochondria and cytoplasm during redox signaling (3).

The key difference in ROS and RSS is their ability to be preserved. When peroxide (ROS) attacks a sulfhydryl group of cysteine in a protein, the peroxide is reduced to oxygen and water. When persulfide attacks the sulfhydryl group, however, the persulfide can be reversed by thiol exchange between the protein-RSS specie and a low molecular weight thiol group as carrier. Consequently, the original persulfide is preserved (116). Coincidentally, oxidation of H_2S produces a stronger antioxidant i.e. persulfide which, if unchecked, may lead to reductive stress (116).

H_2S itself has significant antioxidant capacity but is not as recognized. At high concentrations H_2S can create reducing environments and cytotoxicity, but at low concentrations or from endogenous sources it offers protective and beneficial effects (34, 176). H_2S can inhibit ROS via diverse mechanisms (92, 123, 156, 187, 195). It is known to directly scavenge $\text{O}_2^\bullet$, H_2O_2 and other ROS species (46, 184, 195). It can upregulate SOD and glutathione peroxidase, reducing oxidative stress in conditions like diabetic nephropathy, intestine or lung damage (30, 64, 90, 130). It also upregulates GSH as a potential mechanism to reduce oxidative stress (64). Pathways associated with antioxidant activity of H_2S include activation of nuclear factor erythroid 2-related factor 2 (NRF2) pathway involved in cardio-protection (22, 158), and inhibition of p38/MAPK and NF- κ B pathways which lowers ROS, oxidative stress and inflammation (28, 37, 80, 89, 173). These effects

of H₂S are largely observed with exogenous H₂S based therapies. CSE and CBS knockout mice show reduced GSH levels and are more prone to oxidative stress (137, 198, 204, 208), proving a relationship between oxidative stress and endogenous H₂S. Regarding the relationship between iron, H₂S and oxidative stress, it has been recently shown by Zhou and coworkers that CBS is required for iron homeostasis in mice (208). They noticed that CBS deficient mice had increased iron content in the liver, serum, heart and spleen along with other markers of oxidative stress. Homozygous CBS deficient mice could not survive past 4 weeks (208). Hepcidin was upregulated in the liver and circulation due to decrease in transcriptional factor, NRF2. Increased hepcidin caused a loss of ferroportin and increased iron retention, severely damaging liver. This phenotype was reversed by reintroduction of CBS through adenoviral vector (208).

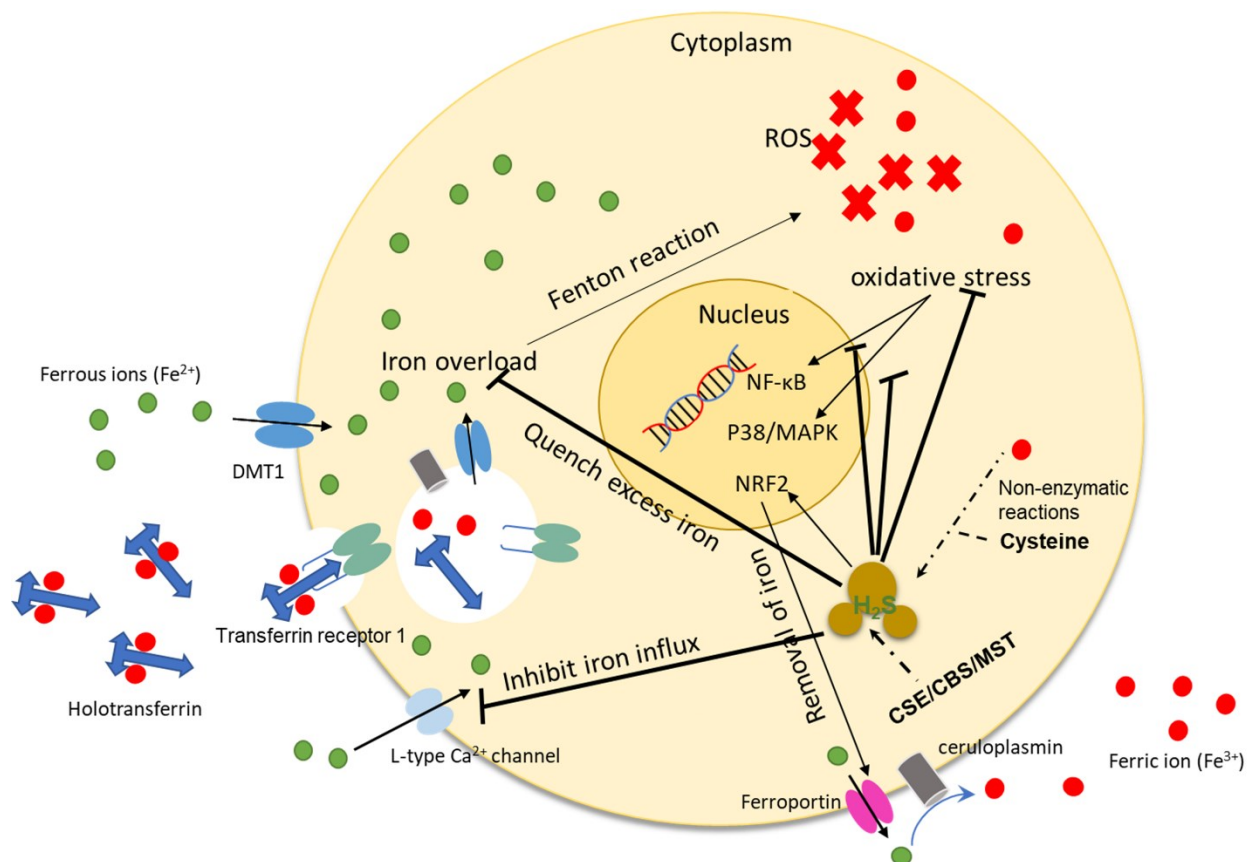


Figure 5. Interaction of H₂S and iron on cell functions. Iron overload causes production of ROS. This will activate NF-κB and P38/MAPK, leading to inflammatory reactions. Excess iron can also lead to non-enzymatic catalysis of cysteine to release H₂S. The latter quenches excess iron and inhibits NF-κB and P38/MAPK pathways and ROS production. The inhibition of L-type Ca²⁺ channels by H₂S decreases iron influx. H₂S also induces NRF2, activating anti-inflammatory genes, upregulating ferroportin, and promoting the efflux of excess iron.

1.6. The effects of iron and H₂S on erythrocytes

The human body produces approximately 200 billion RBCs per day with each containing 300 million hemoglobin molecules with single iron moiety. This feat requires 20 mg of iron daily which is mostly sourced through macrophage recycling of senescent RBCs, body iron stores and dietary iron (161). Transport of oxygen by RBCs is the most prominent role of iron inside hemoglobin in the

body. Low oxygen delivery is sensed by peritubular cells in renal cortex and outer medulla in kidney which release erythropoietin (EPO) in response (143). The EPO drives erythropoiesis by proliferation and differentiation of erythrocytes which requires a large reserve of iron to produce hemoglobin (161). Transferrin bound iron is kept high during each stage of nucleated erythroblast differentiation (141). Once the hemoglobin production ceases, iron uptake is reduced, and transferrin receptors are released as final steps of erythroid differentiation (2).

Iron metabolism and erythropoiesis are linked, and defect in erythropoiesis often leads to iron overload (112, 136). Hepcidin plays an important role in regulating iron and its expression can be controlled by erythropoiesis (93). Hypoxia induced factor (HIF) stimulates EPO and suppresses hepcidin, resulting in an increase in iron concentration. Liu et al. also found that hepcidin suppression indirectly requires EPO induced erythropoiesis (93). Erythroferrone (ERFE), released from erythroblasts, can suppress hepcidin in hepatocytes, strengthening the link between erythropoiesis and iron metabolism (69).

H₂S and iron may interact on erythropoiesis in different ways. It has been recently demonstrated that chicken red blood cells which contains an intact nucleus and mitochondria, produces H₂S, mainly from 3MST enzyme (63). Human RBCs which lack the nucleus and mitochondria may produce limited H₂S by sulfur reduction non-enzymatically (12, 142, 170). Jennings demonstrated that deprotonated H₂S in the form of HS⁻ can be transported into the RBCs in exchange for a chloride ion through AE1 anion exchange protein as part of Jacobs-Stewart cycle. The HS⁻ can be converted to H₂S in cells which can take part in cellular functions or diffuse out (58).

H₂S is a regulator of erythropoiesis. CBS deficient mice had systemic iron overload due to decreased erythropoiesis and iron utilization (204). The absence of CBS caused lower expression of delta-aminolevulinate synthase 2, ferrochelatase, FLVCR, HIF-2 α and EPO, resulting in low erythropoiesis and anemia (204). Exogenous H₂S donor, GYY 4137 can stimulate erythropoiesis through HIF (85). Mouse lacking CSE had reduced EPO and hemoglobin in hypoxia and patients with anemia associated with chronic kidney disease had low H₂S and EPO (84).

1.7. Regulation of cardiovascular functions by iron and H₂S

Iron deficiency or overload has been associated with increased cardiovascular risk with different mechanisms (82). Iron deficiency can be caused by either underutilization of circulating iron or failure to absorb dietary iron (202). As a result of iron deficiency-induced anemia, heart workload increases along with elevated ischemia and myocardial cell death (82). Elevated renal ischemia can activate renin-angiotensin system, causing increased salt and water retention and vasoconstriction, further putting stress on already stressed out heart (51, 82). Anemic mouse models, IRP1/2 knockout or TfR1 knockout, showed decreased cardiac function and mitochondrial respiration which was reversed with aggressive iron therapy (202). These findings have connotation that iron deficiency due to underutilization or improper regulation may result in cardiovascular disturbance.

Iron overload can also produce detrimental effects on the cardiovascular system primarily through ROS generation and enhanced oxidative stress (82). After having tested adult and neonatal cardiomyocytes as well as H9c2 cells (rat myoblasts), Sweeney et al. demonstrated that iron

overload increased ROS via mitochondria and xanthine oxidase. Moreover, iron overload decreased autophagic reflux as seen by an increase in microtubule-associated protein 1A/1B-light chain 3-II (LC3-II) and p62 protein expression which are markers for autophagy (160). This caused a decrease in insulin sensitivity in cardiomyocytes which was confirmed in autophagy deficient H9C2 cells prepared by over expressing dominant negative form of autophagy related 5 protein (Atg5) (157).

Júnior et al. measured effects of chronic iron overload on mesenteric resistance arteries isolated from rats. Chronic iron overload increased wall to lumen ratio and thickness of the blood vessel, along with decreased lumen and external diameters. Decreased distensibility and increased stiffness and pulse wave velocity were also observed. With wire myography recording, Júnior et al. found that iron overload, induced by repeated injections of iron-dextran, increased vasoconstrictor response in isolated mesenteric resistance arteries but did not affect relaxation responses by acetylcholine and sodium nitroprusside. They concluded that iron overload-induced free radicals decreased nitric oxide bioavailability. This conclusion was supported by the observation that L-NAME, an inhibitor of nitric oxide synthase, had no effect on vasoconstriction of mesenteric resistance arteries with iron overload, whereas L-NAME with superoxide dismutase resulted in typical shifting of the phenylephrine contraction curve towards left. In vitro assays also showed a decrease in NO amount in iron overload tissues (65). Interestingly, the same group showed similar results with aortic tissues (103). Kuang et al. reported on acute effects of iron overload on rat aortic tissues (77). They found that ferric ammonium citrate can transiently contract isolated aorta with an increased contraction amplitude at higher calcium concentrations. They also note that incubating tissues with ferric ammonium citrate impairs vasoconstriction by phenylephrine, and this decrease is abolished by adding catalase or glutathione before ferric ammonium citrate. This

suggests that iron overload-caused contraction dysfunction is due to accumulation of ROS (77). Sangartit et al. report an increase in blood pressure and impaired vascular function in iron overload mice (139). Nitric oxide and glutathione levels in the blood were decreased by iron overload but reversed by tetrahydrocurcumin and deferiprone, compounds with antioxidant and anti-inflammatory properties (139). Iron overload increased calcification in human aortic smooth muscle cells and increased interleukin-24, thereby accelerating arteriosclerosis (70). Similarly, iron overload accelerated atherosclerosis and induced endothelial dysfunction in apolipoprotein E deficient mice due to oxidative stress (102). Hypertension as a complication or comorbid factor is also found in patients with sickle cell anemia (4), thalassemia (41), kidney disease (168) and liver disease (36). Recent research has shown that hypertension in sickle cell anemia and thalassemia patients is due to toxic effects of chronic iron overload rather than anemia (4, 41). An increased cardiac output, decreased heart rate variability and vascular damage due to oxidative stress were observed in both diseases (4, 41, 49, 185). These insights warrant further research into mechanisms of iron overload and vascular damage.

ROS may induce vascular damage and activation of p38/MAPK pathway. The consequential endothelial dysfunction, elastin remodeling and vascular stiffness can result in hypertension (105, 166, 179). Inhibitors of p38/MAPK are known to ameliorate vascular damage and reduce endothelial dysfunction and hypertension (179). Iron overload has been shown to stimulate ROS production and p38/MAPK activation in MC3TC-E1 preosteoblast cells (26). Another mediator of inflammation, NF- κ B pathway, also increases vascular damage and is believed to be pivotal in the pathogenesis of hypertension and can be activated by iron overload and ROS (19, 33).

One of the most prominent effects of H₂S is vasorelaxation (198). The discovery that H₂S causing vasorelaxation invoked significant interest into H₂S biology worldwide. One of the earliest papers by our lab found that H₂S at physiologically relevant concentrations opens K_{ATP} channels, which leads to vasorelaxation (206). We subsequently demonstrated that mouse lacking *CSE*-gene had hypertensive phenotype because CSE is the major enzyme for H₂S generation in vasculature (198, 206). Vascular response to H₂S is also endothelium dependent, not inhibited by 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) or NS2028, two potent inhibitors of guanylate cyclase pathway (205). H₂S can mediate the phosphorylation of endothelial nitric oxide synthetase through p38/MAPK and Akt dependent pathway, increasing bioavailable NO for vasorelaxation (5). The interaction of H₂S and NO on vasorelaxation also involves the redox regulation of soluble guanylyl cyclase (sGC). Zhou et al. suggested that H₂S reduced ferric (Fe³⁺) heme center of sGC to its ferrous (Fe²⁺) form in cultured rat aortic smooth muscle cells. By doing so alone, H₂S would not activate sGC. In the presence of H₂S, however, sGC can be conditioned for activation by NO since the latter binds to ferrous (Fe²⁺) heme of sGC to increase its catalytic activity. Consequently, cGMP level was increased and vasorelaxation ensued (209).

H₂S also suppresses the development of atherosclerosis (100) by inhibiting adhesion molecule expression and vessel intimal proliferation. We also observed that decreased endogenous H₂S leads to atherosclerosis by vascular remodeling (99). Estrogen and H₂S both inhibit atherosclerosis in CSE-WT mice fed with atherosclerotic diet, but estrogen cannot inhibit atherosclerosis in CSE-KO mice fed with the same diet. Markers of atherosclerosis, like ICAM-1 and NF-κB, also increased more in CSE-KO than CSE-WT on atherosclerotic diet. Finally the observation that estrogen increases H₂S concentration in livers of CSE-WT but not CSE-KO lead to the conclusion that

estrogen interacts with H₂S to inhibit atherosclerosis (87). We recently reported that insulin like growth factor 1 receptor (IGF-1) interacts with estrogen receptor (ER- α) to cause atherosclerosis but this interaction is inhibited by H₂S, as the latter can inhibit IGF-1R by S-sulphydration (147). Zheng et al. demonstrated that H₂S donor, GYY4137, decreased atherosclerotic plaques in aorta by decreasing intracellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1) and inflammatory markers like tumor necrosis factor (TNF- α), monocyte chemoattractant protein 1 (MCP1), interleukin 6 (IL-6), and IL-1 β in diabetes-accelerated atherosclerotic mice (207). It was concluded that H₂S inhibits NLR family pyrin domain containing 3 (NLRP3) inflammasome which ultimately led to decrease in atherosclerosis (207).

The role of H₂S as an inhibitor of smooth muscle proliferation and viability is well documented by our research team (100, 147, 148, 194, 197, 199) and others. The absence of CSE expression causes VSMCs to over-proliferate (197). Exogenous addition of 100 μ M H₂S significantly increased VSMC apoptosis and phosphorylation of extracellular signal-regulated kinase (ERK1/2) in CSE-deficient mice. ERK regulates apoptosis, growth and cellular response to stress. H₂S also decreased cyclin D1 and increased p21^{Cip}/WAF-1 expression which arrests cell cycle in G1 phase (197). These findings have great implications in pathogenesis of atherosclerosis. We recently demonstrated that estrogen increased endogenous H₂S production to regulate smooth muscle cell proliferation (87). Iron is also associated with atherosclerosis, but this role of iron is often debated (191). Mueller et al. reported the addition of ferrous ions decreased proliferation of smooth muscle cells (110). Conversely, Porreca et al. reports iron chelation and deficiency also inhibits proliferation of vascular smooth muscle cells (129). Although iron accumulation is observed in

atherosclerotic plaques but epidemiological data are conflicting regarding the association of iron overload and atherosclerosis in patients (169). H₂S can offer protection against iron overload, and H₂S maybe released as a consequence of iron overload. This may very well be the reason why it has been difficult to prove link between iron and atherosclerosis, whereas a strong link between H₂S and anti-atherosclerosis already exists. For example, it was found that iron overload can stimulate ERK1/2 in SH-SY5Y neuroblastoma cells (8). Whether the activation of ERK results from iron overload or from the consequential increase in H₂S is unsettled. Epidemiological data also suggest that females are at lesser risk of cardiovascular diseases and iron overload (31). The discovery that estrogen can induce H₂S production may offer a valid explanation on why females are at lesser risk of iron overload and cardiovascular diseases.

H₂S also regulates vascular endothelial proliferation and function (178). H₂S stimulates angiogenesis and endothelial repair (121). H₂S is also an endothelium-derived hyperpolarizing factor (EDHF), along with K_{ATP} channel opener, which establishes role of H₂S in regulation of vascular tone (178). Jian et al. reported that iron deficiency is associated with increased angiogenesis due to upregulation of vascular endothelial growth factor (VEGF) and stabilization of hypoxia-inducible factor-1 α (60). Iron overload, on the other hand, is associated with increased oxidative stress and MAPK activation (60). Interestingly, H₂S is pro-angiogenic under normoxic conditions through phosphorylation of PI3K/AKT, ERK and p38/MAPK pathways, but it is anti-angiogenic under hypoxic conditions by inhibiting translation of HIF-1 α (177, 186). Liu et al. reported that H₂S promoted angiogenesis by stabilizing HIF-1 α in hypoxia by using cobalt to induce a chemical hypoxia (94). The conflicting conclusions may be due to different models of hypoxia,

along with differences in chronic and acute hypoxia. Regardless, there seems to be a link between iron deficiency-induced angiogenesis and H₂S-induced angiogenesis. Figure 6 summarizes potential avenues for interaction between H₂S and iron in the cardiovascular system.

1.8. Molecular mechanisms of interaction between H₂S and iron

As summarized in the previous sections, iron causes oxidative stress and increases markers of inflammation, whereas H₂S can inhibit p38/MAPK, NF-κB, and ROS (79, 156, 158, 173). These are all possible mechanisms where H₂S can potentially decrease deleterious effects of iron overload and ameliorate hypertension. Cysteine can undergo catalysis under the influence of ferric ions to release H₂S as explained before (200). This non-enzymatic H₂S generation is believed to be a protective mechanism to decrease excess iron as H₂S can react with iron to form acid labile iron sulfide, quenching and inhibiting the excess iron from participating in oxidative stress (200). H₂S has been shown to have a cardioprotective role through activation of NRF2 (22) but this role is yet to be established in vasculature. The increased NRF2 upregulates ferroportin 1 (Fpn1), which will lead to efflux of iron and ultimately decrease iron overload (40, 203). CBS knockout mice showed increased iron content in the heart, decreased Trf1, Fpn1 and IRP1 by decrease in hepcidin (208). The effect of H₂S on TfR1 and Fpn1 has only been demonstrated in macrophages and spleen tissue. Should the similar effect occurs in the cardiovascular system, it may mitigate detrimental effects of anemia (203).

To reiterate, iron deficiency or overload causes cardiovascular diseases and H₂S can potentially ameliorate the detrimental effects of abnormal iron metabolism. H₂S can accomplish this through

direct regulation of iron and transport and indirectly through suppressing downstream targets of iron dysfunction like renin-angiotensin system, ROS generation, p38/MAPK and Nf-κB regulation. Further, H₂S can offer enhanced protection through Nrf2 signaling to lower iron induced oxidative stress.

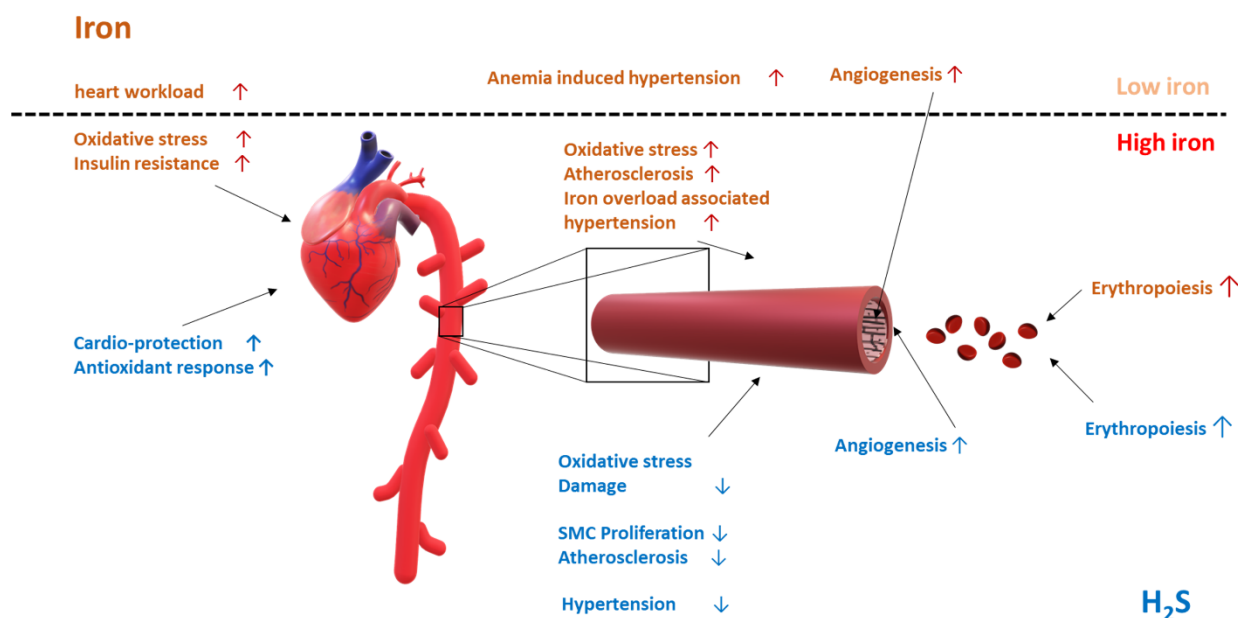


Figure 6. Interactions between iron and H₂S in the cardiovascular system. Iron deficiency can increase heart workload, hypertension, angiogenesis, and erythropoiesis. Iron overload can increase oxidative stress and damage heart and blood vessels. On the other hand, H₂S offers cardio-protection, decreases oxidative stress, inhibits smooth muscle cell proliferation and decreases hypertension. H₂S also increases angiogenesis and erythropoiesis.

1.9. Iron and H₂S interaction in the liver

The liver is the command center for iron metabolism, chiefly through expression of hepcidin. High levels of hepcidin can inhibit ferroportin, decreasing iron release into circulation through

enterocytes and iron recycling by macrophages (13). The liver produces significant amount of H₂S (21, 66) and excrete H₂S-producing enzymes, such as CSE and CBS, into the blood stream (9, 177). Iron and H₂S can interact through different pathways, including BMP/SMAD pathway which governs apoptosis, cellular differentiation, embryogenesis, organogenesis and osteogenesis (115). Another important pathway is JAK/STAT pathway which can affect genes responsible for apoptosis, cell differentiation, proliferation and migration (135). Hepcidin expression is controlled by BMP/SMAD signaling in the liver sinusoidal endothelial cells (150). Activin receptor like kinase 2 and 3 (ALK2 and ALK3) can activate BMP6 and BMP2, respectively, in response to increased tissue iron or basal expression of hepcidin. BMP-SMAD pathway also interacts with JAK/STAT3 pathway which regulates hepcidin produced due to inflammatory response (150).

Hepcidin is also regulated by erythropoietic drive, which stimulates kidney to produce EPO in response to hypoxia. This increased EPO results in erythroblast differentiation, leading them to secrete erythroferrone to suppress hepcidin (175). Although direct regulation of erythroferrone by H₂S is yet to be established, H₂S may increase it indirectly by increasing EPO. Similarly, inflammatory cytokine IL6 can upregulate hepcidin by JAK-STAT3 pathway interacting with SMAD1/5/8 pathway and Activin B (174). It has been shown that H₂S reduced hepcidin expression through JAK-STAT pathway by suppressing pSTAT3 leading to decreased IL6 (203). A similar report revealed that H₂S promoted SIRT1 expression which stabilizes SIRT1-STAT3, reducing STAT3 acetylation. The latter event lowered hepcidin level (190).

It is evident that hepcidin expression is affected by a JAK/STAT and BMP/SMAD axis. The fact that H₂S can also affect these pathways and ultimately hepcidin expression points towards a key role of H₂S in regulating physiological iron levels.

1.10. Iron and H₂S interaction in the brain

As discussed earlier, iron is important for energy metabolism because it is bound to complexes in electron transport chain like FAD. Iron sulfur clusters and heme also serve as cofactors for different enzymes. In the brain, iron serves as a cofactor in synthesis of neurotransmitters, such as tryptophan hydroxylase and tyrosine hydroxylase (109). Absorption of iron through the blood brain barrier (BBB) is analogous to dietary iron absorption in duodenum. Once crossing BBB, it is oxidized to Fe³⁺, bound to transferrin and released into the brain circulation. Transferrin-bound iron is thought to be either broken down in endosomes and released into the cytosol by DMT1 and exported by ferroportin or to be transported into the endosomes from luminal to abluminal surface where endosomes release iron between brain endothelial cells and astrocytes. Further, Fe²⁺ can bind to ATP or citrate to be transported as NTBI in oligodendrocytes and astrocytes which do not express TfR1 (11). Iron concentration in the brain is tightly regulated. Abnormal iron concentrations are associated with neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, Huntington disease and neurodegeneration (62).

The physiological role of H₂S in the nervous system has evolved to include neuroprotection, neuromodulation, and nociception (95). H₂S also induces long term potentiation and increases the sensitivity of NMDA receptor mediated memory response. Administration of NaHS elicited anti-inflammatory response to counter LPS induced inflammation in microglia by inhibiting p38/MAPK (53). H₂S increases [Ca²⁺]_i for neuronal health and function (95). Moreover, H₂S can inhibit NADPH oxidase and decrease oxidative stress in the brain (138). Studies have also shown importance of H₂S in peripheral nerve regeneration and degeneration, significant for Schwann cell proliferation

and dedifferentiation (120). The role of H₂S in various neurodegenerative disease has been reviewed by Snyder et al. (122).

With Parkinson's disease (PD), substantia nigra region of brain has considerable dopaminergic neuronal cell death (153). Symptoms of this disease include postural instability, tremors and rigidity. The neuropathological characteristics include Lewy bodies which are intracytoplasmic inclusions, widely distributed in paralimbic and neocortical regions of substantia nigra. Neuromelanin level, associated with iron storage, decreases with age, leading to high iron accumulation and oxidative stress. DMT1 isoform was upregulated in PD with increased iron uptake and dopaminergic cell death. A decrease in ceruloplasmin in PD is also observed in substantia nigra, which leads to increased iron deposition (7). Iron chelation therapy has shown to be effective in decreasing neurotoxicity in PD (10, 68). H₂S offers a protective role in neurotoxin-induced PD in rat models by reducing oxidative stress, inflammation by regulating different pathways, including PKC, p38, JNK, PI3K/Akt and ERK-MAPK (163). Endogenous H₂S was downregulated in substantia nigra by 6-hydroxydopamine (6-OHDA), whereas exogenous administration of H₂S reversed 6-OHDA induced neuronal death, suggesting a protective role of H₂S in PD (23).

Alzheimer's disease (AD) is a neurodegenerative disease with impairment of memory, reasoning and perception as its prominent features. Pathological features include insoluble amyloid beta protein (ab) aggregates and neurofibrillary tangles (NFT). NFTs contain hyperphosphorylated tau proteins which can reduce Fe³⁺ to Fe²⁺, increasing NFT production (193). Amyloid precursor protein (APP) is a transmembrane protein 1 ubiquitously found in the brain. It is upregulated in the presence of iron and causes iron deposition in amyloid plaques. APP is like ceruloplasmin and aids in efflux

of iron and converts Fe^{2+} to Fe^{3+} . High iron can upregulate paired amino acid cleaving enzyme, resulting in an increase in amyloidogenic peptides (117).

H_2S levels in brain are significantly decreased along with the expression of CBS in patients with AD (35). Exogenous H_2S administration slowed down AD progression in mouse and rat models (47, 48). In these animal models, H_2S lowered ab plaques in hippocampus and proteins associated with AD progression. Also, H_2S was found to modulate inflammation and apoptosis of hippocampus cells and with mice of different ages with AD (167). H_2S may protect against AD by directly reducing hypochlorous acid induced cytotoxicity, protein and lipid peroxidation, along with homocysteine induced oxidative stress (180). There is a positive association with homocysteine and iron level and deposits in arterial plaques, suggesting iron-homocysteine interaction (50). H_2S has been reported to inhibit homocysteine-induced AD-like pathology (67). This raises an interesting question whether iron-homocysteine- H_2S axis exists in regulating brain functions under physiological and pathophysiological conditions.

1.11. Concluding remarks from literature

The interactions between iron and H_2S impact each other's metabolism and functions. Whereas excess iron produces ROS, H_2S can quench excess iron and ROS altogether. Conversely, whereas low iron causes anemia, H_2S can downregulate hepcidin to release more iron. The interaction is also seen at molecular level where iron and H_2S can act on same cellular pathways but with opposite effects. For example, iron catalyzes ROS-activated p38/MAPK and NF- κB and H_2S can counter this effect. Given that excess iron can increase H_2S production non-enzymatically, the

effect of iron on H₂S signaling pathways and targets is much less known. The interactions between iron and H₂S have been observed in numerous systems, including the brain, blood, liver, and cardiovascular system. Atherosclerosis is a case in point. Iron can increase H₂S and the latter would protect from atherosclerosis. On the other hand, iron overload accelerated the development of atherosclerosis. The net outcome of iron overload and induced H₂S production on atherosclerosis development is difficult to speculate yet. The similar dilemma also challenges the contributions of iron overload and H₂S level, respectively and/or integrated, to the development of hypertension. While having acknowledged these challenges, there is no doubt that a better understanding of the interactions between iron and H₂S would shed light on the pathogenesis of and treatment strategies for many diseases. In the future, physiological and pathophysiological importance of H₂S and iron as well as their therapeutic applications should be evaluated jointly, not separately. Future investigation should also expand from iron-rich cells and tissues to the others in which H₂S and iron interaction has not received due attention. In light of these identified gaps, the following work sought to define the mechanistic basis of H₂S–iron interactions and their physiological relevance to vascular function through a combination of molecular, mitochondrial, cellular, and animal studies.

1.12. Hypothesis and Objectives

1.12.1. Hypothesis

This thesis tested the hypothesis that cystathionine γ -lyase–derived hydrogen sulfide (H₂S) safeguards vascular iron homeostasis by integrating ferritin buffering, antioxidant defense,

and autophagy signaling. Specifically, it was hypothesized that endogenous H₂S mitigates iron-induced oxidative injury and vascular dysfunction by:

1. Limiting intracellular iron accumulation through modulation of iron transport and storage proteins.
2. Enhancing antioxidant protection via activation of the NRF2 and AMPK–autophagy pathways.
3. Balancing apoptosis–autophagy signaling to preserve vascular smooth muscle cell (VSMC) integrity and maintain vasomotor function under iron stress.

In the absence of physiological H₂S production (as in CSE deficiency), these adaptive responses were predicted to collapse, leading to mitochondrial dysfunction, heightened oxidative stress, and impaired vascular reactivity during iron overload. This integrated hypothesis was examined across molecular, cellular, and systemic levels to elucidate the protective role of the H₂S pathway in iron-mediated vascular injury.

1.12.2. Objectives

To address the above hypothesis, a series of interrelated objectives were pursued across four experimental chapters encompassing in vitro, ex vivo, and in vivo studies:

1. To delineate the molecular mechanisms linking H₂S to iron regulation and redox balance in vascular smooth muscle cells (**Chapter 2 – Published**).

This objective investigated how H₂S modulates ferritin expression, iron response element (IRE)–dependent signaling, and reactive oxygen species (ROS) production under conditions of iron overload. Comparisons between wild-type (WT) and CSE-knockout (KO) VSMCs identified the dependence of these effects on endogenous H₂S generation.

2. To define the role of CSE-derived H₂S in preserving mitochondrial function during iron-induced stress (**Chapter 3** – Published).

This work assessed mitochondrial respiration, ATP production, and membrane potential in WT and CSE-KO VSMCs, linking H₂S bioavailability to the maintenance of mitochondrial integrity and cellular energy homeostasis under iron challenge.

3. To establish a high-performance liquid chromatography method for accurate quantification of H₂S in plasma (**Chapter 4** – Published).

To enable translational measurement of H₂S levels in animal models, a novel methylene blue/methylene green (MBMG)–based assay was developed and validated for stability, sensitivity, and reproducibility, providing a standardized platform for systemic H₂S assessment.

4. To investigate the vascular and systemic outcomes of CSE deficiency and iron overload in vivo (**Chapter 5** – Published).

Using WT and CSE-KO mice subjected to controlled iron loading, this objective evaluated ferritin expression, vascular remodeling, elastin integrity, inflammatory signaling, and

vasomotor responses. These studies established how physiological H₂S production modulates iron distribution and vascular tone in the intact organism.

1.12.3. Central Aim

Collectively, these objectives aimed to integrate molecular and physiological evidence to establish hydrogen sulfide as a critical regulator of vascular iron homeostasis and as a potential therapeutic target in iron-mediated vascular injury. The findings provide mechanistic insights into how redox-active sulfur and iron systems intersect to determine vascular fate under stress.

1.13. Acknowledgement:

This study has been supported by a Discovery Grant from Natural Sciences and Engineering Research Council of Canada to RW.

1.14. Authorship confirmation statement:

Arif, Z. Qian, and R. Wang have equally contributed to writing, editing and revising of the manuscript and figures

1.15. Author disclosure statement

The authors declare that they have no competing interests.

1.16. References

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Chapter 2: Hydrogen Sulfide (H₂S) Metabolism, Iron Overload, and Apoptosis-autophagy Equilibrium in Vascular Smooth Muscle Cells

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This chapter has been published as:

Arif, H. M., Fu, M., & Wang, R. (2025). Hydrogen Sulfide (H₂S) Metabolism, Iron Overload, and Apoptosis–Autophagy Equilibrium in Vascular Smooth Muscle Cells. *Antioxidants*, 14(5), 560.

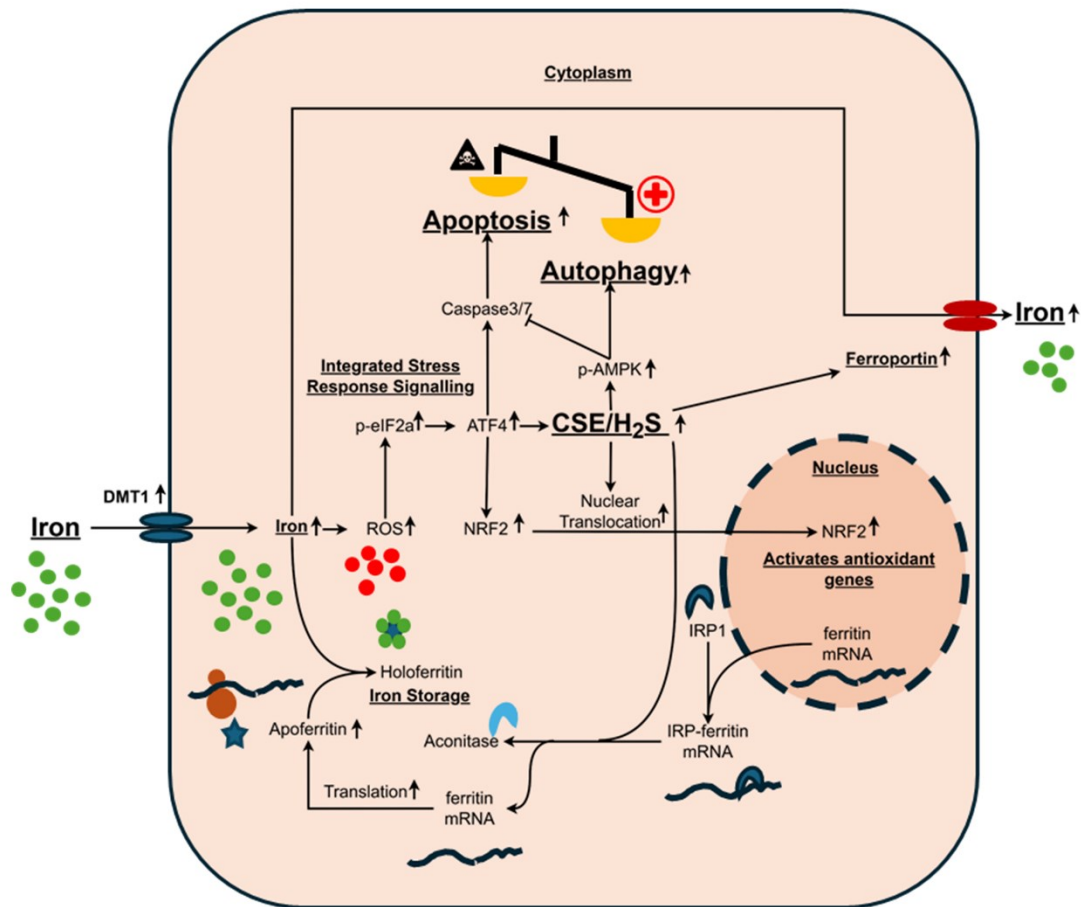
Contributions: H.M.A. conceived the study, developed the methodology, performed data analysis, and wrote the original draft. M.F. contributed to methodology and assisted with data analysis. R.W. provided supervisory input, guidance on data analysis and revision, and support with project administration and funding. H.M.A. carried out all experiments and writing at each stage.

The sections and headings in this chapter are structured to align with the formatting guidelines of Antioxidants journal.

2.1. Abstract

Iron overload contributes to proliferative vascular diseases, yet its interplay with hydrogen sulfide (H_2S) in vascular smooth muscle cell (VSMC) proliferation remains poorly understood. This study elucidates H_2S 's role in mitigating iron overload-induced oxidative stress and cellular damage. Using aortic VSMCs from wild-type (WT) and cystathionine γ -lyase knockout (CSE-KO) mice treated with ferric ammonium citrate (FAC) at the concentrations equivalent to serum levels of iron and citrate, we demonstrate that FAC triggers the integrated stress response (ISR) in WT cells, upregulating CSE to enhance H_2S production. The ISR mediator ATF4 activates caspases but simultaneously induces CSE, which inhibits caspase activity and promotes autophagy via AMPK signaling. In CSE-KO cells, iron overload leads to diminished ferritin upregulation, unchecked caspase activation, and impaired autophagy compared to WT. Exogenous H_2S restored iron homeostasis by enhancing ferritin expression, activating NRF2 antioxidant pathways, and rebalancing apoptosis-autophagy equilibrium in both WT and KO cells. These findings establish H_2S as a critical regulator of iron-induced VSMC dysfunction, highlighting its therapeutic potential in managing vascular pathologies linked to iron dysregulation.

Keywords: H_2S biology; Iron metabolism; iron- H_2S interaction; Smooth muscle cells; Apoptosis; Autophagy



Graphical Abstract

2.2. Introduction

Iron participates in diverse cellular functions from oxygen transport to energy metabolism. These roles of iron are realized by its ferric (Fe^{3+}) or ferrous (Fe^{2+}) status and its binding strength with different macromolecules. For example, its weak conjugation with citrate yields the form of labile iron [1,2]. Diminished iron level is a key pathogenic factor for anemia. Iron overload is an abnormal condition of iron metabolism with more iron present than physiologically required [3]. It contributes to numerous detrimental reactions, such as the Fenton reaction, to produce reactive oxygen species (ROS) that can cause widespread cellular damage [2]. Iron overload can be a direct

complication of genetic diseases, such as heterozygous hemochromatosis and Friedreich ataxia, or secondary to diseases like thalassemia where repeat blood transfusions are required [4]. As a comorbid factor in vascular diseases [2], excess iron exacerbates atherosclerosis with oxidative stress and apoptosis in vascular smooth muscle cells (VSMCs) [5].

Hydrogen sulfide (H_2S) is a key gasotransmitter that is produced mainly via three enzymes, cystathionine gamma-lyase (CSE), cystathionine beta synthase, and 3-mercaptopyruvate sulfurtransferase [2]. Recently, cysteinyl-tRNA synthetases and the non-enzymatic reaction between cysteine and iron were also reported to produce significant H_2S in cells [6]. The discovery that endogenous H_2S can cause endothelium-dependent vasorelaxation [7] paved the way for scientists to explore the role of H_2S in other physiological avenues. Endogenous H_2S in vasculature is primarily CSE derived. Our previous studies have shown CSE ablation exacerbates vascular complications like hypertension and atherosclerosis [8].

The interaction between iron and H_2S in the vasculature has been investigated mostly using toxic levels of extracellular iron concentrations [9,10]. One study on the formation of iron-sulfur clusters or the formation of non-enzymatic H_2S via iron and vitamin B6 used toxic doses, ranging from 50 to 100 μM , of ferric or ferrous iron [2,11,12]. In another study on H_2S -facilitated conversion of iron regulatory protein 1 (IRP1) to its aconitase form in VSMCs used 100 μM of ferric chloride. The modulation of the expression and activity of CSE/ H_2S system by iron at physiological or pathophysiologic level has been unknown [2,11,12]. Vice versa, whether and how H_2S causes cellular iron overload without increasing extracellular iron to toxic levels are not clear.

While iron overload decreases autophagy and triggers cell death mainly via ferroptosis, or apoptosis depending on cell-type and iron overload model [9,13]. H₂S has been shown to trigger apoptosis via p38/MAPK and ERK pathway in VSMCs [14,15]. H₂S has also been shown to regulate autophagy since it can directly influence different autophagic proteins, like Adenosine monophosphate-activated protein kinase (AMPK), Nuclear factor erythroid 2-related factor 2 (NRF2), and SIRT [16–18]. However, the integrated effects of iron overload and H₂S on apoptosis and autophagy in VSMCs remain unexplored. This study aims to investigate the interplay between iron overload and H₂S in modulating apoptotic and autophagic pathways in VSMCs, providing new insights into their potential crosstalk in vascular pathophysiology.

2.3. Material and Methods

2.3.1. Animals and protocols

All protocols were approved by the Animal Care Committee, York University. Briefly, wildtype mice with the background C57BL/6J x129SvEv and in-house created homozygous CSE-KO mice of the same genetic background were used for all experiments. The homozygous CSE-KO mice were produced after backcrossing at least 10 generations of WT mice with heterozygous CSE-KO mice, as described in our previous study [7]. All mice had unlimited access to water and standard rodent chow ad libitum. They were kept in HVAC-ventilated cages on a 12:12 light/dark cycle.

2.3.2. Aortic VSMC culture

Mouse aortic VSMCs were isolated as previously described with modifications [15,19]. Aorta of 8-week-old male mice was excised and kept in ice-cold Krebs's buffer with the following composition in mM: 118 NaCl, 4.7 KCl, 1.18 KH_2PO_4 , 1.17 MgSO_4 , 5 glucose, 25 NaHCO_3 , and 2.5 mM CaCl_2 . The dish containing the isolated aorta in Krebs's buffer was kept on ice and continuously gassed with 95% oxygen and 5% CO_2 . A thin wire was inserted in the lumen to scrape and remove endothelial cells. Surrounding adipose tissue was carefully removed, and the aorta was placed in fresh 1.4 mg/ml collagenase type II solution prepared in serum-free Dulbecco's Modified Eagle Medium (DMEM) for 20 minutes at 37°C in a humidified 5% CO_2 incubator. Supplementing DMEM with 2.05 g of NaHCO_3 helped keep the pH of DMEM at 7.4 in the incubator with 5% CO_2 . After 20 minutes of incubation, the adventitious layer was carefully rolled off the aorta, leaving only the medial layer containing VSMCs. The medial layer was incubated with fresh 1.4 mg/ml collagenase solution prepared in DMEM in a CO_2 incubator for 4 hours to release the VSMCs. Once sufficient cells were released, DMEM with 10% FBS was added to quench collagenase, followed by centrifugation at 350 x g for 10 minutes to pellet VSMCs. The VSMCs were resuspended in DMEM containing 10% FBS and plated in a 35 mm culture dish. Cells were left undisturbed in the incubator for 5 days when colonies of VSMCs started to appear. Media was changed every 48 hours until confluency reached, which took 5 to 7 days. The presence of aortic VSMCs was confirmed by the expression of α -smooth muscle actin and the absence of Platelet endothelial cell adhesion molecule.

Cells were passaged upon 80% confluency, and cells from passages 3-7 were used for experiments. In all experiments, cells were first synchronized by adding serum-free medium for 24 hours, followed by reintroduction of media with 10% FBS with different treatment groups, which

included phosphate buffered saline (PBS) as a control, 0.1 mM ferric ammonium citrate (FAC), 0.1 mM NaHS, and cells pretreated with 0.1 mM NaHS for 4 hours, followed by addition of 0.1 mM FAC (N+F).

2.3.3. Iron source

FAC from MP Biomedicals, catalog number 158040, was used as an iron source for all experiments. Because FAC contains 17% ferric iron, 0.1 mM of FAC is equivalent to 17 μ M of iron. The average plasma iron concentration in C57BL6J mice is 17-23 μ M, and the average serum iron concentration is 19-21 μ M [2]. The average citrate concentration in mouse plasma is between 100 – 130 μ M [20].

2.3.4. Cell viability

Cell viability was assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [19]. Cells were seeded with a density of 5000 cells per well of 96 well plates. After different treatments, MTT (5 mg/ml) was added, and the plates were incubated for 4 hours at 37°C in a 5% CO₂ incubator. Dimethyl sulfoxide (DMSO) (200 μ l) was added post-incubation for 20 minutes to dissolve the insoluble purple formazan. Plates were read on Biotek Synergy HTX multimode plate reader (Agilent) at 570 nm. Viability was expressed as a percentage change in absorbance (purple formazan) relative to controls of the same genotype (WT or CSE-KO).

2.3.5. Cell proliferation

Cell proliferation was assessed by colorimetric Bromodeoxyuridine (BrdU) assay kit (Calbiochem) as described previously [19]. Cells were seeded at a density of 5000 cells per well in a 96-well

plate. After different treatments, the BrdU label was added for 4 hours, after which fixing/denaturing reagents were added and incubated for 30 minutes. Anti-BrdU antibody was diluted 1:100 and added post fixing/denaturing for 1 hour followed by 3 washes with wash buffer provided with the kit. Peroxidase goat anti-mouse IgG HRP conjugate was added and incubated for 30 minutes, followed by 3 more washes. After the final wash, the substrate solution was mixed for 15 minutes, followed by adding a stop solution and measuring absorbance on Biotek Synergy HTX multimode plate reader (Agilent) at 450/540 nm using 540 nm as background.

2.3.6. *Ferrozine Assay*

Intracellular iron content was measured via ferrozine assay as described previously [21]. Cells were seeded at a density of 20,000 cells per well of a 24-well plate. After treatments, cells were lysed with fresh 200 μ L iron releasing agent (mixture of 1.4 M HCl and 4.5% KMnO₄ 1:1). The plates were sealed in aluminum foil and incubated at 60°C for 2 hours. Afterward, the plates were removed from the incubator and cooled to room temperature. Further, 30 μ L iron detection reagent (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 mM ammonium acetate, and 1 M ascorbic acid) was added to each well for a 30-minute reaction. The resulting absorbance was measured on a Biotek Synergy HTX multimode plate reader (Agilent) using 550 nm wavelength.

2.3.7. *H₂S production rate*

H₂S production rate was measured via our previously described method [19]. In brief, lead acetate paper was prepared by soaking filter paper in 1% lead acetate for 1 hour, then placing the filter paper in the oven to dry. Cell lysates prepared in ice cold PBS with final concentration of 100 μ g protein in 50 μ L were reacted with 10 μ L of 10 mM cysteine and 10 μ L of 2 mM pyridoxal 5'-

phosphate in a 96 well plate overlaid with lead acetate filter paper. The 96 well plate was sealed in aluminium foil and placed in a 37°C incubator for 2 hours, after which the lead acetate filter paper was imaged and the resulting darkened, circular bands representing H₂S activity were quantified using ImageJ software version 1.54h (NIH, USA) [22].

2.3.8. *Aconitase activity assay*

Aconitase activity was measured using an aconitase assay kit (Abcam) as described previously [10]. Briefly, after treatment, cytoplasmic aconitase (c-aconitase) was isolated by homogenizing cell pellets in assay buffer, followed by centrifuging at 800g for 10 minutes. The supernatant collected was centrifuged at 20,000g for 15 minutes to pellet the mitochondrial aconitase (m-aconitase). The c-aconitase-containing supernatant was activated with 10 µl of activation solution per 100 µl supernatant and incubated on ice for 1 hour. Afterward, 50 µl of the activated sample was incubated with 50 µl of the reaction mixture for 1 hour at room temperature, and the remaining 50 µl of the activated sample was used to measure the background. After incubation 10 µl of developing solution was added, the resulting absorbance was measured at 450 nm on a Biotek Synergy HTX multimode plate reader (Agilent).

2.3.9. *ROS production*

ROS production was measured using CellROX™ Green Reagent (Invitrogen). Cells were seeded with a density of 100,000 cells per 35 mm plate. After different treatments, CellRox reagent was added at a final concentration of 5 µM and incubated for 30 minutes at 37°C. Plates were washed with PBS 3 times, and Hoechst 33342 was added as a counter stain, which was further incubated for 10 minutes. The plates were immediately imaged using an Olympus x71 fluorescence

microscope (Olympus America Inc.) with FITC (green) for ROS, and DAPI (blue) channels for the Hoechst-stained nuclei. The fluorescence intensity ratio between green and blue channels was calculated using ImageJ.

2.3.10. Lipid peroxidation

Lipid peroxidation levels were measured using BODIPY581/591 C11 dye. Cells were seeded with a density of 100,000 cells per 35 mm plate. After different treatments, 2.5 μ M BODIPY dye was added. Plates were put on an orbital shaker for 5 minutes to evenly distribute the dye and incubated in a 37°C incubator with 5% CO₂ for 20 minutes. The media was removed, and PBS with Hoechst 33342 was added as a counter stain and further incubated for 10 minutes. The plates were immediately imaged using an Olympus x71 fluorescence microscope (Olympus America Inc.) with channels FITC (green) for oxidized, TRITC (red) for the reduced probe, and DAPI (blue) to normalize against Hoechst-stained nuclei. The fluorescence intensity ratio between red and green channels was calculated using ImageJ.

2.3.11. DNA fragmentation (TUNEL) assay

DNA fragmentation was assessed using a fluorescent FragEL DNA fragmentation detection kit (Calbiochem). Cells were seeded at a density of 100,000 cells per 35 mm plate containing a fibronectin-coated coverslip. After treatments, coverslips with cells were permeabilized with 20 μ g/ml of proteinase K for 5 minutes, followed by 3 washes with Tris buffered saline (TBS). Cells were equilibrated with 1X equilibration buffer for 15 minutes, then the equilibration buffer was removed and TdT was added to label the reaction mixture and incubated for 1 hour. The reaction was terminated by washing 3 times with TBS. Coverslips were counter stained with Hoechst,

mounted on slides, and visualized using an Olympus x71 fluorescence microscope (Olympus America Inc.) with a FITC and DAPI channel. Mean fluorescence intensity was calculated using ImageJ.

2.3.12. Caspase 3/7 activity

Cellular apoptosis was assessed by measuring caspase activity via CellEvent™ Caspase-3/7 Green ReadyProbes™ Reagent (Invitrogen). Cells were seeded with a density of 100,000 cells per 35 mm plate. After different treatments, 20 µl of dye was added to each plate and incubated in a 37°C incubator with 5% CO₂ for 10 minutes. Cells were visualized using an Olympus x71 fluorescence microscope (Olympus America Inc.) with a FITC channel. Fluorescence intensity was calculated using ImageJ.

2.3.13. Autophagy assay

Autophagy was assessed using the autophagy assay kit (MAK138, Sigma Aldrich) which produces fluorescence in DAPI channel when bound to autophagosome. Cells were seeded at a density of 100,000 cells per 35 mm plate. After different treatments, cells were incubated with 1x autophagosome detection reagent for 1 hour at 37°C in an incubator with 5% CO₂. Afterward, cells were washed 3 times with PBS and imaged immediately on an Olympus x71 fluorescence microscope (Olympus America Inc.) with DAPI channel. The mean fluorescence intensity was calculated using ImageJ software.

2.3.14. Nuclear and Cytoplasmic extraction

Nuclear and cytoplasmic extracts were separated using NE-PER reagent (ThermoScientific). Harvested cells were centrifuged at 350 x g for 5 minutes, and the supernatant was removed. The resulting cell pellet was resuspended in ice-cold 200 μ L CER I reagent and vortexed for 15 seconds, followed by rest on ice for 10 minutes. Further, 11 μ L of ice-cold CER II was added and vortexed twice for 5 seconds with 1-minute intervals on ice. Lysate was centrifuged at maximum speed at 4°C, and the supernatant containing cytoplasmic extract was transferred to a new tube. The resulting pellet with nuclear extract was resuspended in 100 μ L ice-cold NER reagent.

2.3.15. Integrated stress response (ISR) pathway inhibition

ISR was inhibited using ISRIB (Sigma), a drug that inhibits phosphorylation of eukaryotic translation initiation factor 2 (p-eIF2 α), and Activating transcription factor 4 (ATF4) [23]. ISRIB stock was prepared in DMSO warmed to 65°C and diluted in PBS, which was syringe-filtered prior to use. VSMCs were serum-starved overnight, followed by reintroduction of DMEM with 10% FBS and 0.1 μ M of ISRIB for 1 hour. In the presence of ISRIB, either PBS (control) or 0.1 mM FAC (I+F group) was added to the culture medium for 48 hours.

2.3.16. Western blot

Cells were sonicated in ice-cold, fresh lysis buffer (1 X RIPA buffer with 1% PMSF and 1% protease inhibitor cocktail). Lysates were separated on SDS-PAGE, transferred on PVDF membranes, and incubated with a blocking buffer (5% milk or 5% BSA). Primary antibodies include 1:1000 anti-CSE (proteintech), 1:2000 anti-Ferritin (Novous Biologicals), 1:2000 anti-GAPDH (Novous Biologicals), 1:2000 anti-NCO4A (Novous Biologicals), 1:2000 anti-NRF2 (Novous Biologicals), 1:2000 anti-

Lamin A/C, 1:2000 p-eIF2 α (Cell Signaling Technology), 1:2000 anti-eIF2 α (Cell Signaling Technology), 1:2000 anti- α tubulin (Cell Signalling Technology), 1:1000 anti-cleaved caspase 3 (Cell Signalling Technology), 1:2000 anti-ATF4 (Thermofisher), 1:2000 anti-p-AMPK (Cell Signaling Technology), 1:2000 anti-AMPK (Cell Signaling Technology), 1:2000 LC3B (Novous Biologicals) were incubated in respective experiments for one and a half hours at room temperature. Wash steps were either done with PBS with 0.1% tween or TBS with 0.1% tween. HRP-conjugated goat anti-rabbit (1:2000) or rabbit anti-mouse (1:2000) antibodies were used as secondary antibodies and were incubated for one and a half hours at room temperature. Detection was performed using ECL (Cytiva) or Super Signal West Pico Plus (Thermofisher) and imaged using Microchemi imaging systems (Froggabio).

2.3.17. Quantitative PCR

Total RNA was isolated using the Trizol (Sigma) method. Reverse transcription with 2 μ g RNA to get cDNA was performed using the Superscript First Strand system (Invitrogen). CSE primers included: Forward primer AGCGATTACACCACAAACCAAG and Reverse primer ATCAGCACCCAGAGCCAAAGG. Primers for 18S rRNA included: Forward AGTCCCTGCCCTTTGTACACA and Reverse primer CGATCCGAGGGCCTCACTA. Realtime PCR was performed using SYBR Green PCR master mix in iCycler iQ5 (BioRad) with iCycler optical system software (version 3.1). Cycle conditions included 95°C for 1 minute followed by 38 cycles of 95°C for 15 seconds, 61°C for 20 seconds, and 72°C for 30 seconds, during which an image was taken. Standard melt curve analysis was made between temperatures of 58°C and 95°C with 1°C increments. Relative mRNA quantification was calculated by using the arithmetic formula 2⁻

$\Delta\Delta CT$, where ΔCT is the difference between the threshold cycle of a given target cDNA and an endogenous reference 18S cDNA.

2.3.18. Statistical analysis and reproducibility

All data was expressed as mean $\pm$ standard error (SEM). A p-value of less than 0.05 was considered statistically significant. Experiments utilizing 96-well plates had 6 wells designated per group, and all data was taken from 3 plates recorded in three individual experiments. Protein from 3 plates (35 mm each) was pooled in one tube, representing one sample, and three samples per group were run on gels for western blotting. Data from all western blots was taken from 4 gels recorded in individual experiments. For imaging experiments, 3 random fields were chosen from 6 plates (35 mm each) per group. Unpaired two-tailed Student's t-tests were employed for comparisons between two groups, as appropriate. For analyses involving three or more groups, either one-way or two-way analysis of variance (ANOVA) was conducted, followed by Tukey's multiple comparison post hoc test.

2.4. Results

2.4.1. Iron Loading Decreased Viability and Caused Iron Overload in CSE-KO Cells

Iron loading with FAC significantly decreased the viability of both WT and CSE-KO cells (**Figure 1**). WT cells demonstrated greater iron tolerance than KO cells, as the viability of WT cells remained stable when exposed to 0.01 mM FAC for up to 48 h, whereas KO cells exhibited a significant viability decline within 24 h (Figure 1a,b). At 0.1 mM FAC, WT cells showed a time-dependent

recovery, with viability increasing significantly after 72 h compared to the 24 h incubation. In contrast, KO cells displayed no such adaptive response, maintaining significantly reduced viability at all incubation periods relative to untreated controls.

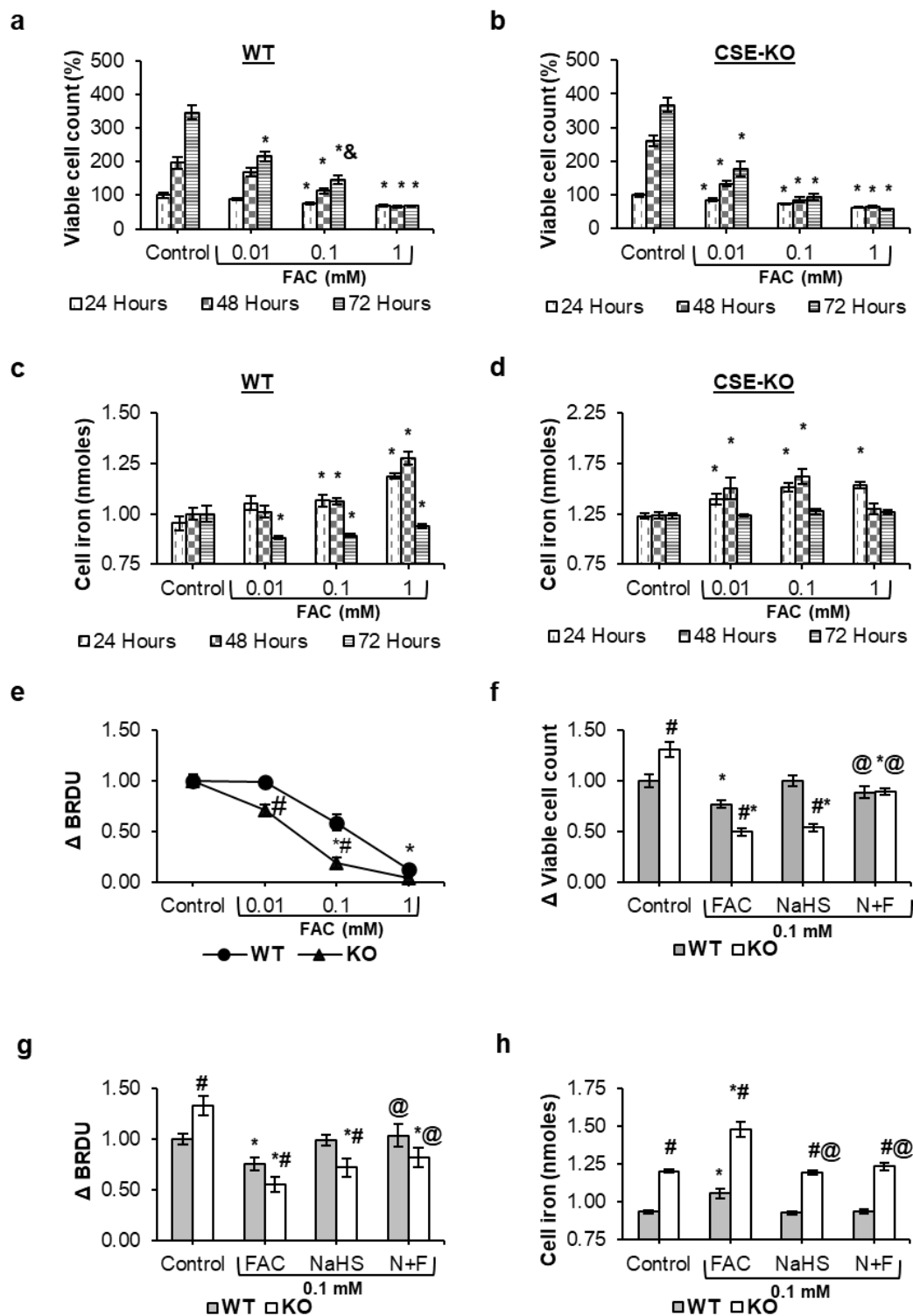


Figure 1. Effects of FAC on cell viability, iron concentration, and proliferation in cultured VSMCs. (a,b) Cell viability measured via MTT assay relative to controls of the same genotype (WT or CSE-KO). (c,d) Cellular iron concentrations. (e) Changes in VSMC proliferation relative to controls of the same genotype measured via BRDU assay after being exposed to FAC for 48 h. (f) Interaction effect of FAC and NaHS on cell viability measured with MTT assay. (g) Interaction effect of FAC and NaHS on cell proliferation measured with BRDU assay. (h) Interaction effect of FAC and NaHS on cellular iron concentrations measured with ferrozine assay. In (f–h), WT and CSE-KO cells were exposed to FAC for 48 h with or without NaHS pretreatment (N+F), and data were normalized to WT controls. * $p < 0.05$ vs. control group with the same genotype, & $p < 0.05$ vs. 24 h group with the same treatment, # $p < 0.05$ vs. WT group with the same treatment, @ $p < 0.05$ vs. FAC treatment with the same genotype group; $n = 6$, with 3 experimental repeats.

The ferrozine assay demonstrated a linear increase in cellular iron concentration in both WT and KO cells, with the maximal iron concentration reached within 24 h of exposure to ferric ammonium citrate (FAC) (Figure 1c,d). In WT cells, treatment with 0.1 mM and 1 mM FAC significantly elevated the cellular iron concentration compared to untreated controls at 24 and 48 h. A sharp decline in intracellular iron was observed in WT cells by 72 h across all FAC concentrations. KO cells exhibited a similar initial trend, with iron concentrations peaking at 24 h. However, KO cells displayed a significant reduction in iron concentration by 72 h compared to earlier timepoints (Figure 1d), mirroring the decline seen in WT cells but with no recovery phase.

Distinct iron-handling dynamics between WT and KO cells emerged at the 0.01 mM FAC concentration. While WT cells exhibited no measurable change in iron concentration over 24–48 h, KO cells displayed a significant increase in iron accumulation within 24 h, sustaining elevated levels through 48 h (Figure 1c,d). The viability of WT cells remained unchanged with 0.01 mM FAC exposure for 24–48 h. In contrast, KO cells suffered a significant viability drop under the same conditions, correlating with their dysregulated iron accumulation. With 0.1 mM FAC, KO cells exhibited exacerbated iron loading and a steeper viability decline compared to WT cells.

With the highest concentration of FAC (1 mM), WT cells maintained an increased cellular iron concentration for 48 h, while KO cells failed to sustain iron levels beyond 24 h. These findings collectively underscore WT cells' iron tolerance, mitigating iron toxicity and sustaining cell viability. The inability of KO cells to survive beyond 48 h in 0.1 mM FAC, coupled with the increase in intracellular iron accumulation within this timeframe, suggests that cell damage or death occurred. It is also interesting to note that KO cells have a higher viability and proliferation rate, as reported in our previous studies [15,24]. KO cells after 48 h of incubation without FAC treatment almost tripled

their viable cell numbers in comparison to those at 24 h. In contrast, the viable cell numbers of the WT only doubled after 48 h of incubation as compared to 24 h of incubation. At 72 h, both WT and KO cells were overconfluent due to the restricted growth space, which is why a significant difference between the viability of WT and KO cells within the control group at this timepoint was not apparent. In all the following studies, 48 h was selected as the incubation time for the comparison of WT and KO cells under different conditions.

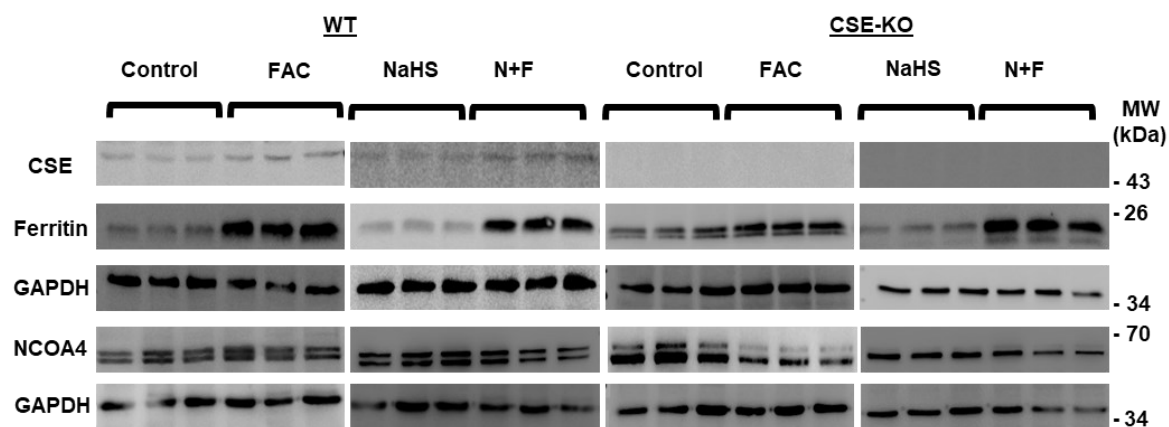
BrdU incorporation assays showed that FAC treatment at all concentrations significantly suppressed the proliferation of KO cells. Incubation with 0.01 mM FAC caused no significant change in WT proliferation, while 0.1 mM FAC induced a less severe decline compared to KO cells (Figure 1e). Next, exogenous H₂S was introduced to KO cells using NaHS, an H₂S salt, to determine whether H₂S mitigates the excessive iron accumulation and restores cell viability and proliferation in FAC-treated KO cells. Pretreating KO cells with 0.1 mM NaHS, followed by adding 0.1 mM FAC, improved cell viability and proliferation as compared to the FAC-alone group (Figure 1f,g). NaHS treatment effectively reduced FAC-induced increase in cellular iron levels in both WT and KO cells (Figure 1h).

2.4.2. H₂S Promotes Ferritin Upregulation

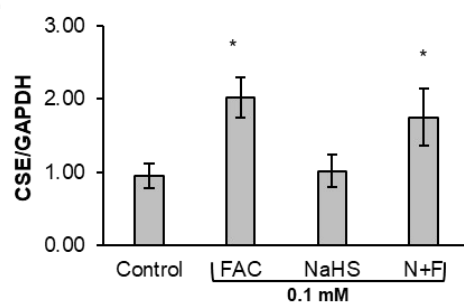
CSE expression at both the protein and mRNA levels was increased in WT cells treated with FAC alone and the N+F group as compared to the control and NaHS-alone groups (Figure 2a–d). KO cells had no band for the CSE proteins with Western blotting, confirming their KO status (Figure 2a). Ferritin protein levels were increased in both WT and KO cells for the FAC-alone and N+F groups as compared to the control and NaHS groups (Figure 2a,e). Western blot results show that FAC-

treated WT cells exhibited 2.8-fold higher Ferritin protein contents than FAC-treated KO cells (Figure 2e). Similarly, the presence of H₂S affects Ferritin levels, as can be observed in Figure 2e. Adding 0.1 mM FAC with or without NaHS significantly increased H₂S production in WT cells. Furthermore, no H₂S production was detected in KO cells with all treatments aforementioned using the lead acetate paper method. This confirms that H₂S production under the control condition and with FAC treatment was catalyzed by CSE.

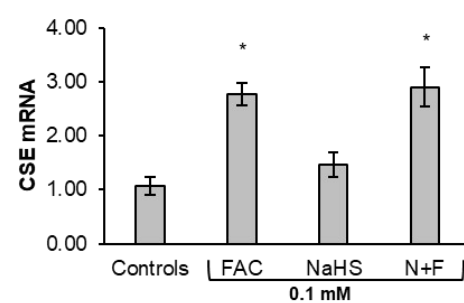
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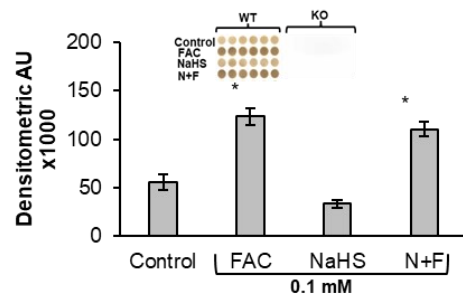
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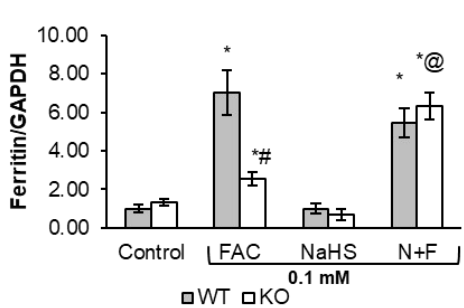
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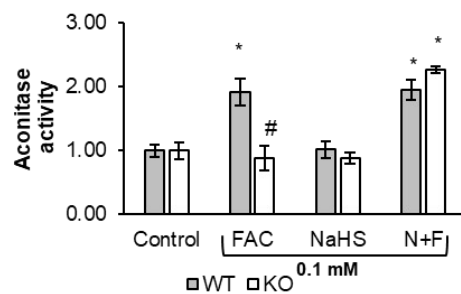
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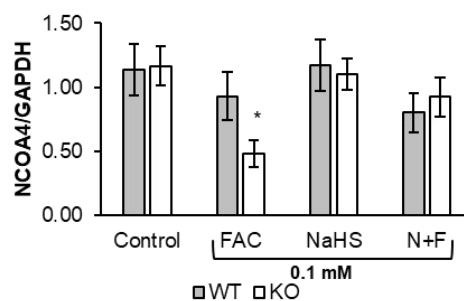


Figure 2. The effects of FAC on the CSE-H₂S pathway and Ferritin expression. (a) Representative Western blots of CSE, Ferritin, and NCOA4 in WT and CSE-KO VSMCs. (b) Relative expression levels of CSE proteins in WT cells with different treatments. (c) Relative CSE mRNA levels in WT VSMCs with different treatments. (d) H₂S production in WT and CSE-KO VSMCs with different treatments. (e) Relative expression levels of Ferritin in WT and CSE-KO cells. (f) Aconitase activities in different groups of VSMCs. (g) Relative expression levels of NCOA4 proteins in different groups of VSMCs. The treatment time for all groups was 48 h. * $p < 0.05$ vs. untreated control group with the same genotype, # $p < 0.05$ vs. WT group with the same treatment, @ $p < 0.05$ vs. 0.1 mM FAC with the same genotype; $n = 3$, with 4 experimental repeats. N+F, 0.1 mM NaHS pretreatment + 0.1 mM FAC.

Since Ferritin has antioxidant properties and sequesters free iron [25], it is possible that the increase in CSE expression and H₂S level may increase iron sequestration via directly or indirectly increasing Ferritin expression. There was a significant, greater than six-fold, increase in Ferritin expression in the FAC-alone and N+F groups of WT cells compared to the control (Figure 2e). In contrast, KO cells showed a meager 2-fold increase in Ferritin expression with FAC treatment alone but a 6-fold increase in the N+F group, where the lack of endogenous H₂S was compensated by exogenous NaHS.

Intracellular iron is primarily regulated via iron regulatory proteins 1 and 2 (IRP1 and IRP2) [26]. C- aconitase is another form of IRP1. The difference in aconitase activity between WT and KO VSMCs is summarized in Figure 2f. FAC treatment alone increased aconitase activity in WT cells but failed to do so in KO cells. However, both KO and WT cells show increased aconitase activity in the N+F groups.

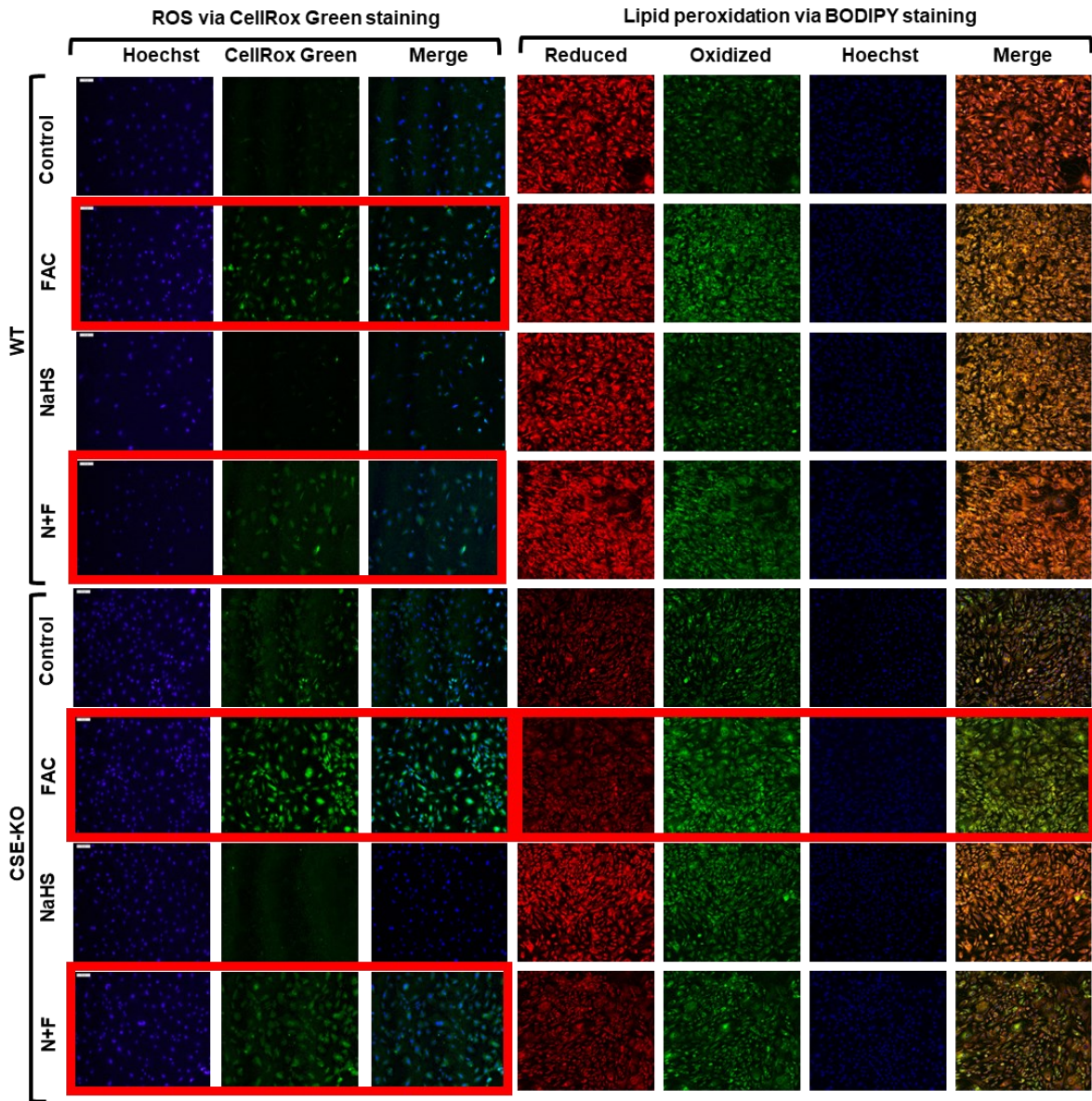
Nuclear Receptor Coactivator 4 (NCOA4) is a selective cargo receptor that mediates ferritinophagy, which is the breakdown of Ferritin via autophagy to release iron in the cell, often leading to iron overload and ferroptosis [27,28]. Figure 2g shows a sharp decrease in NCOA4 in FAC-treated KO cells. This suggests that the lower Ferritin level in KO cells resulted from impaired Ferritin expression rather than breakdown of already expressed Ferritin via ferritinophagy.

2.4.3. H₂S Inhibits Iron-Mediated Oxidative Stress

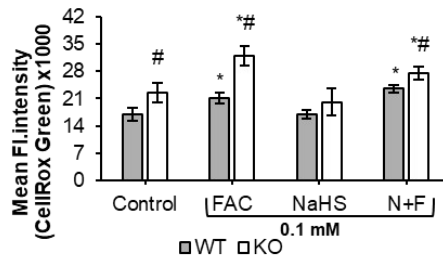
Elevated ROS levels were detected in the FAC and N+F groups of both WT and KO cells (Figure 3a,b). However, only the FAC-treated KO cells, not the WT cells, exhibited significant lipid peroxidation as compared to the controls (Figure 3c). The absence of endogenous H₂S in KO cells

appears to lead to ROS accumulation to an extent that triggers lipid peroxidation. The addition of NaHS lowered lipid peroxidation in FAC-treated KO cells (Figure 3c, N+F group) as compared to the FAC-alone and control groups.

a



b



c

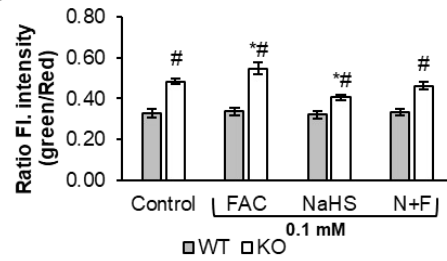


Figure 3. The effects of H₂S on iron-mediated oxidative stress and lipid peroxidation. **(a)** Representative images of oxidative stress measured via CellRox green and lipid peroxidation via BODIPY 581/591 C11 staining in WT and CSE KO cells with different treatments for 48 h. **(b)** Mean fluorescence intensity of CellRox Green normalized to Hoechst. **(c)** Ratio of mean fluorescent intensity in the green (oxidized BODIPY) and red (reduced BODIPY) channels for BODIPY dye. Groups with significant differences are in a red border. **p* < 0.05 vs. control with the same genotype, #*p* < 0.05 vs. WT group with the same treatment. From each 35 mm plate, 3 random fields were chosen, 18 images per group. Scale bar represents 100 µm. Control, PBS treatment; N+F, 0.1 mM NaHS pretreatment + 0.1 mM FAC.

2.4.4. Iron-Treated CSE-KO Cells Undergo Apoptosis

The results from the TUNEL assay (Figure 4a,b) showed increased DNA fragmentation in CSE-KO cells treated with FAC or NaHS only. There was no observable fragmentation in WT cells with any referred treatment. Higher caspase activity was found in KO cells treated with FAC or NaHS alone as compared to controls (Figure 4c). However, CSE-KO cells with the N+F treatment had significantly lower caspase activity than those with the FAC-alone treatment, indicating that exogenous H₂S can lower caspase activity via its interaction with FAC. Moreover, Western blot analysis showed a significant increase in cleaved caspase 3, the active form of caspase, in FAC- and NaHS-treated KO cells (Figure 5a,b). No significant caspase activity was observed in all groups of WT cells, as determined by caspase 3/7 staining. Interestingly, Western blot analysis did not detect cleaved caspase 3 bands in the control, FAC, or NaHS groups. Although faint bands appeared in the N+F group of WT cells, no apoptosis of these cells could be confirmed due to lack of DNA fragmentation and caspase activity. This confirms that CSE-KO cells undergo apoptosis after increased iron overload and oxidative stress as compared to WT cells.

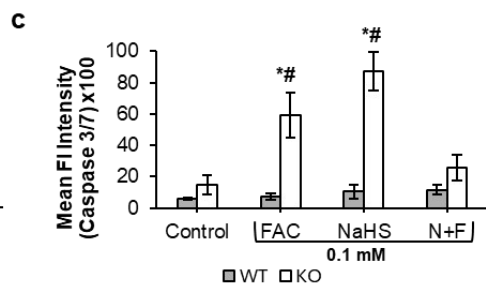
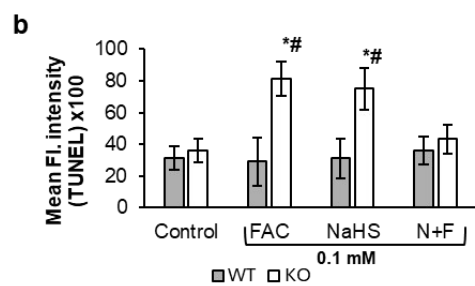
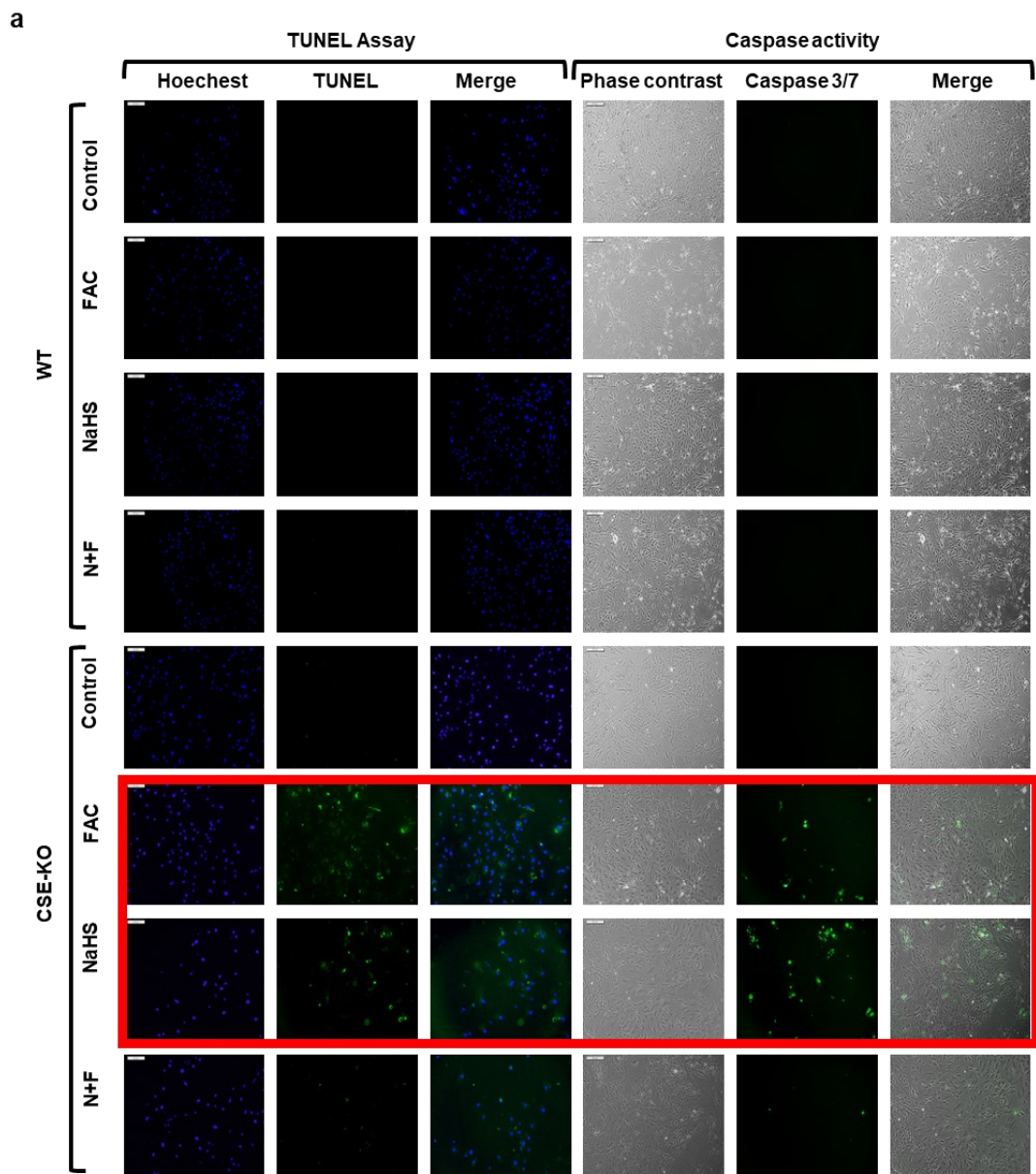


Figure 4. The effects of H₂S on iron-mediated apoptosis. **(a)** Representative images of apoptosis measured via TUNEL and CellEvent caspase 3/7 green staining in WT and CSE KO cells. **(b)** Mean fluorescence intensity of TUNEL-positive cells normalized to Hoechst. **(c)**. Mean fluorescent intensity in the green channels for CellEvent caspase 3/7 dye. Groups with significant differences are in a red border. * $p < 0.05$ vs. control with the same genotype, # $p < 0.05$ vs. WT group with the same treatment. From each 35 mm plate, 3 random fields were chosen, 18 images for each group. Scale bar represents 200 μ m. Control, PBS treatment; N+F, 0.1 mM NaHS pretreatment + 0.1 mM FAC.

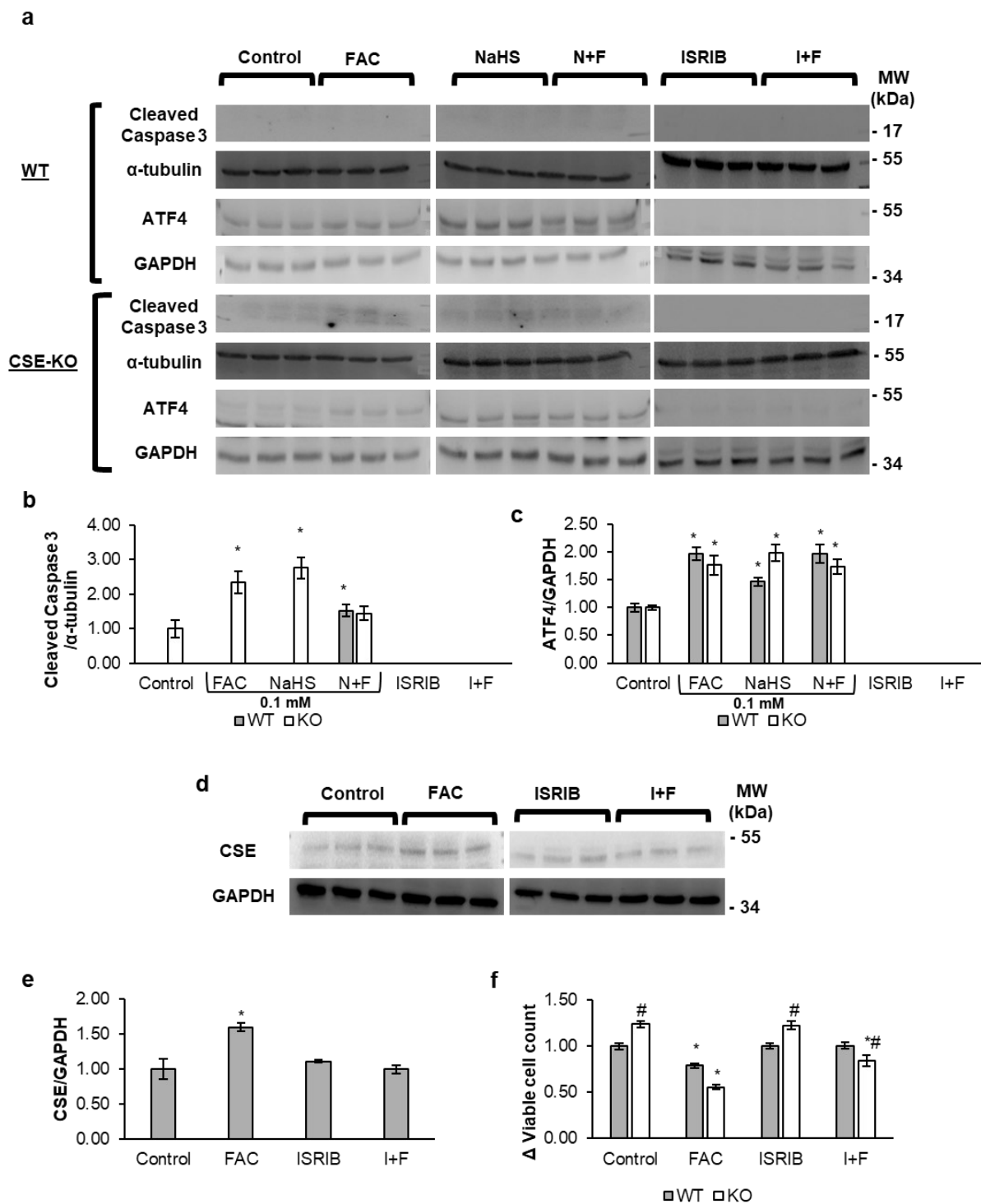


Figure 5. Iron upregulates CSE/H₂S via ATF4, which in turn prevents apoptosis. **(a)** Representative Western blots of cleaved caspase 3, α -tubulin, and ATF4 in WT and CSE-KO VSMCs. **(b)** Relative expression of cleaved caspase 3 proteins. **(c)** Relative expression of ATF4 proteins. **(d)** Representative Western blots of CSE in WT cells. **(e)** Relative expression of CSE proteins in WT cells. **(f)** Viable cell count measured via MTT assay. All treatments were of 48 h duration. * $p < 0.05$ vs. untreated control group, # $p < 0.05$ vs. similar WT group. $n = 3$, with 4 experimental repeats. Control, PBS treatment; N+F, 0.1 mM NaHS pretreatment + 0.1 mM FAC; ISRIB at 0.1 μ M was used; I+F, 0.1 μ M of ISRIB pretreatment + 0.1 mM FAC.

2.4.5. Iron Upregulates CSE Expression via the ROS-eIF2 α -ATF4 Pathway

We further investigated the mechanism whereby iron increases CSE expression. The Western blot data in Figure 5c show that FAC increased ATF4 expression as compared to the controls in both WT and KO cells. ISRIB is a blocker of ATF4 and the integrated stress (ISR) pathway. Figure 5c–e show that the addition of ISRIB inhibited ATF4 expression in the ISRIB-only and I+F groups. ISRIB also blunted the iron-mediated increase in CSE expression in FAC-treated WT cells (I+F). This confirmed that the iron-mediated increase in CSE occurred via ATF4. Interestingly, ISRIB-pretreated KO cells in FAC had no cleaved caspase 3 activation as compared to FAC-treated KO cells (Figure 5b). ISRIB and FAC co-treatment of KO cells showed an increase in cell viability compared to KO cells treated with FAC alone (Figure 5f). Figure 6a,b show an increase in the p-eIF2 α -to-eIF2 α ratio in FAC-treated WT and KO cells. The increase in ROS, expression of ATF4, and phosphorylation of eIF2 α confirm that iron triggers ISR in both WT and KO cells.

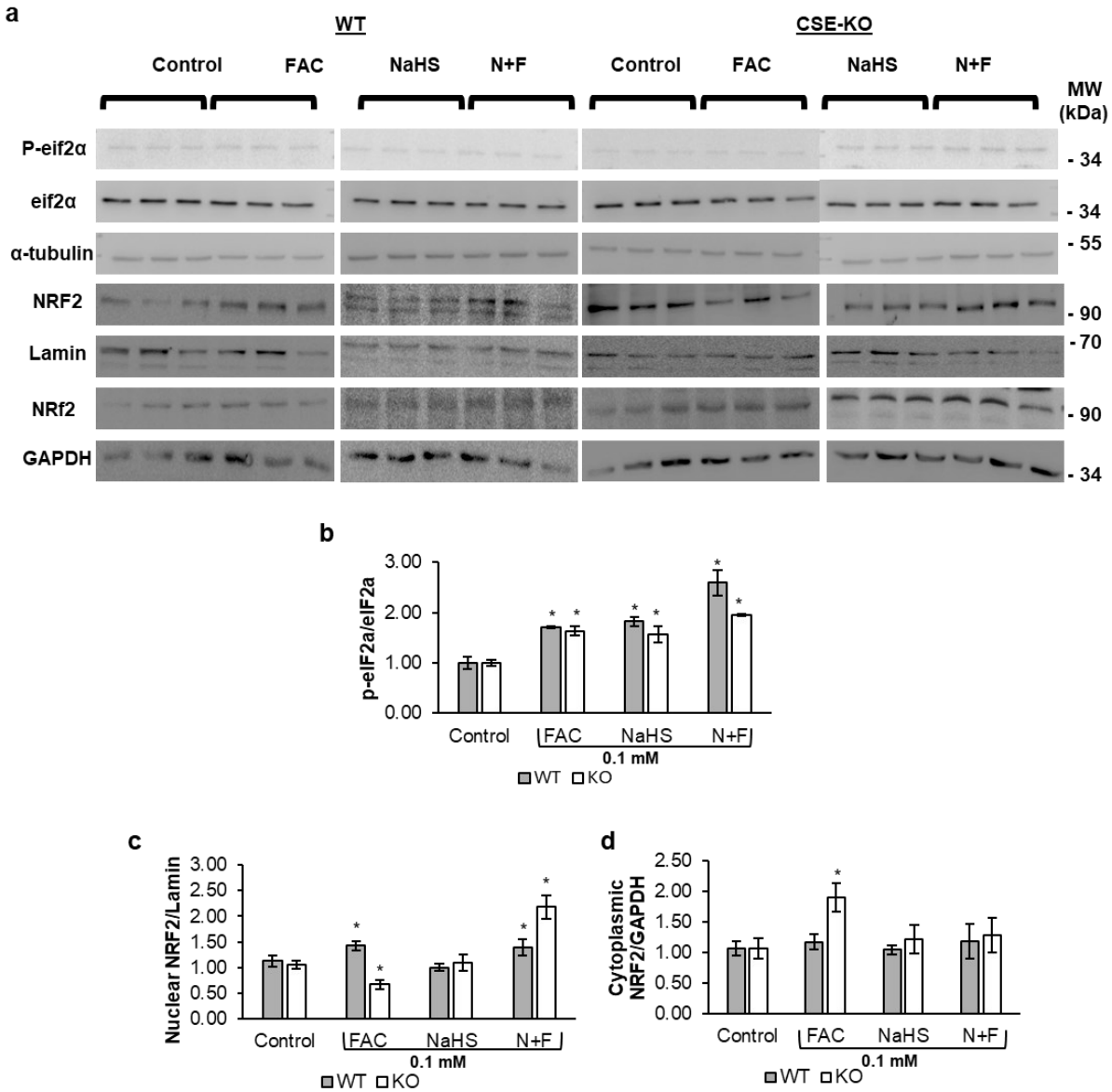


Figure 6. Iron activates the integrated stress response and increases nuclear translocation of NRF2. **(a)** Representative Western blots of NRF2 from nuclear fractions (Lamin) and cytoplasmic fractions (GAPDH) and p-eIF2α and eIF2α in WT and CSE-KO VSMCs. **(b)** Phosphorylation of eIF2α. **(c)** Relative expression of nuclear NRF2. **(d)** Relative expression of cytoplasmic NRF2. * $p < 0.05$ vs. control group with the same genotype. $n = 3$, with 4 experimental repeats. All cells received the treatments for 48 h. Control, PBS treatment; N+F, 0.1 mM NaHS pretreatment + 0.1 mM FAC.

2.4.6. H₂S Increases Nuclear Translocation of NRF2 in Response to ISR

NRF2 expression was elevated in FAC-treated WT and KO cells (Figure 6a–d), indicating that NRF2 is upregulated in iron-treated VSMCs irrespective to the presence of CSE. In WT cells, this increase was observed in nuclear fractions for both the FAC and N+F groups. In KO cells, however, NRF2 upregulation in the FAC group was confined to the cytoplasmic fractions, whereas in the N+F group, NRF2 upregulation was evident in the nuclear fractions. These results suggest that exogenous H₂S facilitates NRF2 nuclear translocation in FAC-treated KO cells.

2.4.7. H₂S Protects Against Iron-Mediated Oxidative Stress via Upregulating AMPK-Mediated Autophagy

Western blot results showed increased AMPK phosphorylation in FAC-treated WT cells but not in FAC-treated KO cells (Figure 7a,b). However, both WT and KO cells with N+F treatments exhibited increased phosphorylation of AMPK. This shows that endogenous and exogenous H₂S can lead to an increase in p-AMPK in iron-treated VSMCs. Further, the increased LC3B expression (Figure 7c) and increased autophagosome activity (Figure 7d,e) also indicated that autophagy is significantly higher in FAC-treated WT cells and NaHS-pretreated WT and KO cells. FAC-treated KO cells did not show significant LC3B expression (Figure 7c), nor any measurable autophagosome activity (Figure 7d,e). This shows that endogenous or exogenous H₂S also upregulates autophagy as a response to iron-catalyzed ISR via ROS.

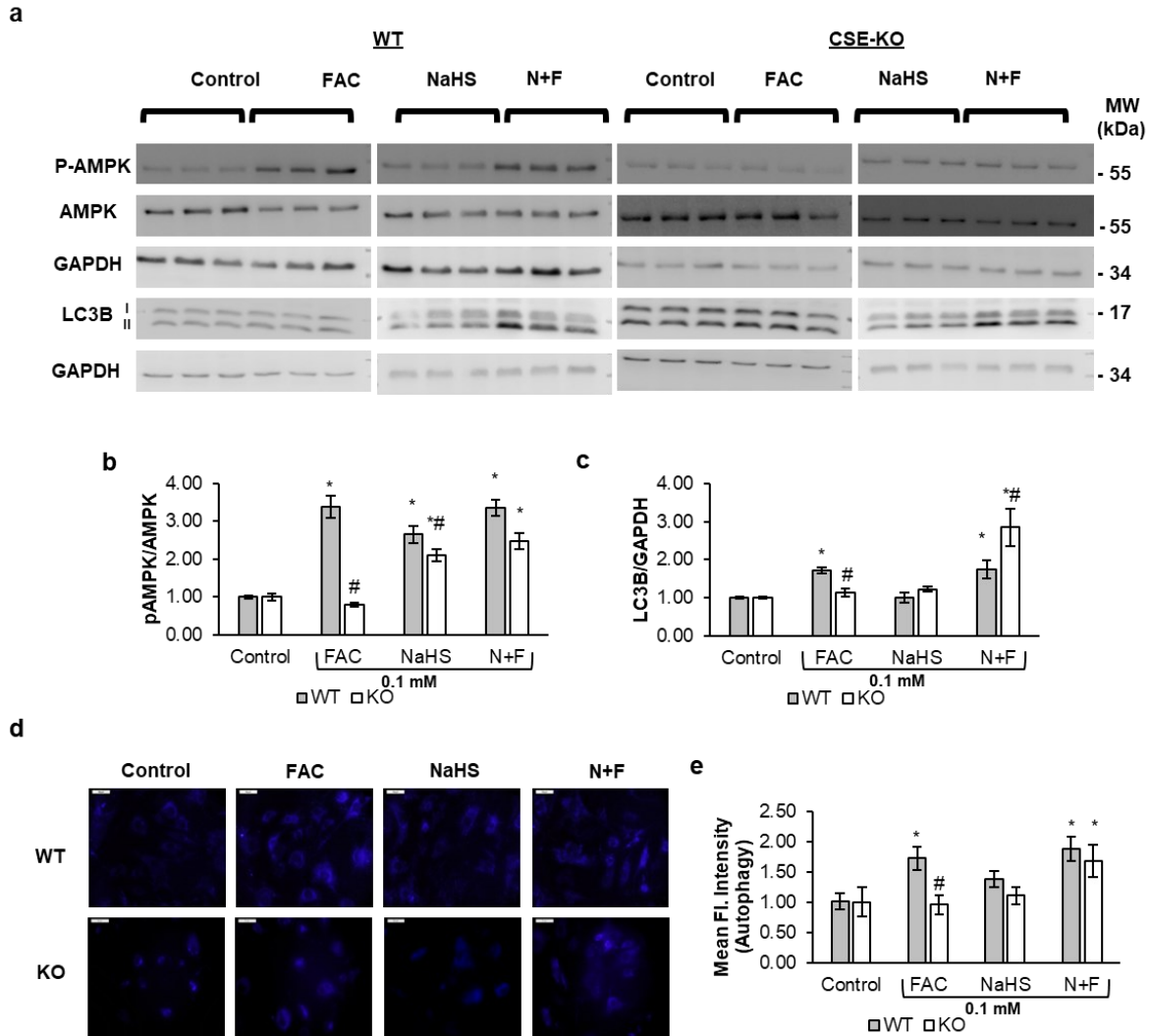


Figure 7. The interaction effect of iron and CSE/H₂S on VSMC autophagy. **(a)** Representative Western blots of p-AMPK and LC3B in WT and CSE-KO VSMCs. **(b)** Relative levels of p-AMPK proteins. **(c)** Relative expression levels of LC3B proteins. **(d)** Representative images of autophagosome staining in treatment groups. **(e)** Mean fluorescent intensity representing autophagosome activity. The treatment duration for all groups of cells was 48 h. Scale bar represents 50 μ m. Control, PBS treatment; N+F, 0.1 mM NaHS pretreatment + 0.1 mM FAC. * $p < 0.05$ vs. control. # $p < 0.05$ vs. WT group with the same treatment. $n = 3$, with 4 experimental repeats.

2.5. Discussion

The interplay between iron accumulation, oxidative stress, and H₂S metabolism constitutes a complex regulatory network critical for iron homeostasis and cell survival. Some previous studies examined the relationship between iron and H₂S by using toxic levels of extracellular iron to induce iron overload [10,29]. Rather than using inorganic sources of iron like FeCl₂, FeCl₃, or other inorganic iron salts [10,30,31], the current study used FAC because 0.1 mM FAC contains 17 μM of iron and 100 μM of citrate, which quantities are in the physiological ranges of iron (17–23 μM) and citrate (100–130 μM) in mouse plasma [2,20]. FAC closely mimics the physiological entry of iron into cells, where iron is weakly complexed with citrate [2,32,33]. In biological systems, non-transferrin-bound iron (NTBI), often termed “labile iron,” exists in loose complexes with molecules like citrate or ascorbate [2,34,35]. Under normal conditions, blood iron is either tightly bound to transferrin or loosely associated with citrate, with no detectable free ferric (Fe³⁺) or ferrous (Fe²⁺) ions [2]. During systemic iron overload, citrate-bound iron gains significance as the primary form facilitating cellular uptake, while inorganic iron forms remain physiologically irrelevant due to their absence in biological environments. Our findings revealed that FAC at physiologically or pathophysiologically relevant concentrations significantly reduced the viability and proliferation of both WT and CSE-KO cells, with a more pronounced effect observed in KO cells compared to WT counterparts. Pretreatment with 0.1 mM NaHS lowered cellular iron levels and rescued the FAC-induced decline in viability and proliferation in KO cells.

FAC-treated WT cells exhibited significantly lower intracellular iron concentrations compared to CSE-KO cells, a difference driven by H₂S-mediated upregulation of ferroportin and Ferritin. While divalent metal transporter 1 (DMT1) facilitates iron import, ferroportin exports excess iron [2].

Building on prior findings by Zhu et al., H₂S specifically upregulates ferroportin in iron-treated WT cells without altering DMT1 expression in either WT or KO cells [10]. This ferroportin induction enhances iron export, thereby reducing intracellular iron levels in WT cells [10]. Concurrently, endogenous H₂S elevates Ferritin expression in WT cells, effectively sequestering labile iron and further diminishing intracellular iron concentrations, as demonstrated in this study. Together, these mechanisms underscore H₂S's dual role in mitigating iron overload through coordinated export and sequestration.

Our study further shows that FAC upregulated CSE expression and activity. Some previous studies did not observe increased CSE expression in iron-overload VSMCs or cardiac cells when they used 0.1 mM of FeCl₂ for 24 h to induce iron overload [10,11]. FeCl₂ at this concentration yields 0.1 mM ferrous iron, which is 5.88 times higher than the 17 µM concentration of ferric iron in 0.1 mM FAC. Iron concentrations and bioavailability significantly differ between the inorganic iron chlorides and FAC, with the latter showing higher bioavailability [32,33]. Additionally, the 24 h incubation period used in previous studies may not have been long enough to induce a detectable increase in CSE proteins, whereas in our study, CSE upregulation was observed after 48 h of FAC treatment.

The mechanism by which iron triggers the upregulation of CSE was investigated in the current study. It has been reported that four transcription factors can increase CSE expression: SP1, NRF2, ELK1, and ATF4 [36]. Iron overload has been shown to downregulate SP1, making SP1 an unlikely candidate for increasing CSE [37,38]. Our previous study showed that nuclear translocation of NRF2 occurs in WT cells but not in KO cells under oxidative stress [39], indicating the stimulatory role of endogenous H₂S for NRF2 nuclear translocation. ELK1 activity in oxidative stress has been shown to occur downstream of NRF2 [40]. Thus, an increase in endogenous H₂S could be a pre-

requirement for ELK1 upregulation and NRF2 nuclear translocation. We have previously shown ATF4-increased CSE expression in response to Golgi stress in murine myoblast (C2C12) cells [41]. Moreover, ATF4 has been shown in different studies to be directly involved in ROS-mediated oxidative stress [42,43,44,45,46]. Sarcinelli et al. showed that ER stress leads to ROS-mediated oxidative stress, which upregulates NRF2 as a protective mechanism via upregulating PKR-like endoplasmic reticulum kinase, which phosphorylates eIF2 α , leading to upregulation of ATF4 [46]. Zhang et al. showed that ER stress-related oxidative stress increased autophagy in mouse spermatocyte-derived cells via phosphorylation of eIF2 α and upregulation of ATF4 [44]. The ER stress response has three arms, ATF4, inositol-requiring enzyme 1, and ATF6. Given that ATF4 is the primary regulator of ROS-related ER stress [47] and a transcription factor for CSE [36], our focus was directed toward ATF4 rather than the other pathways. The interaction of the CSE/H₂S system with inositol-requiring enzyme 1 and ATF6 has been unknown. Our current study shows that ATF4 was upregulated in FAC-treated WT and KO cells via the ROS-mediated ISR. Pretreatment of WT VSMCs with the ATF4 inhibitor, ISRIB, abolished FAC-induced upregulation of CSE, pinpointing ATF4 as the pivotal transcription factor responsible for iron-induced CSE upregulation.

Ferritin stores iron in its redox-inactive form [48]. FAC increased Ferritin levels in both WT and KO cells. However, the increased Ferritin level in FAC-treated KO cells (2 folds) was significantly lower than in FAC-treated WT cells (6 folds), indicating that the stimulatory effect of iron on Ferritin expression is facilitated by endogenous H₂S. Pretreating KO cells with NaHS followed with FAC increased Ferritin levels to a degree comparable to that in FAC-treated WT cells. Zhu et al. also found that iron (Fe²⁺)-treated CSE-KO cells had significantly lower Ferritin upregulation compared

to iron-treated WT cells, but adding NaHS significantly improved Ferritin levels in iron-treated KO cells [10]. They further reported that in the presence of H₂S, IRP1, which is bound to *ferritin* mRNA, is readily converted to cytosolic aconitase. This releases the *ferritin* mRNA, ready to be translated. Our results also showed the same phenomenon. Significant aconitase activity and Ferritin expression were observed in the FAC-treated WT and N+F groups of WT and KO cells, but not in FAC-treated KO cells.

The weaker Ferritin upregulation in KO cells compared to WT cells may also reflect impaired iron buffering via NCOA4-mediated ferritinophagy. Contrary to expectations, NCOA4 expression decreased in FAC-treated KO cells, suppressing ferritinophagy. As shown by Zhao et al., mitochondrial aconitase generates 3Fe-4S clusters under iron-replete conditions, which bind NCOA4 to trigger its degradation—a process distinct from cytosolic aconitase/IRP1 signaling [48,49,50]. In KO cells, elevated ROS likely activated mitochondrial aconitase to produce 3Fe-4S clusters, promoting NCOA4 degradation despite low Ferritin levels and iron overload. This suggests that NCOA4 downregulation serves as an adaptive response to excessive labile iron, further exacerbating dysregulation.

In parallel, ferroptosis—an iron-dependent, caspase-independent cell death pathway marked by lipid peroxidation [9,51,52,53,54]—emerged as a secondary mechanism in KO cells. At physiological iron levels (0.1 mM FAC), KO cells exhibited caspase-3 activation and TUNEL-positive apoptosis, aligning with studies linking iron dysregulation to mitochondrial lipid peroxidation [55,56]. The absence of CSE/H₂S disrupted Ferritin-mediated iron sequestration, enabling gradual oxidative damage and apoptosis (Figure 1). While ferroptosis typically requires acute iron overload, partial viability rescue in KO cells despite caspase inhibition (ISRIB; Figure 5f)

implied residual ferroptosis. At 1 mM FAC, both WT and KO cells showed rapid, caspase-independent death consistent with ferroptosis, mirroring Wang et al.'s findings in myoblasts [9]. Thus, H₂S deficiency shifts iron-induced death from apoptosis (low iron) to ferroptosis (high iron), underscoring H₂S's role in maintaining iron homeostasis and suppressing oxidative cell death.

Oxidative stress is a common result of iron overload [2]. Hydrogen peroxide generated in mitochondria or cytoplasm can react with the excess free iron in labile iron pools of cells to produce ROS in toxic amounts via the Fenton reaction [2]. The excess ROS lead to oxidative stress and widespread cell damage. Our previous studies have shown that KO cells have higher basal ROS levels than WT cells due to the absence of CSE and high homocysteine levels in KO cells [8]. Our current study reaffirmed the higher ROS basal level in KO cells compared to WT cells (Figure 3a,b). Moreover, higher ROS levels were observed in the FAC and N+F groups as compared to controls, in both WT and KO cells. Interestingly, NaHS lowered basal ROS in KO cells, which confirmed our previous finding [57]. When the accumulation of ROS overwhelms the natural antioxidant capacity of the cell, oxidative stress occurs [58], leading to detrimental chain reactions, such as lipid peroxidation. While ROS production increased in both FAC-treated WT and KO cells, only the FAC-treated KO cells produced sufficient ROS to induce lipid peroxidation. It was also noticed that pretreating FAC-treated KO cells with NaHS significantly reduced lipid peroxidation compared to untreated FAC-treated KO cells. This suggests that, in the absence of endogenous H₂S, iron-treated VSMCs experience a pronounced increase in oxidative stress, which can be effectively mitigated by the addition of exogenous H₂S. Further, only FAC-treated KO cells exhibited DNA fragmentation detected with the TUNEL assay and caspase3/7 activity, which could be reversed by pretreating the KO cells with exogenous H₂S. The effect of 0.1 mM NaHS on apoptosis has been reported [14].

Our previous studies showed that 0.1 mM NaHS decreases the viability and proliferation of KO cells but not WT cells [19]. KO cells are devoid of endogenous H₂S and are more sensitive to apoptotic triggers as compared to WT cells, which require a much higher H₂S concentration (0.5 mM) to induce significant apoptosis [14]. It is interesting to note that KO cells with N+F treatment exhibited a significant decrease in DNA fragmentation as compared to FAC-treated KO cells. This shows that exogenous H₂S offers protection against iron-overload-mediated DNA fragmentation.

H₂S mitigates iron-overload damage in FAC-treated WT cells by promoting the nuclear translocation of NRF2, a master regulator of antioxidant gene expression [39,59]. Mechanistically, H₂S directly modifies Kelch-like ECH-associated protein 1 (KEAP1)—an NRF2 inhibitor—via S-sulfhydration, destabilizing the KEAP1-NRF2 complex and enabling NRF2 to translocate into the nucleus, as demonstrated in our prior work [39]. This activation of NRF2 drives the transcription of antioxidant genes, counteracting oxidative stress. In the current study, FAC-treated WT cells exhibited robust NRF2 nuclear accumulation, whereas KO cells showed cytoplasmic retention of NRF2, correlating with their heightened oxidative damage. Exogenous H₂S supplementation rescued this deficit in KO cells, restoring NRF2 nuclear localization. This aligns with findings by Kasai et al. [60], who linked NRF2 activation to the ROS-p-eIF2 α -ATF4 pathway during integrated stress responses. Thus, H₂S orchestrates a dual protective mechanism: direct KEAP1 S-sulfhydration to liberate NRF2 and indirect amplification of antioxidant defenses via NRF2-driven gene expression. This pathway not only buffers iron-induced oxidative stress but also highlights H₂S's central role in maintaining redox homeostasis.

H₂S critically modulates the balance between autophagy and apoptosis in vascular smooth muscle cells. In FAC-treated WT cells, H₂S enhances AMPK phosphorylation, driving autophagy via

mTORC1/ULK1/PIK3C3 pathways [61,62,63,64], while suppressing apoptosis through reduced cleaved caspase-3. Conversely, CSE-KO cells under FAC exhibit AMPK inactivation, failed autophagy, and elevated apoptosis—phenotypes rescued by exogenous H₂S. This aligns with studies linking AMPK suppression to apoptosis in stressed VSMCs [13,65,66]. The interplay extends to iron–H₂S dynamics: iron overload upregulates CSE, boosting H₂S to sequester labile iron into Ferritin, thereby mitigating oxidative damage. In CSE-KO cells, excess iron triggers ATF4-mediated ISR, activating caspases and apoptosis [67,68]. WT cells, however, leverage H₂S to activate AMPK, counterbalancing ATF4's pro-apoptotic effects. ISRIB inhibition of ATF4 in KO cells prevents caspase activation, underscoring ATF4's dual role as both an apoptotic driver and CSE upregulator. Thus, H₂S orchestrates survival by coupling iron detoxification (via Ferritin) to AMPK-driven autophagy, while its absence shifts the balance toward ATF4-caspase apoptosis. These findings position H₂S as a molecular fulcrum, harmonizing iron metabolism and stress responses to prioritize cell survival. A detailed overview of the proposed mechanism is presented in the graphical abstract, summarizing the key interactions explored in this study.

H₂S-mediated autophagy may play an important role in cellular homeostasis beyond apoptosis. For instance, in senescence, H₂S can mitigate oxidative stress and promote cellular longevity, potentially delaying aging-related dysfunction. Additionally, its influence on autophagy may affect cell proliferation by modulating metabolic pathways and nutrient availability, impacting tissue regeneration and repair. These broader effects highlight the need for further investigation into H₂S's role in complex cellular processes.

We acknowledge that our study utilized isolated VSMCs to examine the direct interaction between free iron and H₂S within the cell. This interaction is likely more complex in vivo, where hormones,

signaling molecules, transferrin-bound iron, and hepcidin may influence the process. However, our findings provide a foundational basis for future research exploring the interaction between iron and H₂S in more intricate in vivo environments.

Our findings hold key implications for diseases like type 2 diabetes, where low blood H₂S levels [69] and iron overload [70] coexist. H₂S-based interventions, such as H₂S donors; dietary restriction [71] to boost endogenous H₂S production; and the application of metformin, which elevates tissue H₂S [72,73] and lowers iron via AMPK [72], may mitigate iron-caused pathological damage. Metformin's iron-lowering effects may hinge on H₂S, as our study identifies CSE as being central to AMPK activation under iron stress. Low H₂S levels also correlate with cerebrovascular diseases (e.g., Alzheimer's), where iron exacerbates progression [74]; restoring H₂S could slow such decline. In hypertension, iron overload's vascular risks may amplify without CSE/H₂S [2]. This synergy suggests that patients with low levels of endogenous H₂S face heightened iron-related damage, warranting strategies to elevate H₂S (e.g., dietary/metformin therapies). Our work underscores the need to clinically explore H₂S–iron interplay for therapeutic breakthroughs.

2.6. Conclusions and Perspectives

Reduced endogenous H₂S production in VSMCs disrupts iron homeostasis, causing iron overload at physiological or pathological extracellular iron levels via impaired ferroportin-mediated export and diminished Ferritin sequestration. This disruption tilts the apoptosis–autophagy balance toward apoptosis. In contrast, H₂S safeguards VSMCs by enhancing iron export, upregulating Ferritin to buffer excess iron, activating NRF2 antioxidant pathways, and promoting AMPK-dependent autophagy. Collectively, these actions inhibit apoptosis and reduce vascular injury,

underscoring H₂S's pivotal role in iron regulation. These insights highlight H₂S-boosting therapies as promising strategies for iron-related vascular pathologies.

2.7. Author Contributions

H.M.A.: conceptualization, methodology, data analysis, writing—original draft. M.F.: methodology, data analysis. R.W.: data analysis, writing, funding acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

2.8. Funding

This study was supported by a Discovery grant to RW (RGPIN-2023-05099) from the Natural Science and Engineering Research Council of Canada.

2.9. Institutional Review Board Statement

The animal study protocol was approved by The Animal Care Committee, York University (2019-11 R1).

2.10. Informed Consent Statement

Not applicable.

2.11. Data Availability Statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

2.12. Conflicts of Interest

The authors declare no conflicts of interest.

2.13. References

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Chapter 3: Hydrogen Sulfide Mitigates Iron-induced Mitochondrial Dysfunction in Vascular Smooth Muscle Cells

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This chapter has been previously published as:

Arif, H. M., Fu, M., & Wang, R. (2025). Hydrogen Sulfide Mitigates Iron-induced Mitochondrial Dysfunction in Vascular Smooth Muscle Cells. *Biochemical and Biophysical Research Communications*, 152279.

Contributions: H.M.A. conceived the study, developed the methodology, performed data analysis, and wrote the original draft. M.F. contributed to methodology and assisted with data analysis. R.W. provided supervisory input, guidance on data analysis and revision, and support with project administration and funding. H.M.A. carried out all experiments and writing at each stage.

The sections and headings in this chapter are structured to align with the formatting guidelines of Biochemical and Biophysical Research Communications journal.

3.1. Abstract

Mitochondrial dysfunction is a key contributor to cellular stress, impaired metabolism, and disease progression. Oxidative stress and iron overload further exacerbate mitochondrial impairment. While wild-type (WT) vascular smooth muscle cells (VSMCs) maintain redox balance under iron exposure, cystathionine γ -lyase knockout (CSE-KO) cells have impaired iron-regulating mechanisms, making them more susceptible to iron overload and oxidative stress. This study investigates how iron exposure affects mitochondrial respiration and whether hydrogen sulfide (H_2S) can mitigate these effects. Seahorse XF96 analysis revealed that iron-treated CSE-KO cells exhibited impaired mitochondrial respiration, reduced maximal respiratory capacity, and diminished spare respiratory capacity. In contrast, WT cells maintained a higher spare respiratory capacity, suggesting a protective role of endogenous H_2S . JC-1 staining confirmed that CSE-KO cells undergo mitochondrial dysfunction upon iron exposure with disrupted membrane potential. However, H_2S treatment of CSE-KO cells restored mitochondrial function and prevented membrane depolarization. These findings enhance our understanding of the iron- H_2S interaction in mitochondrial regulation, which can be leveraged for therapeutic strategies to enhance mitochondrial resilience and mitigate iron-induced cellular damage.

Highlights

1. Iron exposure induces mitochondrial dysfunction in the absence of endogenous H_2S in VSMCs
2. Exogenous H_2S restores mitochondrial respiration and membrane potential in iron stressed VSMCs with diminished endogenous H_2S

3. H₂S offers protection of iron-induced mitochondrial dysfunction.

Keywords: H₂S, Iron overload, Mitochondrial dysfunction, Metabolic stress, Vascular smooth muscle cells

3.2. Introduction

Mitochondria are essential for cellular energy production and homeostasis, playing a crucial role in regulating cell survival and stress responses [1]. Their dysfunction is closely linked to oxidative stress, metabolic imbalances, and disease progression. Iron, a vital element for numerous biological processes, becomes detrimental when its homeostasis is disrupted, contributing to oxidative damage and mitochondrial impairment [2]. Hydrogen sulfide (H₂S) has emerged as a key regulator in cellular protection, influencing redox balance and iron metabolism [2–4].

Our previous findings [4] demonstrated that wild-type (WT) vascular smooth muscle cells (VSMCs) maintain redox balance under iron exposure. In contrast, cystathionine γ -lyase knockout (CSE-KO) cells, with impaired iron-regulating mechanisms, are more susceptible to iron overload and oxidative stress. Given the critical role of mitochondria in cellular energy metabolism and stress adaptation, understanding how iron exposure affects mitochondrial function in the presence or absence of endogenous H₂S is essential.

This study builds on these observations by examining the effects of iron overload on mitochondrial activity and assessing the potential of H₂S to mitigate iron-induced dysfunction. By investigating

this interaction, we aim to clarify the role of H₂S in mitochondrial regulation, which could provide valuable insights for therapeutic strategies targeting iron-related metabolic disorders.

3.3. Materials and Methods

3.3.1. Animals and Protocols

All experimental protocols were reviewed and approved by the Animal Care Committee at York University. This study used wild-type mice with a C57BL/6J x129SvEv background and in-house-generated homozygous CSE-KO mice with the same genetic background. The homozygous CSE-KO mice were obtained through at least 10 generations of backcrossing WT mice with heterozygous CSE-KO mice, as previously described [5]. All mice had unrestricted access to water and standard rodent chow ad libitum and were housed in HVAC-ventilated cages under a 12:12-hour light/dark cycle.

3.3.2. Aortic VSMCs culture

Mouse aortic VSMCs were isolated following previously described protocols [4]. Aorta from 8-week-old male mice were excised, kept in ice-cold Krebs's buffer, and continuously gassed with 95% oxygen and 5% CO₂. Endothelial cells were removed using a thin wire, and the surrounding adipose tissue was carefully dissected. The medial layer, isolated after enzymatic digestion with collagenase type II in serum-free Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, Oakville, ON, Canada), was further incubated to release VSMCs. After centrifugation (350 × g for 10 min), VSMCs were plated in DMEM containing 10% Fetal Bovine Serum (FBS) and cultured until

confluency (5–7 days), with medium changes every 48 h. VSMC identity was confirmed by α -smooth muscle actin expression and the absence of endothelial marker PECAM. Cells were passaged at 80% confluency, and passages 3–7 were used for experiments. Prior to treatments, cells were synchronized in serum-free medium for 24 h, followed by exposure to 10% FBS under different conditions: Phosphate Buffered Saline (PBS) (control), 0.1 mM ferric ammonium citrate (FAC), 0.1 mM NaHS, or 0.1 mM NaHS pretreatment for 4 h followed by 0.1 mM FAC exposure (N+F).

3.3.3. *Mitochondrial Respiration Assays*

Assays were conducted using the Seahorse XF96 Cell Mito Stress Kit (Agilent Technologies, Mississauga, ON, Canada). Briefly, VSMCs were seeded at a density of 10,000 cells per well in a 96-well Seahorse microculture plate and incubated overnight. In the following day, treatments were conducted and lasted for 48 hours as described above. After treatment, cells were washed with XF assay media containing 10 mM glucose and 2 mM glutamine and incubated in a CO₂-free environment at 37°C for 1 hour. Oligomycin (1.5 μ M), carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) (1.0 μ M), and rotenone + antimycin A (0.5 μ M each) were sequentially added, and oxygen consumption rate (OCR) measurements were recorded using a calibrated Seahorse XF96 Extracellular Flux Analyzer. Basal respiration was measured before the addition of any inhibitors. ATP-linked respiration was determined from the decrease in OCR following oligomycin injection, while proton leak was assessed from the residual OCR after ATP synthase inhibition. Maximal respiration was measured after the addition of FCCP, which uncouples oxidative phosphorylation, and spare respiratory capacity was calculated by subtracting basal respiration from the FCCP-stimulated maximal respiration. Finally, non-mitochondrial respiration

was determined after the addition of rotenone and antimycin A, which inhibit Complex I and III, respectively. Following OCR analysis, wells were washed twice with DMEM and incubated with Hoechst 33342 stain (Invitrogen, Burlington, ON, Canada) for 30 minutes. Fluorescence was measured using a Biotek Synergy HTX multimode plate reader (Agilent Technologies Mississauga, ON, Canada) and OCR values were normalized using Hoechst. Data were analyzed using XF Wave software (Agilent Technologies Mississauga, ON, Canada).

3.3.4. Mitochondrial membrane potential

Following the manufacturer's guidelines, the mitochondrial membrane potential was assessed using the JC-1 Mitochondrial Membrane Potential Assay Kit (Abcam, Cambridge, MA, USA). Cells were cultured in 35 mm plates at a density of 100,000 cells per plate. After treatment, JC-1 was diluted to a working concentration of 5 μ M and incubated for 10 minutes. Imaging was performed using an Olympus IX71 fluorescence microscope (Olympus America Inc., Center Valley, PA, USA), with the TRITC (red) channel detecting JC-1 aggregates and the FITC (green) channel detecting JC-1 monomers. Fluorescence intensity ratios of red to green channels were quantified using ImageJ software (version 1.54h, NIH, Bethesda, MD, USA) [6].

3.3.5. Statistical Analysis and Reproducibility

All data were expressed as means $\pm$ standard errors (SEMs). A *p*-value less than 0.05 was considered statistically significant. Unpaired two-tailed Student's *t*-tests were employed for comparisons between two groups, as appropriate. For analyses involving three or more groups, either one-way or two-way analysis of variance (ANOVA) was conducted, followed by Tukey's multiple-comparison post hoc test.

3.4. Results

3.4.1. *Iron induces mitochondrial stress in CSE-deficient VSMCs*

In WT cells, mitochondrial respiration largely remained unchanged with different treatments. However, KO cells treated with FAC exhibited a significant decline in overall mitochondrial respiration parameters. KO cells pretreated with NaHS before FAC exposure did not show this decline (Figure 1a).

KO VSMCs in the control group exhibited a higher basal respiration rate than their WT counterparts (Figure 1b). While basal respiration remained similar across WT treatment groups, FAC-treated KO cells significantly reduced basal respiration. In contrast, KO cells treated with NaHS alone had higher basal respiration, and those pretreated with NaHS before FAC exposure also exhibited elevated basal respiration compared to both FAC-treated and control KO cells.

FAC treatment increased maximal respiration in WT cells relative to controls (Figure 1c). However, WT cells pretreated with NaHS prior to FAC exposure exhibited lower maximal respiration than WT controls. In KO cells, FAC treatment led to a significant decline in maximal respiration. In contrast, NaHS treatment—both alone and as a pretreatment before FAC exposure—resulted in higher maximal respiration compared to KO controls. A similar trend was observed in spare respiratory capacity, where FAC treatment enhanced spare capacity in WT cells but led to a significant decline in KO cells (Figure 1d). No difference in spare capacity was observed between control and NaHS-only treatment groups. However, NaHS pretreatment with FAC increased spare respiratory capacity in KO cells but reduced it in WT cells compared to their respective controls.

ATP production remained unchanged in WT cells treated with FAC or NaHS alone, but WT cells pretreated with NaHS before FAC exhibited lower ATP production than WT controls (Figure 1e). FAC-treated KO cells showed reduced ATP production, whereas both NaHS-only and NaHS-pretreated KO cells displayed higher ATP production than WT cells. Similarly, an increased proton leak was also only observed in FAC-treated KO cells as compared to the control group; however, pretreatment with NaHS prior to FAC restored proton leak to control levels in KO VSMCs.

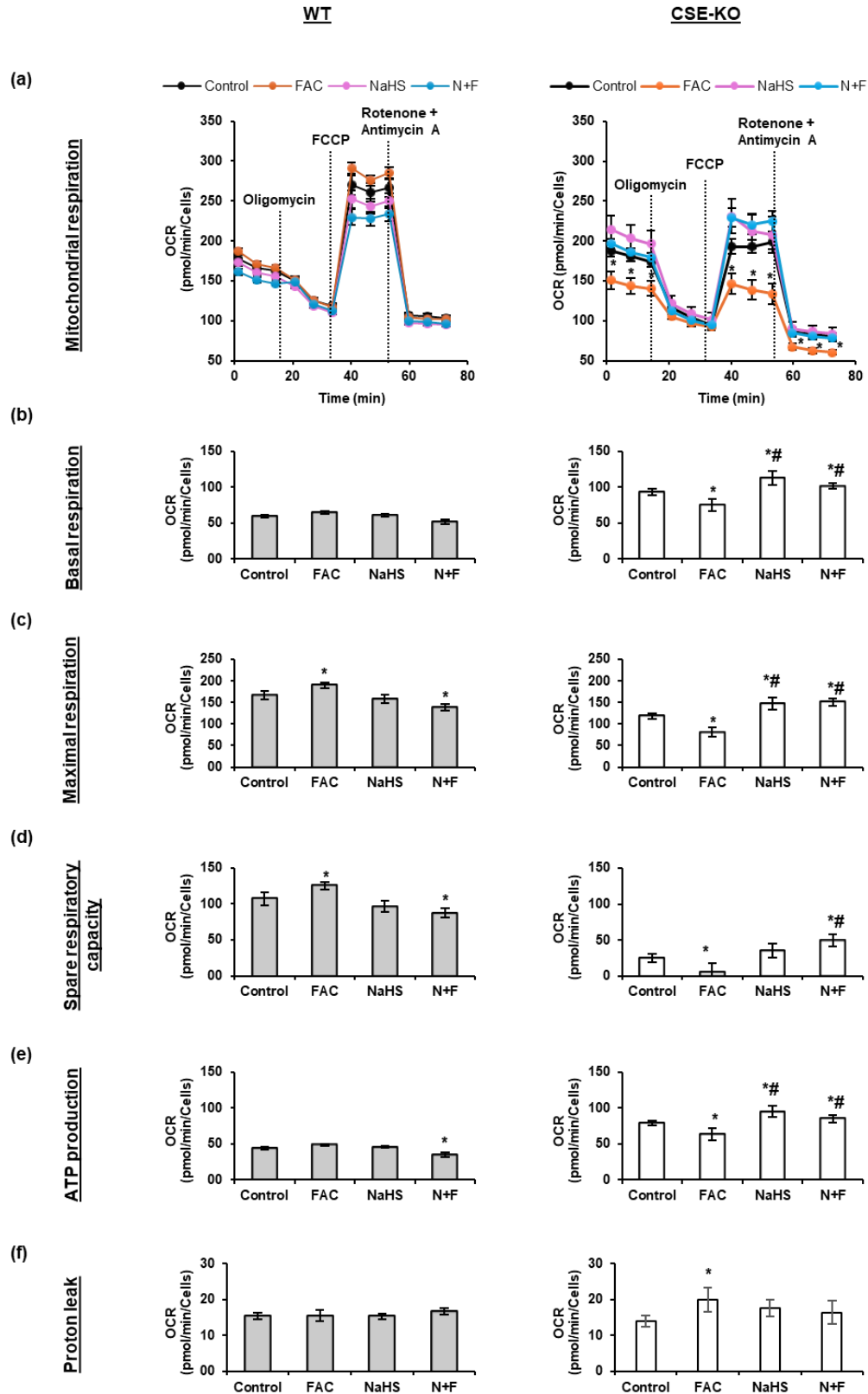


Figure 1. Mitochondrial Respiration and Stress Assessment Using Seahorse XF96 Cell Mito Stress Kit. (a) Mitochondrial respiration, (b) basal respiration, (c) maximal respiration, (d) Spare respiratory capacity, (e) ATP production, and (f) Proton leak between treatment groups of WT and CSE-KO. * $p < 0.05$ vs Control. $n = 6$ with 3 experimental repeats.

3.4.2. Iron-mediated mitochondrial membrane depolarization is higher in CSE-deficient cells

There was no significant change in mitochondrial membrane potential in FAC- or NaHS-treated VSMCs compared to WT control cells (Figure 2 a&b), assessed with JC-1 staining. However, NaHS pretreatment prior to FAC exposure resulted in lower mitochondrial membrane potential, indicating increased mitochondrial membrane depolarization.

In KO cells, FAC treatment led to the highest degree of membrane depolarization, as evidenced by the lowest aggregate-to-monomer ratio (Figure 2c). NaHS treatment alone also induced significant membrane depolarization. However, NaHS pretreatment before FAC exposure rescued KO VSMCs from the membrane depolarization observed in the FAC-only treatment group. Notably, the NaHS pretreatment with FAC group in KO cells exhibited a mitochondrial membrane potential similar to that of KO control cells.

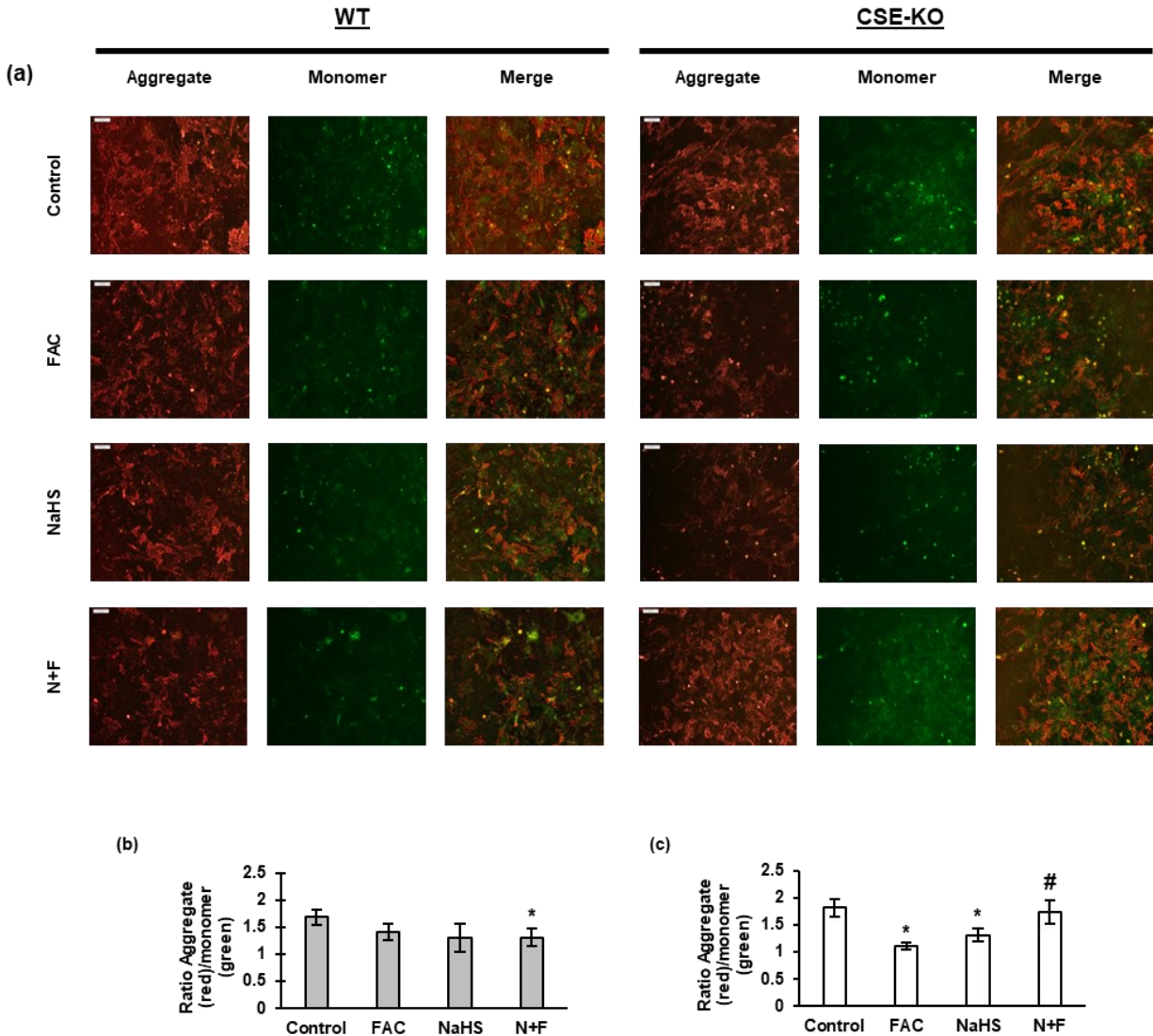


Figure 2. Mitochondrial membrane potential in WT and KO VSMCs measured via JC-1 Staining. (a) Representative images showing mitochondrial membrane potential across different WT and KO treatment groups. (b) Summary of the changes in the ratio of fluorescence intensity of the red channel (Aggregate) to the green channel (Monomer) in WT cells. (c) Summary of the changes in the ratio of fluorescence intensity of the red channel (Aggregate) to the green channel (Monomer) in KO cells. * $p < 0.05$ vs Control; # $p < 0.05$ vs 0.1 mM FAC group. From each 35 mm plate, 3 random fields were chosen, 18 images for each group. Scale bar represents 100 μm . Control, PBS treatment; N+F, 0.1 mM NaHS pretreatment + 0.1 mM FAC. Scale bar represents 100 μm .

3.5. Discussion

The interaction of iron and H₂S regulates the redox status of VSMCs and modulates autophagy and apoptosis of these cells [4]. Mitochondria play an important role in cell energy production, and its health status can be an indicator of metabolic efficiency, and susceptibility to diseases related to oxidative stress and aging [1].

Mitochondria generate reactive oxygen species (ROS) as part of metabolic processes, whereas iron overload-induced Fenton reaction [2] can be curbed through either regulating iron levels or increasing the antioxidant capacity of the cells [7]. Previous studies have shown that H₂S plays a central role in regulating labile iron levels via promoting the conversion of iron to redox inactive ferritin form and exporting excess iron out of the cells via ferroportin [3]. Our recent study confirmed this mechanism, demonstrating that H₂S reduces iron-mediated ROS and oxidative stress by enhancing ferritin formation [4]. While ROS levels increased in both FAC treated WT and CSE-KO cells, only the CSE-KO cells displayed sustained ROS levels that triggered lipid peroxidation [4]. NaHS pretreatment prior to FAC exposure greatly improved the cellular outcome by reducing ROS levels and mitigating lipid peroxidation in CSE-KO cells [4].

In the current study we explored mitochondrial status in VSMCs when exposed to 0.1 mM FAC with and without the influence of H₂S. In the absence of CSE generated endogenous H₂S and without application of exogenous H₂S, 0.1 mM FAC treated CSE-KO cells display a significant decrease in mitochondrial respiration as compared to control. This decrease alone signifies mitochondrial impairment or a shift in cellular metabolism. NaHS pretreatment of the CSE-KO cells followed by FAC restores the mitochondrial respiration to normal levels.

Further, it was found that basal respiratory levels of CSE-KO cells (93.2 ± 4.3 pmol/min/cells) were higher than those of WT cells (59.2 ± 1.8 pmol/min/cells) as shown in Figure 1b. This observation aligns with our several previous studies that show higher proliferative rates of CSE-KO cells as compared to WT cells [4,8,9]. H₂S, whether exogenously supplied or endogenously produced, limits the over proliferation of VSMCs, which in turn do not require the elevated metabolic activity observed in proliferating CSE-KO cells.

FAC treatment did not affect basal respiration in WT cells as compared to WT control group, whereas FAC treatment significantly decreased basal respiration in CSE-KO cells as compared to KO control group. This is another evidence of mitochondrial dysfunction in the FAC treated KO cells. Iron treatment for 48 hours significantly damages mitochondria in KO cells which is why they have lower basal respiration (75.0 ± 8.5 pmol/min/cells) than control (93.2 ± 4.3 pmol/min/cells) and other treatment groups.

FAC treatment significantly increased the maximal respiration and spare respiratory capacity in WT cells as compared to control groups, whereas FAC treatment decreased maximal respiration and spare respiratory capacity for CSE-KO cells. FCCP increases mitochondrial respiration by uncoupling oxidative phosphorylation, specifically disrupting the proton gradient between the mitochondrial matrix and the intermembrane space, which forces the electron transport chain to consume more oxygen in an attempt to restore the membrane potential [10]. The difference between FCCP-induced maximal respiration and basal respiration defines the spare respiratory capacity, which reflects how much additional stress mitochondria can handle. Interestingly, the spare respiratory capacity for CSE-KO cells became virtually non-existent. This shows the extent

of damage and mitochondrial dysfunction FAC treated CSE-KO cells experience as compared to FAC treated WT cells.

The ability of FAC treated WT cells to preserve spare respiratory capacity suggests a higher resilience to oxidative damage induced by iron, allowing them to meet increased energy demands when needed. In contrast, the loss of spare respiratory capacity in FAC treated CSE-KO cells highlights their impaired mitochondrial function and reduced adaptability to stress conditions, making them more vulnerable to metabolic dysfunction.

Proton leak occurs when protons re-enter the mitochondrial matrix without passing through ATP synthase, resulting in oxygen consumption that doesn't produce ATP [11]. It reflects mitochondrial membrane inefficiency and is estimated in Seahorse assays as the residual respiration after oligomycin treatment [12]. The decrease of ATP production and membrane potential, with an increase in proton leak in FAC treated CSE-KO cells as compared to the non-treated KO cells, are strong indicators of iron mediated mitochondrial dysfunction (Figure 1d,e and Figure 2a,c) in CSE-KO cells. Similar results were reported by Gordan et al. which show that iron overload depolarizes mitochondrial membranes in mouse ventricular myocytes and contributes towards mitochondrial dysfunction [13].

NaHS pretreatment prior to FAC exposure increased maximal respiration, spare respiratory capacity, ATP production, and proton leak, while also restoring the mitochondrial membrane potential to control levels in CSE-KO cells. These findings suggest that exogenous H₂S can partially rescue mitochondrial function in CSE-deficient cells, highlighting its potential therapeutic value in mitigating oxidative damage and preserving metabolic resilience under conditions of iron overload.

An interesting thing to note is that the spare respiratory capacity of control CSE-KO cells (24.9 ± 5.5 pmol/min/cells) is significantly lower than control WT cells (107.2 ± 9.3 pmol/min/cells). This shows that CSE-KO cells are near their limit to take additional metabolic stress, due to the previously mentioned reason that CSE-KO cells have a higher proliferation rate. This also explains why CSE-KO cells are more sensitive to oxidative stress, as observed in our previous studies [14,15]. While NaHS alone and NaHS pretreatment prior to FAC increases the spare respiratory capacity of CSE-KO cells, it does not increase it to WT levels. This may be attributed to the transient nature of NaHS, as its volatility reduces bioavailability over time, potentially diminishing efficacy within 48 hours [16]. A more stable and compound that can slowly release H₂S may offer better protection. This also underscores the importance of checking the status of endogenous H₂S when targeting diseases correlated with iron overload with simultaneously decrease in H₂S levels, like Alzheimer's disease [17,18] or type 2 diabetes [19,20]. In such cases, increasing H₂S via slow releasing H₂S donors [16] or therapeutic interventions that increase H₂S like dietary restriction [21] or using metformin [22] should be considered to counter the iron overload complication.

How does H₂S protect cells from iron mediated mitochondrial dysfunction? We previously found that iron upregulates CSE/H₂S pathway via the integrated stress response (ROS-p-eIF2 α -ATF4) [4]. The upregulated CSE increases autophagy via the AMPK pathway, clearing damaged mitochondria. FAC-treated KO cells did not show autophagy, but increased apoptosis via caspase 3 that is likely due to mitochondrial dysfunction noted in this study. NaHS pretreatment prior to FAC exposure restored autophagic activity of KO cells, suggesting a protective role for H₂S in maintaining mitochondrial quality. Furthermore, H₂S regulates cellular iron levels by enhancing ferritin and exporting excess iron, thereby eliminating the root cause of the mitochondrial dysfunction.

3.6. Conclusions and Perspectives

Our findings underscore the critical role of H₂S in mitigating iron-induced mitochondrial dysfunction in VSMCs. This discovery positions H₂S not merely as an antioxidant, but as a key regulator of mitochondrial resilience, affecting iron sequestration, mitochondrial dynamics, and the maintenance of mitochondrial health and integrity. Importantly, we show that CSE-KO cells, even at baseline, operate near their metabolic ceiling, making them uniquely vulnerable to iron-induced damage—a vulnerability rescued by H₂S.

Future investigations should explore how H₂S modulates mitochondrial iron trafficking and whether it influences the assembly or stability of electron transport chain complexes. Additionally, it will be important to determine whether H₂S directly impacts mitophagy signaling or supports mitochondrial biogenesis in stressed cells. Expanding this work into in vivo models could clarify the therapeutic potential of sustained H₂S delivery in iron overload–related vascular and metabolic diseases.

3.7. Author Contributions:

H.M.A.: conceptualization, methodology, data analysis, writing original draft. M.F.: methodology, data analysis. R.W.: data analysis, writing, funding acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

3.8. Funding:

This study was supported by a Discovery grant to RW (RGPIN-2023-05099) from the Natural Science and Engineering Research Council of Canada.

3.9. Institutional Review Board Statement:

The animal study protocol was approved by The Animal Care Committee, York University (2019-11 R1)

3.10. Data Availability Statement:

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

3.11. Conflicts of Interest:

The authors declare no conflicts of interest.

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Chapter 4: A HPLC-based Methylene Blue/Methylene Green Method for Accurate Measurement of Hydrogen Sulfide in Plasma

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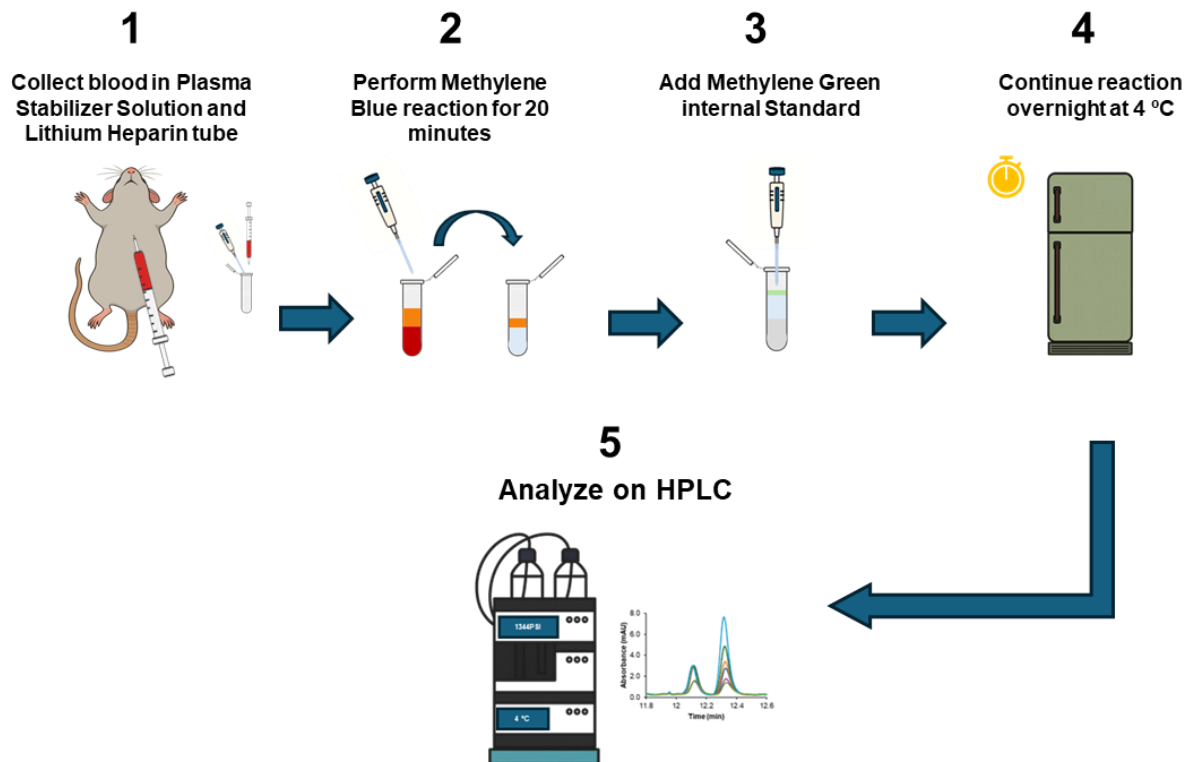
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This chapter has been published as:

Arif, H. M., Fu, M., Amanpour, T., & Wang, R. (2025). A HPLC-based Methylene Blue/Methylene Green Method for Accurate Measurement of Hydrogen Sulfide in Plasma. *Biochemical and Biophysical Research Communications*, 152557.

Contributions: H.M.A. conceived the study, developed the methodology, performed data analysis, and wrote the original draft. M.F. contributed to methodology and assisted with data analysis. T.A. assisted with methodology. R.W. provided supervisory input, guidance on data analysis and revision, and support with project administration and funding. H.M.A. carried out all experiments and writing at each stage.

The sections and headings in this chapter are structured to align with the formatting guidelines of Biochemical and Biophysical Research Communications journal.



Graphical Abstract

4.1. Abstract

Hydrogen sulfide (H_2S) is an endogenously produced gasotransmitter with important physiological roles in cardiovascular, neurological, and metabolic health. Accurate quantification of circulating H_2S remains technically challenging due to its volatility, reactivity, and rapid oxidation. Here, we present a high-performance liquid chromatography (HPLC)-based methylene blue method using methylene green as an internal reference (MBMG) for plasma H_2S quantification with improved sensitivity, reproducibility, and compatibility. Using this approach, a linear standard curve was generated for NaHS concentrations between 0.05–10 μM . The method successfully detected significantly higher plasma H_2S levels in wild-type (WT) mice ($\sim 0.5 \mu\text{M}$) compared to CSE-knockout (KO) mice ($\sim 0.15 \mu\text{M}$), with both groups showing expected increases following NaHS intraperitoneal injection. Specificity testing revealed minimal interference from biologically relevant sulfur species; only cysteine at supraphysiological concentrations generated a negligible signal. Plasma cysteine levels remained unchanged following NaHS injection. Post-reaction samples remained stable for at least six months at -80°C , facilitating remote analysis and long-term storage. Collectively, the MBMG method provides a practical and sensitive platform for reliable plasma H_2S measurement, with broad applicability in basic, translational, and clinical research settings.

Keywords: H_2S detection, methylene blue, HPLC, mouse plasma, cysteine

4.2. Introduction

Hydrogen sulfide (H_2S) is a gasotransmitter with well-documented physiological roles in the nervous, cardiovascular, and metabolic systems [1]. Dysregulated H_2S levels have been implicated in numerous pathologies, including Alzheimer's disease, hypertension, and diabetes [1,2].

Given its diverse functions and clinical relevance, the ability to accurately and sensitively quantify circulating H_2S is essential. Several methods are currently used to measure H_2S , including the methylene blue (MB) spectrophotometry assay [3], monobromobimane (MBB) derivatization [4], sulfide-sensitive electrodes [5], and fluorescent probes. Among the latter, the P3 probe [6], a phosphine-based fluorogenic sensor, and 7-Azido-4-methylcoumarin (AzMC) [7], a reduction-sensitive coumarin derivative, are commonly employed in live-cell or tissue-based imaging applications. Additionally, the lead acetate strip method [8] has been used to detect gaseous H_2S released from biological samples, based on the formation of black lead sulfide. However, each approach has significant limitations and often produces inconsistent or non-comparable results or not suitable for measure plasma H_2S level.

The HPLC-based detection of the sulfide–MBB adduct is highly sensitive but requires specialized equipment, such as nitrogen-purged chambers, and strict anaerobic handling, making it technically demanding. Sulfide-selective electrodes, while offering real-time measurements, are costly, prone to drift and fouling, and require frequent calibration [9]. Fluorescent probes, though useful for imaging intracellular H_2S dynamics, often lack sufficient specificity and are not ideal for quantitative plasma analysis [10].

The MB spectrophotometry assay remains one of the more accessible and cost-effective approaches for H₂S detection. However, it is frequently criticized for producing variable results and low sensitivity. Reported plasma H₂S levels using this method vary widely between laboratories, with some studies reporting concentrations as high as 40 μM [11], while another group detected levels closer to 8 μM [12].

In this study, we present a HPLC-based MB method using methylene green (MG) as an internal reference (MBMG), tailored for measuring H₂S in plasma. Our goal was to develop a method that is simple to implement, does not require specialized equipment, and improves both sensitivity and reproducibility—ultimately contributing to a standardized approach for H₂S quantification in biological samples.

4.3. Material and methods

4.3.1. Animals and Protocol

All experimental procedures were reviewed and approved by the Animal Care Committee at York University. The study utilized wild-type mice on a C57BL/6J × 129SvEv background, along with in-house-bred homozygous cystathionine gamma-lyase knockout (CSE-KO) mice maintained on the same genetic background. Homozygous CSE-KO mice were generated through at least 10 generations of backcrossing heterozygous CSE-KO mice with wild-type mice, as previously described [13]. All animals were housed in HVAC-ventilated cages under a 12:12-hour light/dark cycle, with unrestricted access to water and standard rodent chow.

Eight-week-old wild-type and CSE-KO male mice were randomly assigned to experimental groups. Control animals received an intraperitoneal injection of 100 μ L phosphate-buffered saline (PBS), while treatment groups were administered sodium hydrosulfide (NaHS) (Sigma Aldrich, Oakville, Canada) at a dose of 39 μ mol/kg body weight—an effective dose to prevent atherosclerosis in CSE-KO mice, as described in our earlier study [14]. Two hours after injection, mice were euthanized, and blood was collected via cardiac puncture for subsequent hydrogen sulfide measurement.

4.3.2. Plasma Processing and H₂S Measurement

Blood collected via cardiac puncture was immediately transferred into lithium heparin-coated Microtainer® tubes (BD, Mississauga, Canada) preloaded with a plasma stabilizer solution (PSS) to preserve endogenous H₂S levels. The PSS was prepared by mixing equal volumes of 11 mM diethylenetriaminepentaacetic acid (DTPA) (Sigma Aldrich, Oakville, Canada) and 11 mM ascorbic acid (Sigma Aldrich, Oakville, Canada), yielding a final concentration of 5.5 mM of each component. To maintain a final in-tube concentration of 0.5 mM DTPA and ascorbic acid, PSS was added to the Microtainer® at 100 μ L/ml of the collected blood volume. The stabilizer maintains a reducing environment and chelates iron, thereby minimizing oxidative loss of H₂S. This approach was based on the effectiveness DTPA in preserving H₂S by chelating trace metals [11], and the known ability of ascorbic acid to maintain a reducing environment and prevent H₂S oxidation by reactive oxygen species [15]. Blood was carefully added on top of the PSS without mixing and the tube was sealed immediately to minimize air exposure. To ensure accurate measurement of endogenous H₂S, all blood samples were processed within 15 minutes of collection.

Samples were centrifuged at $845 \times g$ for 3 minutes to separate plasma. A 100 μL aliquot of plasma was then mixed with 300 μL of trapping solution composed of 1% zinc acetate (Sigma Aldrich, Oakville, Canada) and 0.05 M Na_2EDTA , pH 12.8 (Sigma Aldrich, Oakville, Canada) in a 1.5 mL Eppendorf tube. To this mixture, 100 μL of 0.02 M *N,N*-dimethyl-*p*-phenylenediamine (DPD) (Sigma Aldrich, Oakville, Canada) prepared in 6 N hydrochloric acid (HCL) (Fisher Scientific, Mississauga, Canada) and 25 μL of 30 mM ferric chloride (FeCl_3) (Acros Organics, Mississauga, Canada) were added. The acidic environment precipitated plasma proteins, while DPD and FeCl_3 reacted with H_2S to form methylene blue. The reaction mixture was protected from light and incubated for 20 minutes to ensure complete precipitation, after which 40 μL of 10 mM Basic Green 5 (methylene green, MG) (Santa Cruz Biotechnology, Mississauga, Canada) was added to each sample as an internal reference due to its close structural similarity to methylene blue [16], while being distinctly separated by HPLC, allowing simultaneous and accurate detection at a single wavelength. Following this, 200 μL of chloroform (Fisher Chemicals, Mississauga, Canada) was added, and the tube was gently inverted to mix. Samples were then centrifuged at $21,000 \times g$ for 5 minutes to separate the phases. Proteins formed a pellet at the interface, while lipids partitioned into the organic (chloroform) layer. The upper aqueous layer, now free of proteins and lipids, was carefully transferred (500 μL) to an HPLC vial (Thermo Fisher Scientific, Mississauga, Canada) and allowed to react overnight (16 hours) to enhance signal. H_2S concentration calibrated using NaHS at different concentrations against MG/MB ratio in HPLC analysis. NaHS was freshly prepared in trapping solution and directly measured without the addition of plasma samples but under identical measurement conditions.

4.3.3. Instrumentation and HPLC Conditions

Quantification of H₂S was performed using a Dionex UltiMate 3000 UHPLC system (Thermo Scientific, Mississauga, Canada) equipped with a quaternary pump (LPG-3400SD), temperature-controlled autosampler (WPS-3000TSL), and diode array detector (DAD-3000). Chromatographic separation was achieved using a Thermo Scientific Hypersil GOLD C18 column (250 × 4.6 mm, 5 µm particle size) with a 20 µL injection volume. The mobile phase consisted of HPLC grade acetonitrile (Fisher Scientific, Mississauga, Canada) and 0.1% trifluoroacetic acid (TFA) (Sigma Aldrich, Oakville, Canada) in HPLC-grade water (Alfa Aesar, Mississauga, Canada), delivered at a constant flow rate of 1.0 mL/min over a 24-minute run. The gradient program began with 5% acetonitrile, which was held constant for the first 3 minutes. The acetonitrile concentration was then increased linearly to 50% over the next 6 minutes (3–9 min) and held at 50% from 9 to 15 minutes. This was followed by a return to 5% acetonitrile over 3 minutes (15–18 min), and a final re-equilibration at 5% acetonitrile from 18 to 24 minutes.

The column temperature was maintained at 30 °C, and the autosampler was kept at 4 °C. Detection of the analyte methylene blue and the internal standard methylene green was performed at 660 nm using a bandwidth of 2 nm, with a reference wavelength set at 540 nm and a reference bandwidth of 10 nm. In this report, the detected signal refers to the ratio of methylene blue to methylene green peak areas.

4.3.4. Detection of Interference from Sulfur-Containing Compounds

To evaluate whether other sulfur-containing compounds generate ratiometric signals using the MBMG method, several biologically relevant thiols and related species were tested under identical assay conditions. Cysteine, cystine, reduced glutathione (GSH), and 3-mercaptopyruvate (3-MP) (Santa Cruz Biotechnology, Inc., Mississauga, Canada) were tested at 1 mM, and potassium polysulfide (K_2S_x) (Sigma Aldrich, Oakville, Canada) at 1.36 mg/mL. Except 3-MP, all other compounds were from Sigma Aldrich (Oakville, Canada). Each compound was freshly prepared in trapping solution and directly measured without the addition of plasma samples but under identical measurement conditions.

4.3.5. Detection of Cysteine Levels in Plasma

L-cysteine was quantified as previously described with minor modifications [17]. Briefly, 200 μ L of plasma collected as described above was mixed with 60 μ L of 6% perchloric acid (Sigma Aldrich, Mississauga, Canada) to precipitate proteins. After centrifugation, 200 μ L of the resulting supernatant was combined with equal volumes of glacial acetic acid (Caledon, Georgetown, Canada) and ninhydrin reagent (prepared by dissolving 250 mg of ninhydrin (Sigma Aldrich, Oakville, Canada) in 6 mL of glacial acetic acid and 4 mL of 12 N HCl). The mixture was incubated at 100 °C for 10 minutes, then cooled on ice for 3 minutes and diluted to 1.0 mL with 95% ethanol (Fisher Chemicals, Mississauga, Canada). Absorbance was measured at 560 nm using a Biotek Synergy HTX multimode plate reader (Agilent Technologies Mississauga, ON, Canada). Cysteine concentrations were determined using a standard curve generated from known concentrations of

L-cysteine treated under identical conditions. Reference controls contained all buffer and reaction components except L-cysteine or its precursors.

4.3.6. Hemolysis protocol

Plasma samples from WT mice were divided into low hemolysis index (Low HI) and high hemolysis index (High HI) groups. The hemolysis index (HI) was calculated as the absorbance difference $A_{571} - A_{658}$, expressed in arbitrary units (AU), following the previously described method [18]. A cut-off of 0.25 AU was applied, with samples ≤ 0.25 AU classified as Low HI and those > 0.25 AU as High HI. High HI samples were generated by collecting blood through a 27-gauge, 1 mL syringe to induce shear-related hemolysis [19], whereas Low HI samples were collected using a 25-gauge needle with minimal manipulation.

4.3.7. Statistical Analysis and Reproducibility

All data were expressed as means $\pm$ standard errors (SEMs). A p-value less than 0.05 was considered statistically significant. Unpaired two-tailed Student's t-tests were employed for comparisons between two groups, as appropriate. For analyses involving three or more groups, either one-way or two-way analysis of variance (ANOVA) was conducted, followed by Tukey's multiple-comparison post hoc test. Each data point (n) represents a biologically independent sample, specifically plasma collected from an individual mouse.

4.4. Results

A linear standard calibration curve was established for NaHS concentrations ranging from 0.05 to 10 μM , demonstrating sensitive and reliable quantification of H_2S , by plotting the ratio of methylene blue to methylene green peak areas, with retention times of 12.3 and 12.1 minutes, respectively (figure 1a, b). The limit of quantification (LOQ)—the lowest concentration that could be measured with acceptable precision and accuracy—was determined to be 0.05 μM , and the limit of detection (LOD)—the lowest concentration distinguishable from background noise—was as low as 0.01 μM . Using the MBMG method, WT mice exhibited significantly higher endogenous plasma H_2S levels ($0.50 \pm 0.04 \mu\text{M}$) compared to CSE-KO mice ($0.15 \pm 0.04 \mu\text{M}$). Intraperitoneal injection of NaHS markedly increased plasma H_2S concentrations in both genotypes, reaching $0.98 \pm 0.10 \mu\text{M}$ in WT and $0.65 \pm 0.03 \mu\text{M}$ in KO mice (figure 1c, d). Among tested sulfur-containing compounds, only 1 mM cysteine produced a signal comparable to 1 μM NaHS solution. Cystine and GSH showed detectable but unquantifiable signals below the limit of quantitation, while 3-MP and polysulfide produced no detectable signal, confirming assay specificity for H_2S (figure 1e). Plasma cysteine levels were lower in CSE-KO mice than that in WT; however, NaHS injection did not alter cysteine concentrations in either genotype (figure 1f). Hemolysis classification by HI was consistent with plasma appearance (High HI: red–pink; Low HI: golden–yellow). High HI samples showed elevated H_2S compared with Low HI, but this difference was markedly reduced in the presence of PSS, indicating that PSS mitigates hemolysis-related interference in the MBMG method (figure 1g). Further, no change in H_2S levels was observed in either WT or CSE-KO mice when MBMG-processed plasma samples were stored at -80°C for six months, indicating strong long-term stability of the measurement (figure 1h).

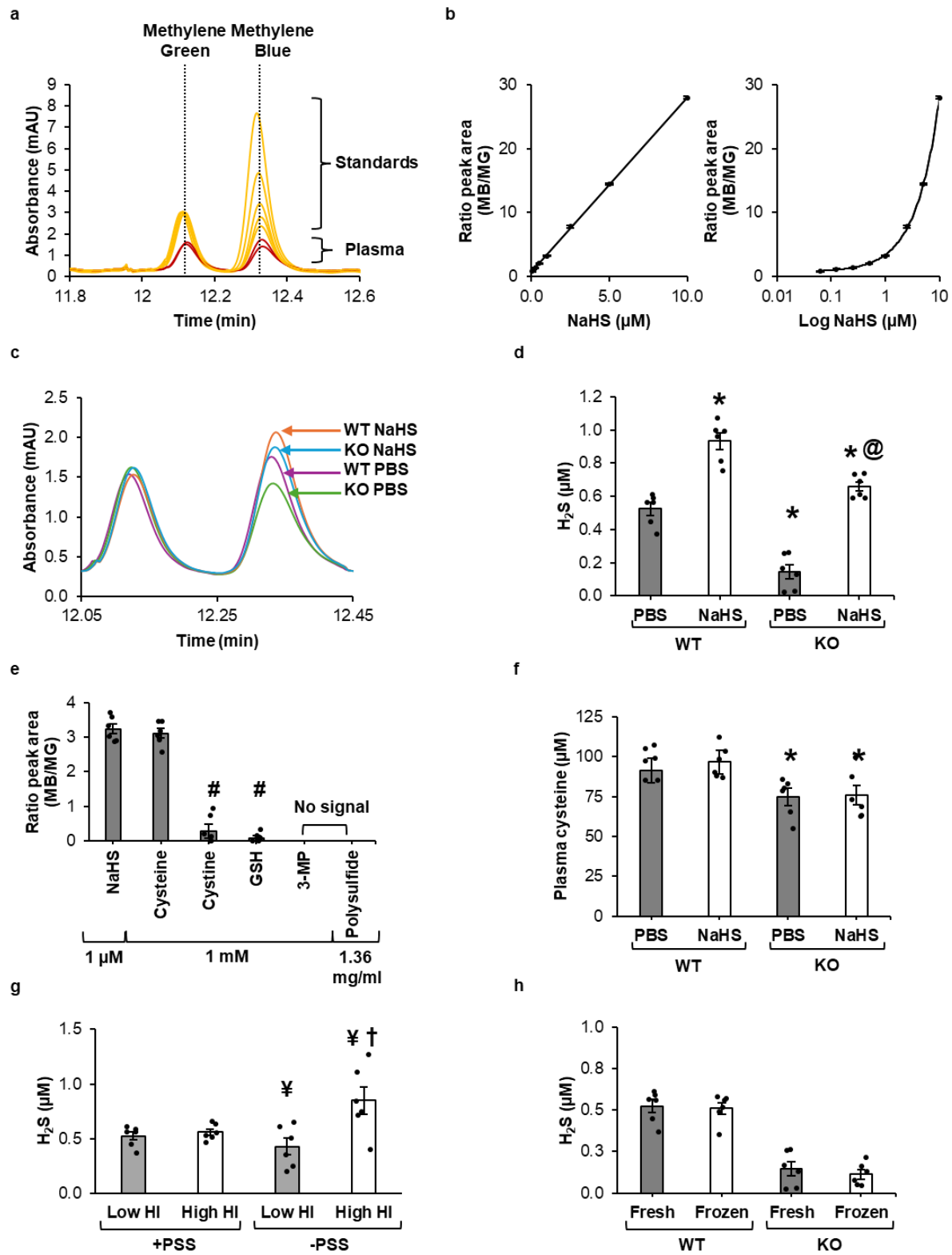


Figure 1. Validation and application of the MBMG method for plasma H₂S quantification. (a) Representative chromatogram showing peaks corresponding to methylene green (12.1 min) and methylene blue (12.3 min) detected at 660 nm with NaHS solutions (standards) at 0.05–1 μ M. The two exemplary plasma samples were from WT (top) and KO (bottom) mice. (b) Standard calibration curves for NaHS from 0.05 to 10 μ M. The linear regression of NaHS calibration is shown on the left. The logarithmic transformation of the NaHS calibration curve is shown on the right to better present the calibration of NaHS concentrations over multiple orders. $R^2 = 0.999$ for both linear regression and its $\log_{10}$ transformation. (c-d) Representative chromatogram and corresponding bar graph showing plasma H₂S levels in WT and CSE-KO mice intraperitoneally injected with PBS or NaHS. (e) Comparison of methylene blue/methylene green peak area ratios between NaHS and various sulfur-containing compounds: cysteine, cystine, GSH, 3-MP, and polysulfide. (f) Plasma cysteine concentrations in WT and KO mice with PBS or NaHS intraperitoneal injection. (g) Effect of hemolysis on plasma H₂S measurements from WT mice with and without PSS. (h) Comparison of freshly prepared plasma samples and that after 6-month storage at -80°C from WT and CSE-KO mice. $n=6$ for each group. * $p<0.05$ vs WT PBS, # $p<0.05$ vs NaHS, @ $p<0.05$ vs KO PBS group, ¥ $p<0.05$ vs Low HI + PSS, † $p<0.05$ vs Low HI - PSS. MG, Methylene green; MB, Methylene blue; HI, hemolysis index.

4.5. Discussion

In this study, we established a MBMG method to measure plasma H₂S concentrations with improved reproducibility and sensitivity. To address key limitations in traditional methylene blue (MB) spectrophotometry assay, including volatility, low sensitivity and low specificity, and matrix interferences, we introduced several critical modifications in the MBMG method. 1) We preloaded lithium heparin tubes with a plasma stabilizer solution (PSS) composed of ascorbic acid and DTPA. This innovation minimizes oxidative loss of H₂S immediately upon blood collection by maintaining a reducing environment and chelating trace metals. 2) We incorporated methylene green (MG) as an internal standard for ratio-metric quantification, as its close structural similarity to MB, differing only by a nitro substituent on the phenothiazine core [16]. Both compounds absorb strongly at 660 nm, enabling simultaneous detection at a single wavelength, and were distinctly separated by HPLC as shown in figure 1a, allowing accurate and interference-free quantification. These features of MG ensure effective correction for matrix effects, injection variability, and instrumental drift. 3) A chloroform-based phase separation step was introduced for an efficient reaction clean-up, thereby enhancing chromatographic clarity and reducing background signal. 4) We allowed the methylene blue–methylene green chromophores to develop overnight (16 hours) before analysis, as extended incubation was required to generate a reproducible and detectable signal. 5) HPLC used in MBMG versus spectrophotometry used in the traditional MB assay. HPLC offers higher sensitivity—allowing reliable detection of H₂S below 1 µM—and effectively separates methylene blue and methylene green, which have overlapping absorbance spectra. This separation enables accurate ratiometric quantification using methylene green as an internal standard, something not achievable with spectrophotometric methods. These modifications collectively address the core

issues of volatility, matrix interference, and signal instability, enabling more accurate and standardized detection of plasma H₂S.

To test the biological validity of the MBMG method, we applied it to the plasma derived from CSE-KO mice, a well-characterized genetic mouse model of diminished H₂S generation. Using this method, we generated a linear calibration standard curve for NaHS in the range of 0.05–10 μM. Notably, the calibration standard chromatograms were not matrix-corrected, as they were generated in trapping solution without plasma components. Matrix effects refer to the influence of non-analyte components on the assay signal, which can suppress or enhance the response of the target analyte and lead to inaccurate quantification [20,21]. In the context of plasma H₂S, it is impossible to generate a H₂S-free plasma because H₂S is continuously produced endogenously and exists in free and/or bound forms that cannot be removed without altering the plasma matrix [4,22]. While it may be tempting to use CSE-KO plasma as a “blank,” KO mice still retain measurable H₂S, likely through CSE-independent and non-enzymatic production pathways [23].

The validity of matrix interference is further supported by our observation that methylene green (MG)—added as an internal standard at equal concentrations to all samples—showed consistent peak areas in calibration standards but significantly decreased in plasma. This suggests that plasma has a markedly different matrix effect compared to the NaHS calibration standard in trapping solution alone. Consequently, matrix correction cannot be accurately applied to either the calibration standard curve or the plasma samples, as it could lead to under- or overestimation of H₂S concentrations. Without accounting for this matrix effect, endogenous plasma H₂S levels are likely underestimated. With the internally normalized results from MBMG method, H₂S level in circulation can be more accurately measured.

The MBMG method yielded a plasma H₂S concentration of approximately 0.5 μM in WT, and 0.15 μM in KO mice. This value aligns well with a conservative estimation that “plasma H₂S levels in healthy humans or animals are in the higher nanomolar to lower micromolar range” [24]. Similarly, a previous study used the MBB-HPLC method also reported plasma free H₂S levels of >0.7 μM in WT, which was significantly diminished in KO mice, further validating our current findings [4]. Higher plasma H₂S levels were reported using the classic MB spectrophotometry assay around 40 μM [11]. The classic MB spectrophotometry assay often overestimates plasma H₂S due to interference from plasma components—such as proteins and lipids—that absorb at the same wavelengths and react with the dye, leading to artificially elevated readings [25]. On the other hand, MBB-HPLC method, performed under strict anoxic conditions, selectively derivatizes free and certain labile sulfane-sulfur species, producing more specific and accurate H₂S measurements [4,11]. In comparison, our MBMG method stabilizes the pre-existing free H₂S through the PSS (ascorbate + DTPA), which maintains a reducing environment and chelates trace metals to limit oxidative loss. Further, MBB-HPLC method requires nitrogen chambers and other specialized anoxic equipment, whereas MBMG achieves reliable detection with far less technical complexity. Consequently, our MBMG method is highly adaptable and accessible to laboratories lacking specialized infrastructure. An additional advantage of the MBMG method is its compatibility with sample storage and remote analysis. We found that plasma samples remained stable for at least six months when frozen, without any noticeable loss of signal. This feature allows labs that do not have immediate access to HPLC systems to prepare and store samples, then ship them on dry ice to centralized facilities for analysis.

To assess the specificity of the MBMG method, we tested several biologically relevant sulfur-containing compounds under identical measurement conditions. Of these, only 1 mM cysteine produced signal comparable to 1 μ M NaHS calibration standard. However, the potential contribution of cysteine to the overall signal in plasma samples is negligible. This is because endogenous plasma cysteine concentrations are about 10-fold lower than the concentration of one mM used in our in vitro test, and the endogenous levels of cysteine remained unchanged after NaHS injection. The weak, nonstoichiometric signal likely arises from low-efficiency and non-enzymatic conversion of cysteine to H₂S under the influence of iron [23]. Even at the observed conversion rate, physiological cysteine concentrations would yield a signal equivalent to only ~0.09 μ M NaHS in WT and ~0.07 μ M in KO, confirming its negligible impact. Cysteine's minimal interference with endogenous H₂S detection is further supported by our experimental data: NaHS injection significantly elevated plasma H₂S levels (from 0.50 ± 0.04 μ M to 1.0 ± 0.1 μ M in WT, and from 0.15 ± 0.04 μ M to 0.65 ± 0.03 μ M in KO), without altering plasma cysteine concentrations. If cysteine interference were substantial, such increases in H₂S would not be observed. Additionally, GSH, another abundant thiol, produced a detectable but unquantifiable signal below the limit of quantitation, while 3-MP and polysulfide produced no detectable signal. Other thiol sources, such as persulfides, are highly transient and occur at very low concentrations in plasma under both physiological and pathological conditions [26,27]. Together, these results confirm that the MBMG method is selective for H₂S under physiological conditions, with minimal confounding from other thiol or sulfide donors.

Hemolyzed plasma without PSS showed markedly higher H₂S concentrations (0.85 ± 0.13 μ M) compared to non-hemolyzed samples (0.43 ± 0.08 μ M), likely due to ferric ions in hemoglobin from

lysed RBCs reacting with cysteine to generate non-enzymatic H₂S [23]. The presence of PSS minimized this effect, with low-HI and high-HI samples showing comparable H₂S levels (0.52 ± 0.04 vs. 0.56 ± 0.03 μ M). This likely reflects the combined action of ascorbic acid in reducing ferric to ferrous ions and DTPA in chelating iron, thereby preventing non-enzymatic H₂S formation. These findings underscore the benefit of PSS in mitigating hemolysis-related bias and variability, a critical factor for patient sample handling where hemolysis may occur during collection and processing.

It is important to note that, due to the volatile nature of H₂S, the MBMG method as currently designed is best suited for measuring H₂S in plasma or other biological fluids, potentially including cerebrospinal fluid or urine. When applying this method to tissues, extra care must be taken during homogenization and sample processing to minimize H₂S loss through volatilization. Rather than measuring the absolute concentration of endogenous H₂S in cells or tissues, measuring and comparing changes in enzymatic H₂S production rates remains an alternative and useful approach, which utilizes the classic MB assay with incubation of cell/tissue extracts in sealed flasks with cysteine and pyridoxal 5'-phosphate (PLP) over a period. The generated H₂S is accumulated as a function of incubation time, and trapped in a center vial, and the formed MB is thereafter measured [1].

Given its simplicity, sensitivity, compatibility with long-term storage, and reproducibility, our MBMG method could serve as a practical standard for plasma H₂S quantification across research labs. Its ease of implementation makes it particularly attractive for clinical and translational studies. Hospitals and diagnostic laboratories lacking advanced instrumentation can adopt the MBMG method for sample preparation and outsource the analytical phase, ensuring reliable and standardized results across institutions.

4.6. Conclusion and Perspective

H₂S plays a significant role in metabolic conditions such as obesity [28], iron overload [29,30], and cardiovascular dysfunction [31]. Accurate monitoring of circulating levels of H₂S will be essential for understanding its contribution to disease pathogenesis and therapeutic response. The MBMG method provides a practical, sensitive, and reproducible method for quantifying plasma H₂S. By incorporating a stabilizer and internal standard, the method overcomes many limitations of traditional assays while maintaining technical simplicity. Its compatibility with frozen sample storage and standard HPLC equipment enables broad applicability across research and clinical settings. This standardized approach will help unify H₂S measurement efforts and accelerate our understanding of its physiological and pathological roles.

4.7. Author Contributions:

H.M.A.: conceptualization, methodology, data analysis, writing original draft. M.F.: methodology, data analysis. T.A. methodology, data analysis. R.W.: data analysis, writing, funding acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

4.8. Funding:

This study was supported by a Discovery grant to RW (RGPIN-2023-05099) from the Natural Sciences and Engineering Research Council of Canada.

4.9. Institutional Review Board Statement:

The animal study protocol was approved by The Animal Care Committee, York University (2019-11 R1)

4.10. Data Availability Statement:

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

4.11. Conflicts of Interest:

The authors declare no conflicts of interest.

4.12. References

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Chapter 5: CSE-derived hydrogen sulfide safeguards vascular iron homeostasis by coupling ferritin buffering to vasoprotection

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This chapter has been accepted for publication as:

Arif, H. M., Fu, M., Verma, R., & Wang, R. (2025). CSE-Derived Hydrogen Sulfide Safeguards Vascular Iron Homeostasis by Coupling Ferritin Buffering to Vasoprotection. *The FASEB Journal*, 39(20), e71162.

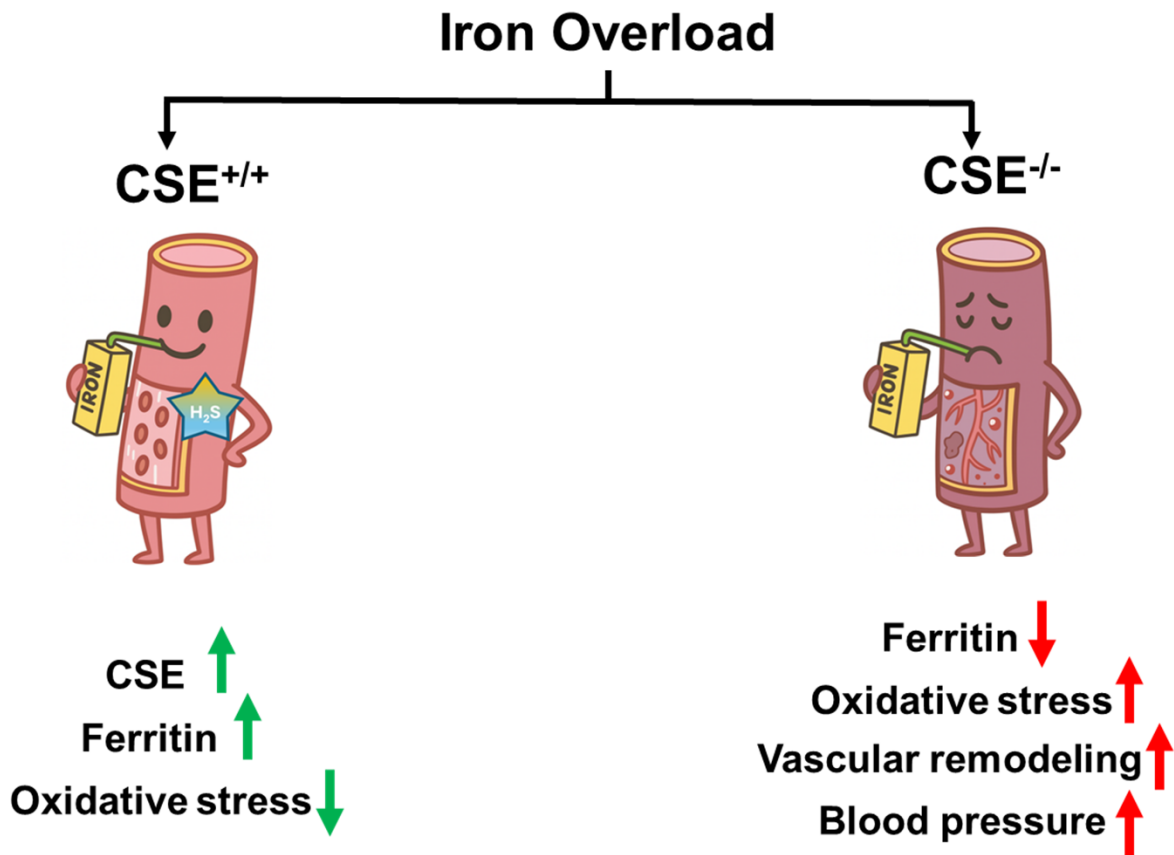
Contributions: H.M.A. conceived the study, developed the methodology, performed data analysis, and wrote the original draft. M.F. contributed to methodology and assisted with data analysis. R.V. assisted with methodology. R.W. provided supervisory input, guidance on data analysis and revision, and support with project administration and funding. H.M.A. carried out all experiments and writing at each stage.

The sections and headings in this chapter are structured to align with the formatting guidelines of FASEB journal.

5.1. Abstract

Iron overload drives oxidative stress and vascular injury, yet the role of hydrogen sulfide (H₂S) in systemic and vascular iron regulation remains unclear. We investigated how cystathionine γ -lyase (CSE)-derived H₂S influences iron handling and vascular responses following acute iron overload. Wild-type (WT) and CSE-knockout (CSE-KO) mice received PBS, low-concentration (1 $\times$) or high-concentration (3 $\times$) iron dextran intravenously. CSE-KO mice exhibited elevated serum iron and transferrin saturation but impaired ferritin upregulation compared to WT. KO aortas showed greater iron deposition, elastin degradation, and inflammatory remodeling under high iron. Iron overload impaired phenylephrine-induced vasoconstriction and H₂S-mediated vasorelaxation, with CSE-KO mice displaying the most severe vascular dysfunction and elevated blood pressure. WT mice compensated via CSE upregulation and increased H₂S production. Our findings demonstrate that CSE/H₂S deficiency disrupts ferritin-mediated iron storage, exacerbates vascular iron accumulation, and worsens vasomotor dysfunction during iron overload. The CSE/H₂S pathway may represent a therapeutic target for mitigating iron-induced vascular damage.

Keywords: Hydrogen sulfide, Iron overload, Vascular dysfunction, Oxidative stress, Hypertension



• **Graphical Abstract:** With iron overload, wild-type (CSE^{+/+}) mice upregulate their CSE and ferritin expression, limiting oxidative stress and maintaining vascular health. In contrast, CSE-deficient (CSE^{-/-}) mice fail to induce ferritin, leading to elevated oxidative stress, vascular remodeling, and hypertension. These findings highlight the protective role of the CSE/H₂S pathway against iron-induced vascular injury.

5.2. Introduction

Iron is an essential micronutrient required for critical biological processes such as oxygen transport, mitochondrial energy production, and maintenance of redox balance (1). However, excessive iron can catalyze the generation of reactive oxygen species (ROS) via Fenton chemistry, leading to oxidative stress, cellular damage, and tissue injury. To prevent iron-mediated toxicity, iron homeostasis is tightly regulated by systemic mechanisms—primarily the hepcidin–ferroportin axis—and by cellular sequestration of iron in ferritin (2). Dysregulation of these pathways, as seen in hereditary hemochromatosis, transfusion-related iron loading, or intravenous iron therapy in chronic kidney disease, has been linked to vascular dysfunction, arterial stiffness, and increased cardiovascular risk (1).

Hydrogen sulfide (H_2S), a gaseous signaling molecule generated endogenously by cystathionine γ -lyase (CSE), has emerged as a key regulator of vascular tone, redox balance, and anti-inflammatory signaling (3). Beyond its vasorelaxant properties, H_2S is increasingly recognized as a modulator of iron metabolism, influencing iron regulatory proteins (IRPs), ferritin synthesis, and labile iron pools (4). Our previous studies in vascular smooth muscle cells (VSMCs), along with recent findings by Zhu et al. demonstrated that H_2S deficiency amplifies iron-induced oxidative stress, limits ferritin upregulation, and increases cell death (4, 5). Despite these insights, the *in vivo* role of the CSE/ H_2S axis in systemic iron regulation and vascular iron handling remains poorly understood.

Acute iron overload, such as that caused by intravenous iron administration or transfusion therapy, transiently exposes vascular tissues to elevated circulating iron before hepatic clearance occurs (1), potentially inducing oxidative damage. Whether H_2S signaling counteracts this acute vascular

iron stress in vivo, and how its deficiency impacts systemic iron homeostasis and vascular remodeling, is unknown. Addressing this gap may uncover novel therapeutic strategies to prevent iron-induced vascular injury.

The aim of this study was to investigate how CSE/H₂S deficiency influences systemic iron regulation, vascular iron accumulation, and functional vascular responses during acute iron overload in vivo. We hypothesized that the CSE/H₂S pathway plays a critical role in modulating both systemic and vascular responses to iron loading.

5.3. Material and Methods

5.3.1. Animals and Protocol

All experimental procedures were approved by the Animal Care Committee at York University and followed the Canadian Council on Animal Care (CCAC) guidelines. Wild-type (WT) mice on a C57BL/6J × 129SvEv background and in-house-bred homozygous cystathionine gamma-lyase knockout (CSE-KO) mice were used. Homozygous CSE-KO mice were obtained by at least 10 generations of backcrossing heterozygous CSE-KO mice with WT mice, as previously described [6]. All animals were housed in HVAC-ventilated cages (4–5 mice per cage) maintained on a 12:12-h light/dark cycle, at 21°C–23°C and 40%–60% humidity, with unrestricted access to autoclaved water and standard rodent chow. Cages were randomly assigned to experimental groups to reduce allocation bias.

Eight-week-old male WT and CSE-KO mice were divided into three treatment groups (n = 6–8 per group):

- 1) control, which received a phosphate buffered saline (PBS) injection (tail vein);
- 2) low-concentration iron (1× iron), which received a single 15 mg/kg tail-vein injection of iron dextran;
- 3) high-concentration iron (3× iron), which received 15 mg/kg iron dextran administered three times, 2 h apart. The 3× iron dextran concentration approximates the pharmacological doses of intravenous iron used in the treatment of iron-deficiency anemia (7).

All animals were euthanized 24 h after the initial injection for blood and tissue collection.

Mice exhibiting signs of tail-vein occlusion, injection failure, or signs of distress were removed from the study. The sample size was chosen based on prior studies assessing H2S responses in atherosclerosis using our WT and CSE-KO mice [8], which showed that a group size of 6–8 animals was sufficient to detect biologically significant differences with a power of 80% and $\alpha = 0.05$.

All injections (PBS and iron dextran) were prepared using reagents from Sigma-Aldrich (Oakville, Canada).

5.3.2. Serum Iron, TIBC, and Transferrin Saturation Measurement

Mice were euthanized 24 hours after the initial injection and blood was collected by performing cardiac puncture into plain Eppendorf tubes (without anticoagulant). The collected blood was allowed to clot for 30 minutes at room temperature, then centrifuged at $845 \times g$ for 3 minutes to separate the serum, which was carefully transferred into fresh tubes for analysis. Serum iron and

total iron-binding capacity (TIBC) were determined using the TIBC and Serum Iron Assay Kit (Abcam, Concord, Canada; ab239715), following the manufacturer's protocol. Absorbance for all standards and samples was recorded at 570 nm using a Biotek Synergy HTX multimode plate reader (Agilent, Mississauga, ON, Canada). All measurements were performed in duplicate, and any sample showing visible hemolysis was excluded to avoid interference.

5.3.3. *Serum Ferritin Measurement*

Serum ferritin was measured using the Mouse Ferritin (FTL) ELISA Kit (Abcam, Concord, Canada; ab157713), following the manufacturer's instructions. Serum samples were diluted 1:40 in 1× assay diluent, and 100 µL of each standard (12.5–400 ng/mL), zero control, or diluted sample was added to designated wells in duplicate. The plate was incubated at room temperature for 60 minutes, washed four times with 1× wash buffer, and incubated with 100 µL of enzyme-antibody conjugate for 10 minutes in the dark. After an additional wash, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added, followed by a 10-minute incubation. The reaction was stopped with 100 µL of stop solution, and absorbance was recorded at 450 nm using a Biotek Synergy HTX multimode plate reader.

5.3.4. *Plasma H₂S Measurement*

Plasma H₂S was measured using a Dionex Ultimate 3000 UHPLC system equipped with a diode array detector set to 660 nm (Thermo Scientific) as previously described (9). Aliquots (20 µL) of prepared plasma samples were injected onto a Hypersil GOLD C18 column (250 × 4.6 mm, 5 µm), with a 20 min gradient elution of acetonitrile and 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. NaHS standards were prepared by diluting stock solutions in 1% zinc acetate. N,N-

dimethyl-p-phenylenediamine (DPD) and FeCl_3 were subsequently added, and the mixture was incubated in the dark for 20 min. The reaction was then cleaned up by chloroform extraction, and the aqueous phase was mixed with methylene green as an internal standard. Samples were incubated overnight at 4 °C to ensure complete methylene blue generation. H_2S concentrations were determined from the methylene blue peak areas in the chromatograms.

Unless specified separately before, all chemicals used for H_2S stabilization and detection were obtained from Sigma-Aldrich (Oakville, Canada).

5.3.5. H_2S Production Rate

The H_2S production rate was assessed using a previously described lead acetate assay (4). Briefly, lead acetate paper was prepared by soaking filter paper in 1% lead acetate solution for 1 hour and drying it in an oven. Liver lysates (1% w/v) prepared in ice-cold PBS (50 μL per well) were incubated with 10 μL of 10 mM cysteine and 10 μL of 2 mM pyridoxal 5'-phosphate in a 96-well plate, which was overlaid with the lead acetate paper. The plate was sealed with aluminum foil and incubated at 37 °C for 2 hours. The resulting darkened circular bands on the lead acetate paper, representing enzymatic H_2S production, were imaged and analyzed using ImageJ software (version 1.54h, NIH, Bethesda, MD, USA) (10). Lead acetate, cysteine, pyridoxal 5'-phosphate, and PBS components were all obtained from Sigma-Aldrich (Oakville, Canada).

5.3.6. Histology and Immunofluorescence

Excised aortas were submerged in 10% neutral-buffered formalin overnight, rinsed thoroughly with PBS, and embedded in Optimal Cutting Temperature (OCT) compound. Tissues were rapidly frozen in liquid nitrogen, stored at -80 °C, and cryosectioned at 10 μm thickness using a Leica CM1850

UV microtome-cryostat (Leica Biosystems, Concord, Canada). Sections were mounted onto Superfrost Plus slides (Fisherbrand, Ottawa, Canada) and incubated at 37 °C for 30 minutes to enhance tissue adhesion. Before staining, slides were equilibrated to room temperature and rinsed with PBS to remove residual OCT.

For Prussian blue staining, tissue sections were incubated with freshly prepared Prussian blue solution (2% potassium ferrocyanide and 2% hydrochloric acid, 1:1) for 20 minutes at room temperature, rinsed thoroughly with distilled water, and counterstained with nuclear fast red for 5 minutes. After additional rinsing, sections were dehydrated through graded ethanol, cleared with xylene, and mounted in Permount (Fisher Scientific, Ottawa, Canada). Images were acquired using an Olympus X71 fluorescence microscope (Olympus America Inc., Center Valley, PA, USA), and the percentage of positive staining area was quantified using ImageJ.

EVG staining was performed using in-house-prepared reagents. The working elastin solution (Verhoeff's hematoxylin) was prepared by mixing 20 mL of 5% alcoholic hematoxylin, 8 mL of 10% ferric chloride, and 8 mL of Lugol's iodine. Tissue sections were stained in this solution for 10 minutes, rinsed with deionized water, and differentiated in 2% ferric chloride solution for up to 2 minutes with microscopic monitoring. After rinsing in tap water for 5 minutes, sections were treated with 95% alcohol to remove residual iodine and rinsed again with deionized water. Counterstaining was performed for 1–3 minutes in Van Gieson's solution (5 mL of 1% acid fuchsin in 95 mL saturated picric acid). Finally, sections were rinsed in 95% alcohol, dehydrated, cleared in xylene, and mounted in Permount. Images were acquired using an Olympus X71 fluorescence microscope, and the percentage of positive staining area was quantified using ImageJ.

Aortic cryosections were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature and incubated overnight at 4 °C in humidified chambers with primary antibodies: CD68 (1:200; Cell Signaling Technology, Whitby, ON, Canada), α -Smooth Muscle Actin (α SMA, 1:400; Alexa Fluor® 488 Conjugate; Cell Signaling Technology, Whitby, ON, Canada), or MMP9 (1:200; Thermo Fisher Scientific, Mississauga, ON, Canada). After washing with TBST, sections were incubated for one hour at room temperature with Alexa Fluor® 488 Goat anti-Rabbit IgG (H + L) (1:200; Thermo Fisher Scientific, Mississauga, ON, Canada) or Alexa Fluor® 594 Goat anti-Rabbit IgG (H + L) (1:400; Thermo Fisher Scientific, Mississauga, ON, Canada), as appropriate. Sections were mounted with 10% glycerol in PBS containing DAPI (1 μ g/mL) and covered with coverslips. Imaging was performed using an Olympus X71 fluorescence microscope (Olympus America Inc., Center Valley, PA, USA) with DAPI, FITC, and TRITC channels, and mean fluorescence intensity was quantified using ImageJ.

All chemicals used for Prussian blue and EVG staining, including potassium ferrocyanide, ferric chloride, hydrochloric acid, picric acid, and acid fuchsin, were obtained from Sigma-Aldrich (Oakville, Canada). Tween-20 was obtained from Fisher Chemicals (Mississauga, Canada)

5.3.7. Measurement of Isometric Tension of Vascular Tissues

Isometric tension was measured using our previously defined method (11). Thoracic aortas from treated mice were excised and immediately placed in ice-cold modified Krebs bicarbonate solution (in mM: 115 NaCl, 5.4 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 5 D-glucose, and 10 HEPES; pH 7.4) continuously aerated with 5% CO₂ and 95% O₂. Under a dissection microscope, all extraneous fat and connective tissue were carefully removed. Aortic rings (3–4 mm) were

mounted on 0.22 mm stainless steel wires (Harvard Apparatus, Saint-Laurent, Canada) connected to a force transducer (AD Instruments, Mississauga, Canada) and organ bath (Harvard Apparatus, Saint-Laurent, Canada) maintained at 37 °C with continuous aeration. Isometric tension was recorded using LabChart 8 software (AD Instruments, Mississauga, Canada).

Danish MyoTechnologies (DMT) normalization was used to ensure vessels were set at their optimal passive tension, corresponding to the optimal length–tension relationship for smooth muscle contraction. This approach enhances reproducibility and comparability across vessels and experiments (12). Aortic rings were incrementally stretched between 1–4 mN and challenged with 60 mM KCl, with maximum active tension recorded. After three KCl challenges, the DMT curve was plotted using the DMT module in LabChart 8, and optimal passive tension was established. A fourth KCl challenge confirmed this setting, followed by three washes (5-minute intervals) and a 30-minute equilibration period (with a buffer exchange at 15 minutes).

Contractile responses were assessed by a single-concentration challenge with 1 μ M phenylephrine (PE), followed by relaxation with 10 μ M acetylcholine (ACh). After four washes (5-minute intervals), rings were incubated with 0.1 mM N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma-Aldrich, Oakville, Canada) for 15 minutes to inhibit endothelial nitric oxide synthase, then challenged with 1 μ M PE and relaxed with 10 μ M hydrogen sulfide (H₂S), prepared by diluting a 0.8 M stock solution (Sigma-Aldrich, Oakville, Canada) in PBS. After washing and 15 minutes of re-equilibration, a final 1 μ M PE challenge was performed, followed by relaxation with 10 μ M sodium nitroprusside (SNP) to evaluate smooth muscle integrity. Contraction was defined as the increase in force from baseline following PE addition, and relaxation was expressed as the percentage decrease in force relative to the maximum stable contraction induced by PE. All

pharmacological agents (PE, ACh, SNP, H₂S) and the salts used to prepare the Krebs bicarbonate solution were purchased from Sigma-Aldrich (Oakville, Canada).

5.3.8. Blood Pressure Measurement

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were assessed using a noninvasive tail-cuff system (CODA-6, Kent Scientific, Torrington, CT, USA) as previously described (13). Mice were acclimated to the restrainers and tail-cuff apparatus for 2–3 sessions before measurements to minimize stress-related artifacts. For each animal, more than six consecutive readings were recorded, and the mean value was used as the final measurement.

5.3.9. Western Blotting

For aortic samples, three thoracic aortas were pooled, pulverized into powder in liquid nitrogen, and lysed in ice-cold lysis buffer (1× RIPA supplemented with 1% Phenylmethylsulfonyl fluoride and 1% protease inhibitor cocktail). Liver samples from individual mice (1% w/v) were also homogenized in ice-cold lysis buffer. . Protein lysates were resolved on 7.5% or 10% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked with 5% non-fat milk in TBST for 1 hour at room temperature and incubated overnight at 4 °C with primary antibodies: CSE (1:1000; Proteintech, San Diego, CA, USA), ferritin (1:2000; Novus Biologicals, Etobicoke, ON, Canada), GAPDH (1:2000; Novus Biologicals, Etobicoke, ON, Canada), DMT1 (1:1000; Thermo Fisher Scientific, Mississauga, ON, Canada), transferrin receptor 1 (TfR1, 1:1000; Thermo Fisher Scientific, Mississauga, ON, Canada), and ferroportin (FPN, 1:1000; Thermo Fisher Scientific, Mississauga, ON, Canada). After washing with TBST, membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:2000; Thermo Fisher Scientific, Mississauga,

ON, Canada) for 1.5 hours at room temperature. Detection was performed using ECL (Cytiva, Montreal, QC, Canada) or Super Signal West Pico Plus (Thermo Fisher Scientific, Mississauga, ON, Canada), and images were captured using a Microchemi imaging system (Froglab, Concord, ON, Canada). GAPDH was used as the loading control, and each blot was performed in triplicate to ensure reproducibility.

All other general chemicals used for lysis and washing (e.g., RIPA buffer components, PMSF, and protease inhibitor cocktail) were obtained from Sigma-Aldrich (Oakville, Canada).

5.3.10. Quantitative PCR

Total RNA was isolated from 1% liver homogenates using TRIzol reagent (Sigma-Aldrich, Oakville, ON, Canada), following the manufacturer's protocol. Reverse transcription was performed with 1 µg of total RNA using the Superscript First-Strand cDNA Synthesis System (Invitrogen, Burlington, ON, Canada). Quantitative PCR (qPCR) was conducted using SYBR Green PCR Master Mix (BioRad, Mississauga, ON, Canada) on an iCycler iQ5 (BioRad) with optical system software (version 3.1). Hepcidin primers were forward: GCACCACCTATCTCCATCAACA and reverse: TTCTTCCCCGTGCAAAGG. The endogenous reference gene was 18S rRNA (forward: AGTCCCTGCCCTTTGTACACA, reverse: CGATCCGAGGGCCTCACTA).

The cycling program consisted of an initial denaturation at 95 °C for 1 min, followed by 38 cycles of 95 °C for 15 s, 58 °C for 20 s, and 72 °C for 30 s, with real-time data acquisition during the extension step. A standard melt curve was performed from 58 °C to 95 °C in 1 °C increments to confirm amplification specificity. Relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method,

with ΔCT representing the difference between the target gene and the 18S reference threshold cycle (1).

5.3.11. Statistical Analysis and Reproducibility

All data are presented as means $\pm$ standard error (SEM). Unless specified otherwise, individual data points for the mean value of each experimental group were included, representing biological replicates (or the average of technical replicates where applicable). For plasma, serum, blood pressure, liver qPCR, and H₂S production rate assays, n represents the number of mice (6–8 per group). For Western blotting, each biological replicate consisted of a pooled sample of three thoracic aortas (n = 1), with six independent pooled samples prepared per group; each sample was run in duplicate across three separate gels, and band intensities were averaged for analysis. For histological analyses (Prussian blue, EVG, IF), three randomly selected fields per section were quantified and averaged to generate one value per mouse. For experiments involving two factors (genotype and iron loading concentration), two-way ANOVA was used to evaluate the main effects and their interaction, followed by Tukey's post hoc test where appropriate, whereas one-way ANOVA was applied in cases where one factor showed no measurable values or changes (e.g., non-detectable expression or no visible activity). A p -value < 0.05 was considered statistically significant. A complete summary of tests, F-statistics, degrees of freedom, and p-values is provided in Table S1.

5.4. Results

5.4.1. CSE Deficiency Impairs Systemic Iron homeostasis

The Total iron-binding capacity (TIBC) remained unchanged between control, 1× iron, and 3× iron groups for both WT and KO (Figure 1a). In contrast, serum iron level increased significantly with iron loading, with a stronger rise in KO than WT (Figure 1b). In WT, serum iron levels increased moderately only with 3× iron (38.4 μM vs. 28.0 μM in controls), whereas KO serum iron doubled (81.4 μM vs. 35.8 μM in controls). Transferrin saturation (%TSAT) mirrored serum iron levels, rising to 65.9% in KO 3× iron compared to 27.4% in KO controls (Figure 1c). Serum ferritin levels increased markedly in WT (181 ng/mL in controls to 429 ng/mL with 3× iron), while serum ferritin levels in KO showed only a mild change (Figure 1d). Plasma H₂S levels increased in WT following iron injections (0.52 μM in controls to 0.77 μM in 3× iron), whereas KO exhibited consistently low levels with no iron-induced changes (Figure 1e).

Representative liver blots (Figure 1f) showed that CSE and ferritin proteins were upregulated in WT, while KO displayed no detectable CSE. Quantification confirmed significant induction of liver CSE and ferritin with iron in WT (5-fold ferritin increase at 3× iron) but not in KO (Figure 1h–i). H₂S production rates were elevated in WT with iron loading but remained undetectable in KO (Figure 1g, j). Hepcidin expression increased after 1× iron in both genotypes but rose significantly higher in WT than KO with 3× iron (9.5-fold vs. 4.9-fold) (Figure 1k).

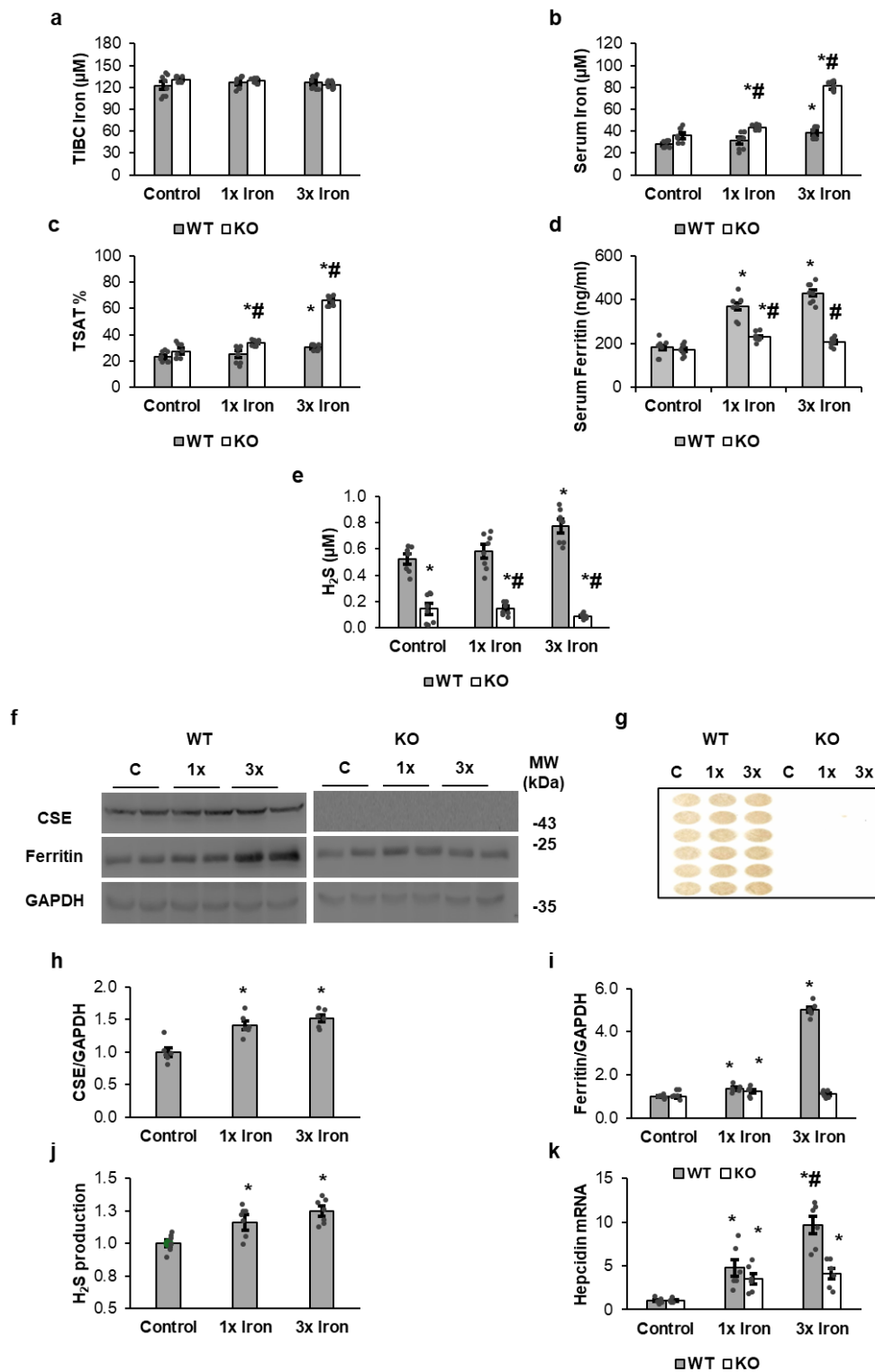


Figure 1. H₂S and iron regulation parameters in plasma and liver following acute iron loading in WT and CSE-KO mice. (a) Total iron-binding capacity (TIBC) in serum. (b) Serum iron levels. (c) Transferrin saturation (TSAT) calculated from serum iron and TIBC. (d) Serum ferritin concentrations. (e) Plasma H₂S levels measured by the MBMG method. (f) Representative Western blots of hepatic CSE, ferritin, and GAPDH. (g) Representative images of hepatic H₂S production rates assessed by the lead acetate assay. (h) Relative hepatic CSE expression normalized to GAPDH. (i) Relative hepatic ferritin expression normalized to GAPDH. (j) Hepatic H₂S production rates normalized to control. (k) Hepatic hepcidin mRNA levels relative to 18S rRNA. Effects of mouse genotypes, iron loading concentrations, and their interaction were assessed by two-way ANOVA. Significance symbols represent Tukey's post-hoc comparisons. * $p < 0.05$ vs. WT control; # $p < 0.05$ vs. WT group with the same iron treatment condition. $n = 6$ per group for Western blot and H₂S production rate; $n = 8$ per group for the measurement of TIBC, serum iron, TSAT, serum ferritin and plasma H₂S.

5.4.2. *CSE Deficiency Impairs Vascular Iron homeostasis*

Aortic CSE protein levels increased with iron loading in WT but were absent in KO (Figure 2a, b). Ferritin expression levels increased in both genotypes with 1× iron; However, with 3× iron, WT ferritin increased 2.5-fold, while KO ferritin dropped to 0.53-fold of the control levels (Figure 2a, c). DMT1 and TfR1 mediate cellular iron uptake, whereas FPN functions as the iron exporter, and their expression patterns are shown in Figure 2d–g. DMT1 remained stable in WT aortic tissues but DMT1 increased nearly 3-fold in KO aortic tissues with 3× iron (Figure 2e), while TfR1 progressively decreased in WT with iron loading (to 28% of control levels at 3× iron) but showed minimal reduction in KO (Figure 2f). FPN also decreased in both genotypes, with a greater reduction in WT (Figure 2g).

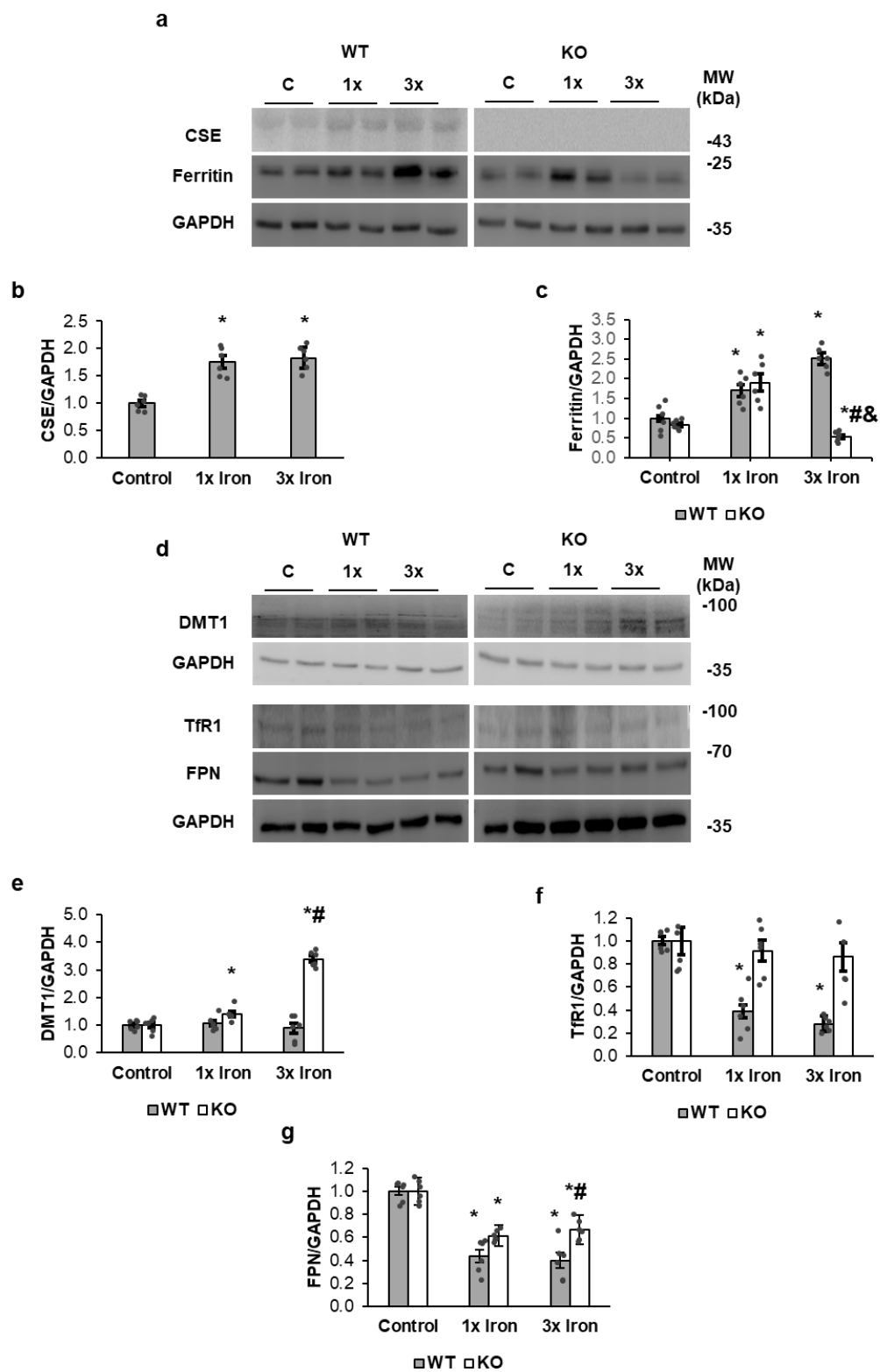


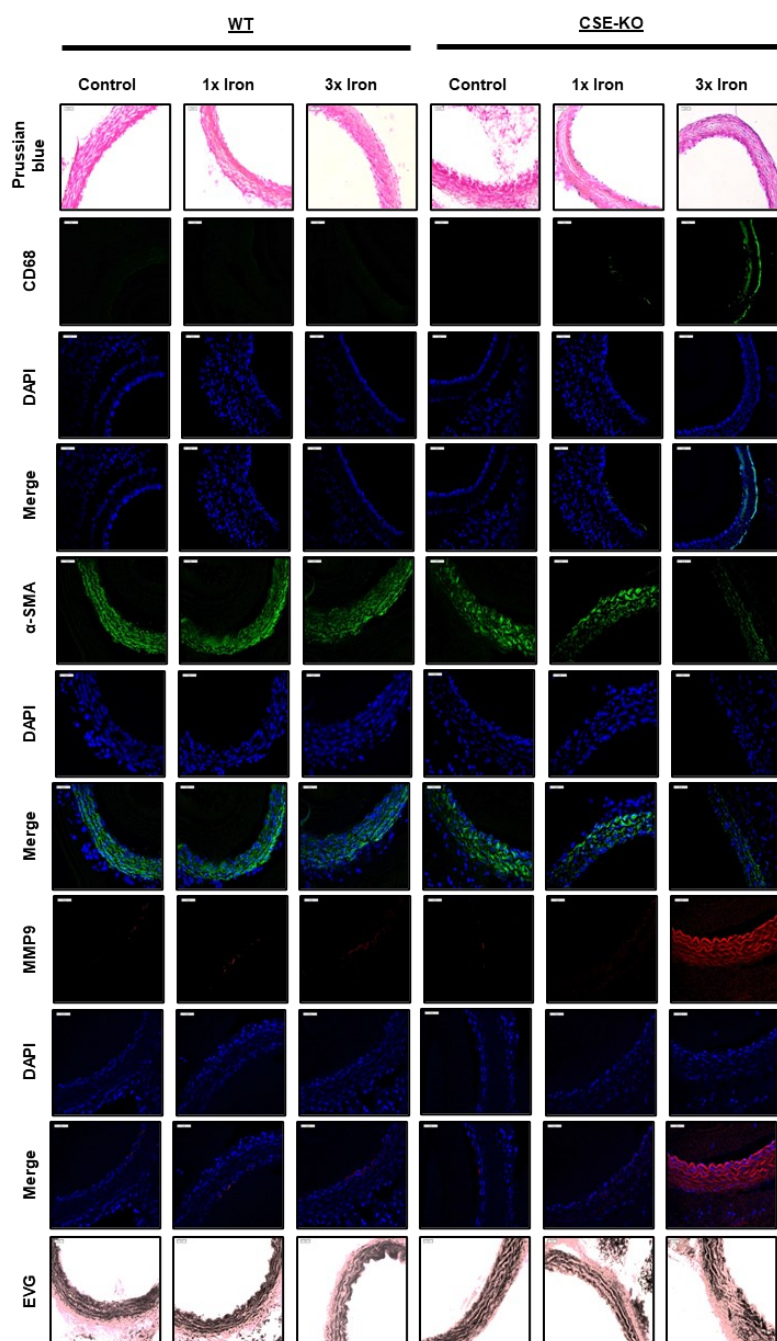
Figure 2. Aortic expression of CSE, ferritin, and iron transporters following acute iron loading in WT and CSE-KO mice. (a) Representative Western blots of aortic CSE, ferritin, and GAPDH. (b) Relative expression levels of aortic CSE normalized to GAPDH. (c) Relative expression levels of aortic ferritin proteins normalized to GAPDH. (d) Representative Western blots of DMT1, transferrin receptor 1 (TfR1), ferroportin (FPN), and GAPDH. (e) Relative expression levels of DMT1 normalized to GAPDH. (f) Relative expression levels of TfR1 normalized to GAPDH. (g) Relative expression levels of FPN normalized to GAPDH. Effects of mouse genotypes, iron loading concentrations, and their interaction were assessed by two-way ANOVA. Significance symbols represent Tukey's post-hoc comparisons. * $p < 0.05$ vs. WT control; # $p < 0.05$ vs. WT group at the same iron concentration; & $p < 0.05$ vs. KO control. $n = 6$.

5.4.3. *Iron Deposition and Vascular Remodeling Are Exacerbated in KO*

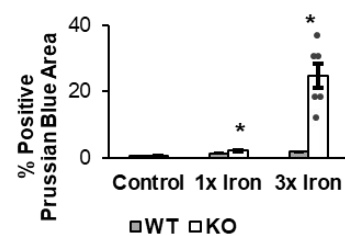
Prussian blue staining revealed significant iron deposits only in vascular tissues from KO mice with 3× iron treatment (Figure 3a, b). CD68-positive macrophage infiltration was also observed exclusively in 3× iron group of KO mice (Figure 3c). αSMA staining, a marker of smooth muscle integrity, decreased greatly in 3x iron group of KO mice with iron loading (0.22 ± 0.11 αSMA/DAPI ratio vs. 0.67 ± 0.12 in KO controls) but showed only a mild and insignificant reduction in WT (Figure 3d). MMP9 expression increased significantly only in KO mice with 3× iron treatment (Figure 3e). The percentage of EVG-positive staining decreased by ~2.1-fold in KO vascular tissues (from 66% positive stain area in controls to 31% with 3× iron), whereas WT vascular tissues showed ~1.5-fold decrease (from 73% to 50%) (Figure 3f).

High-resolution images for Prussian blue and EVG staining are provided in Supplementary Figures S1 and S2.

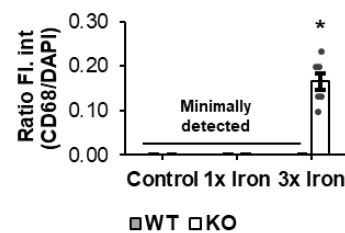
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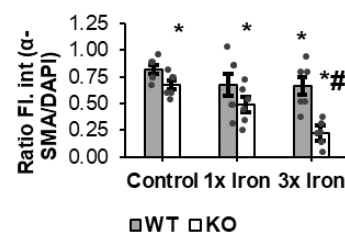
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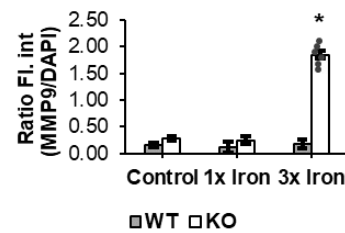
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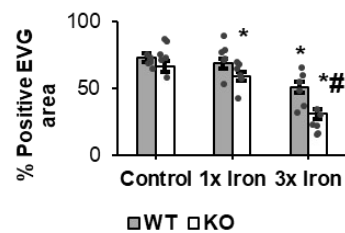


Figure 3. Histological and immunofluorescence analysis of vascular remodeling following acute iron loading in WT and CSE-KO mice. (a) Representative images of Prussian blue staining, CD68, α SMA, MMP9, and EVG staining in aortic sections. (b) Quantification of Prussian blue–positive area. (c) Mean fluorescence intensity ratio of CD68 to DAPI. (d) Mean fluorescence intensity ratio of α SMA to DAPI. (e) Mean fluorescence intensity ratio of MMP9 to DAPI. (f) Percentage of EVG-positive elastin content. Effects of mouse genotypes, iron loading concentrations, and their interaction were assessed by two-way ANOVA. Significance symbols represent Tukey’s post-hoc comparisons. * $p < 0.05$ vs. WT control; # $p < 0.05$ vs. WT group at the same iron concentration. $n = 6$ per treatment group.

5.4.4. CSE Deficiency Increases Vascular Stiffness and Blood Pressure with Iron Loading

A representative isometric tension development recording for determining optimal passive tension through DMT normalization is shown in Figure 4a. A representative original isometric recording of sequential applications of PE, ACh, L-NAME, H₂S, and SNP is shown in Figure 4b. Optimal passive tension increased in KO mice with 1× iron treatment (4.14 mN vs. 3.84 mN in WT tissues) and in both genotypes with 3× iron treatments, with KO tissues showing a modest (~8%) higher tension compared to WT tissues (Figure 4c). Representative complete traces are provided in Supplementary Figure S3.

PE-induced vasocontraction increased notably after L-NAME conditioning (Figure 4d). The second PE response rose by ~43% in control WT tissues and ~38% in control KO tissues compared to the pre-L-NAME levels. The third PE response remained elevated at ~70% higher than pre-L-NAME level in WT and ~120% higher in KO tissues. This demonstrates that L-NAME, by inhibiting endothelial NO production, increased PE-induced contractions, providing a higher baseline tone.

Without L-NAME treatment, iron loading caused a wide variation in PE-induced contraction magnitudes among control, 1×, and 3× iron groups. For example, in WT aortic rings, contraction forces dropped by ~28% with 1× and ~64% with 3× iron compared to control. A similar but more pronounced decrease was seen in KO aortic rings (–45% with 1× iron and –76% with 3× iron). After L-NAME conditioning (second and third PE stimulations), these group differences became less pronounced due to the overall increase in contractile force.

With L-NAME treatment, the third PE-induced contraction of KO tissues remained more sensitive to iron, with reductions of ~35% (1× iron) and ~54% (3× iron) compared to KO control. In contrast,

WT aortic tissues exhibited smaller decreases of ~27% (1× iron) and ~33% (3× iron) (Supplementary Figure S4). These findings confirm that vascular smooth muscle contractility is more sensitive to iron loading in the absence of CSE. This interpretation was supported by two-way ANOVA, which revealed a significant interaction of mouse genotypes with iron loading concentrations for PE-induced contractions.

ACh-induced vasorelaxation was examined in the absence of L-NAME treatment. With different baselines of PE-induced contractions in aortic tissues, ACh relaxed all the tissues almost completely within 10 min for both WT tissues (Figure 4e) and KO tissues (Figure 4f). In the following experiments with H₂S and SNP, the aortic rings were all pre-treated with L-NAME in order to block eNOS-derived NO. SNP induced the same extents of vasorelaxation among all WT (Figure 4g) and KO aortic tissues (Figure 4h), confirming that the vasorelaxant responses of smooth muscles to the NO donor was not changed by iron treatments. In contrast, in WT aortae, H₂S-induced relaxation decreased by ~32% with 1× iron and ~58% with 3× iron compared to controls (Figure 4i). KO aortic tissues showed a greater loss, with reductions of ~64% (1× iron) and ~76% (3× iron) relative to controls (Figure 4j).

Different vasorelaxation responses were further quantified in Figure 4k as a percentage of the preceding PE-induced contraction under each treatment condition, allowing comparison based on the same contractile baseline tone. It is clear that while ACh- and SNP-induced vasorelaxations were not affected by iron treatments, H₂S-induced vasorelaxation was severely decreased by iron treatment and more so in KO vascular tissues. Two-way ANOVA revealed significant main effects of both mouse genotype (WT vs. KO) and iron loading concentrations (control, 1×, 3×), as well as a significant interaction of mouse genotypes and iron loading concentrations. This means that while

mouse genotypes and iron loading concentrations each independently influenced the extent of H₂S-induced vasorelaxation, the decrease of vasorelaxation was disproportionately greater in KO tissues with iron loading.

WT mice at the age of eight weeks had stable systolic and diastolic pressures, which were not significantly affected by iron treatments. In contrast, KO mice of the same age exhibited significant increases in both SBP and DBP with 3× iron loading (>10% above control). Two-way ANOVA confirmed main effects of genotype (WT vs. KO) and iron concentration (control, 1×, 3×), as well as a significant genotype × concentration interaction (SBP: $F_{\text{genotype}(1,42)}=2151.8$, $F_{\text{concentration}(2,42)}=416.8$, $F_{\text{interaction}(2,42)}=147.3$; DBP: $F_{\text{genotype}(1,42)}=37.9$, $F_{\text{concentration}(2,42)}=3.2$, $F_{\text{interaction}(2,42)}=5.4$; all $p < 0.05$). These results demonstrate that while both genotype and iron concentration independently influence blood pressure, the hypertensive effect of iron loading is disproportionately greater in KO mice, whereas WT mice maintained relatively stable pressures across treatments.

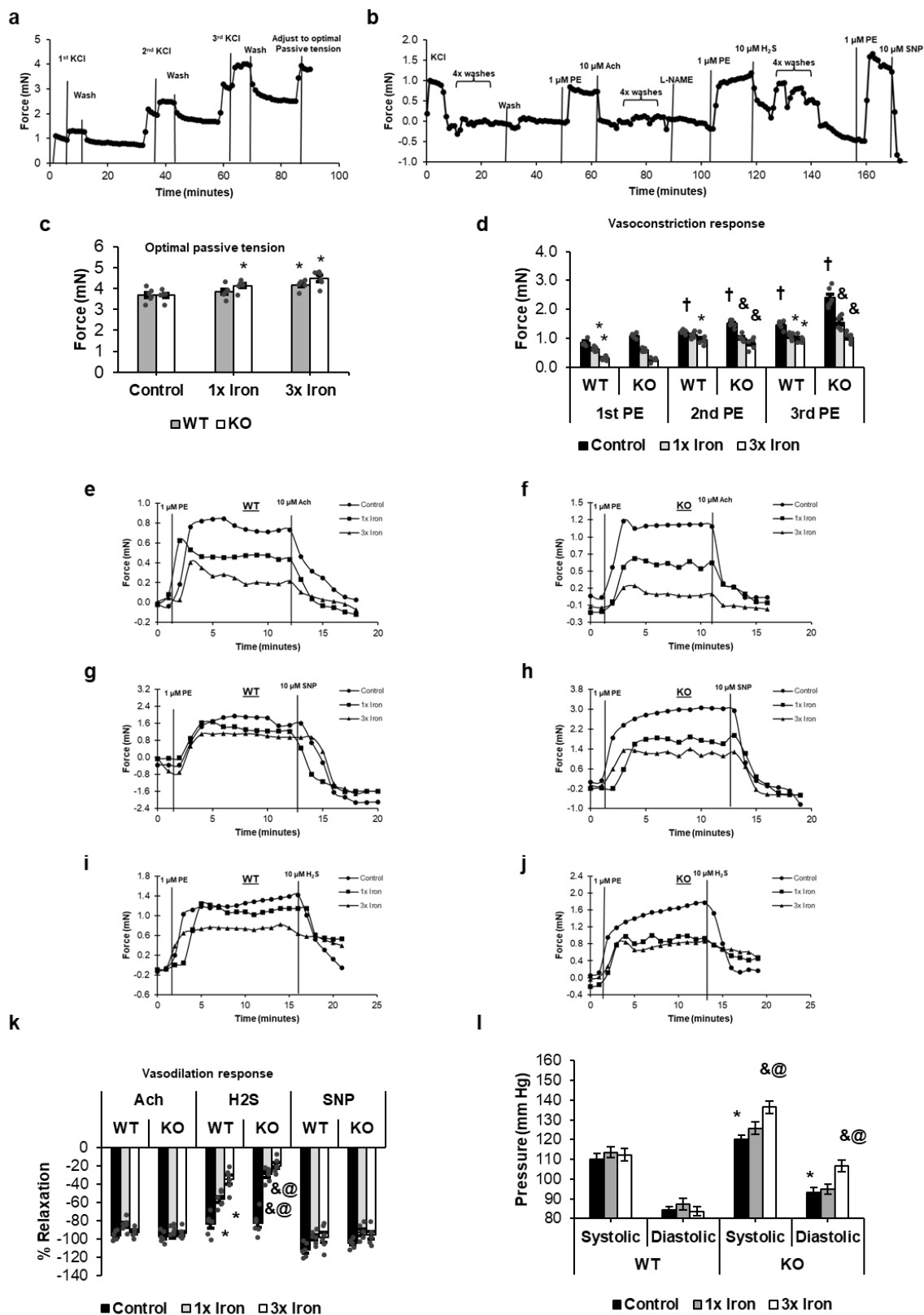


Figure 4. Vasomotor responses and blood pressure changes following acute iron loading in WT and CSE-KO mice. (a) Time–force trace showing DMT normalization for determining optimal passive tension. (b) Representative recording of the contraction–relaxation sequence (PE, ACh, H₂S, SNP) in aortic rings. (c) Optimal passive tension determined from DMT normalization. (d) Peak contractile responses to three consecutive PE challenges, with the first measured before L-NAME treatment and the second and third measured after L-NAME conditioning. (e, f) Snapshots of PE contraction followed by ACh-induced relaxation in WT and KO aortae. (g, h) Snapshots of PE contraction followed by SNP-induced relaxation in WT and KO aortae. (i, j) Snapshots of PE contraction followed by H₂S-induced relaxation in WT and KO aortae. (k) Summary of the vasorelaxation responses to ACh, H₂S, and SNP. (l) Systolic and diastolic blood pressure measured via tail-cuff method. Scattered individual points are not shown for the mean blood pressure means due to the high number of replicate readings per mouse. Effects of mouse genotypes, iron loading concentrations, and their interaction were assessed by two-way ANOVA. Significance symbols represent Tukey’s post-hoc comparisons. * $p < 0.05$ vs. WT control; & $p < 0.05$ vs. KO control; † $p < 0.05$ vs. the 1st PE response of the control group (same genotype); @ $p < 0.05$ vs. WT with the same iron treatment conditions. $n = 6$ with 2 replicates per treatment group for vascular tension studies and $n = 8$ per treatment group for blood pressure measurements

5.5. Discussion

Our study reveals a previously unrecognized role of the CSE-derived H₂S in systemic and vascular iron regulation, showing that CSE deficiency leads to impaired ferritin upregulation, abnormal iron accumulation, and pronounced vascular remodeling following acute iron loading. CSE-KO mice exhibited elevated serum iron and transferrin saturation with a blunted hepcidin response, accompanied by a decreased ferritin induction compared to WT. This suggests that CSE-KO mice fail to effectively sequester excess iron in as ferritin which is a redox-inactive storage form (14). In iron-treated KO aortas, ferroportin failed to be downregulated, and DMT1 expression was elevated. Combined with impaired ferritin upregulation, this leads to labile iron accumulation and oxidative stress. The increase in DMT1 expression in KO mice is likely due to impaired IRP1-to-aconitase conversion, leaving more active IRP1 bound to the DMT1 iron response elements (IRE) in DMT1 mRNA. With high iron loading, IRP1 should convert to aconitase and detach from DMT1 mRNA as occurred in WT tissues, causing its breakdown (15). In CSE-KO mice, this regulation fails due to impaired IRP1-to-aconitase conversion (4, 5) resulting in stable DMT1 expression (15). These findings are consistent with our earlier cell-based study (4), where H₂S deficiency in VSMCs disrupted IRP1-to-aconitase conversion, resulting in low ferritin expression and increased iron accumulation. Zhu et al. similarly demonstrated that H₂S stabilizes iron homeostasis through IRP1/IRE interactions (5). Extending these in vitro results, our in vivo data show for the first time that tail-vein iron injection in WT mice upregulates ferritin expression in both livers and aortae, while KO mice fail to mount a comparable response, particularly with high-iron loading conditions.

CSE expression in liver and aortic tissues was also elevated by iron loading in WT mice, in line with our previous finding that iron upregulates CSE through the integrated stress response via ATF4 (4).

The associated rise in plasma H₂S levels and hepatic H₂S production in WT mice likely reflects compensatory activation of liver CSE, a major H₂S-producing tissue (3). In contrast, iron-loaded KO mice exhibited persistently low plasma H₂S, correlating with impaired vasorelaxant responses and systemic iron dysregulation. Histological results support the idea that impaired ferritin upregulation enhances iron toxicity, as shown by strong Prussian blue staining in KO aortae after 3× iron loading, despite lower ferritin. This parallels observations in H-ferritin knockout mice, where the absence of functional ferritin led to increase in labile iron pools, and significant iron accumulation in spleen that was observable via Prussian blue staining (16). Our findings suggest that failure to upregulate ferritin in KO mice keeps iron in reactive, labile forms rather than safely stored as ferritin, amplifying oxidative injury in vascular tissues.

Vascular remodeling has been observed in KO mice, including loss of elastin (EVG), reduced αSMA, and increased CD68 and MMP9 staining, reflects oxidative stress and inflammation (4). Previous studies also report that immune activation caused by vascular oxidative stress leads to fibrosis and elevated blood pressure (17). Moreover, Martínez-Revelles et al. showed that lysyl oxidase-mediated oxidative stress alters elastin structure and increases vascular stiffness via p38MAPK activation (18). These structural changes are linked to vascular functional changes observed in our current study. Phenylephrine-induced contraction was decreased with iron loading, particularly in CSE-KO mice, reflecting smooth muscle depletion. This is consistent with findings by Kuang et al., who demonstrated that ferric ammonium citrate impairs PE-induced vasoconstriction of rat aortic tissues, an effect reversed by antioxidants (19). H₂S-mediated vasorelaxation was markedly decreased in iron-loaded CSE-KO mice compared to WT, an effect driven by iron-induced ROS production that was further exacerbated in KO mice due to its inherently elevated oxidative stress.

ROS can inhibit vascular K_{ATP} channels in mesenteric artery via S-glutathionylation (20), and our previous study confirmed that KO VSMCs generated substantially more ROS than WT cells when exposed to physiologically relevant concentrations of iron (4). Since H_2S -induced vasorelaxation depends on K_{ATP} channel activation (21, 22), ROS-induced inhibition of K_{ATP} channels in KO cells may account for the greater loss of the vasorelaxant effects of H_2S in these cells with iron overloading.

Interestingly, we did not observe any significant changes in SNP-mediated relaxation among all groups. SNP, as an NO donor, promotes vasorelaxation through activation of soluble guanylate cyclase (sGC), leading to increased intracellular cGMP levels and subsequent activation of protein kinase G (PKG), which decreases vascular smooth muscle tone (23). Chronic oxidative stress is known to impair the NO–cGMP signaling pathway by suppressing PKG activation and inhibiting vasodilator-stimulated phosphoprotein (VASP) phosphorylation, as shown in a study where no significant difference in cGMP-dependent relaxation was observed after 1 week of oxidative stress in rat mesenteric artery, but a marked decrease occurred after 3 weeks (24). In contrast, our acute model (24 hours post-iron loading) revealed a decrease in H_2S -mediated relaxation, suggesting that the H_2S signaling pathway is more vulnerable to early oxidative perturbations than the NO–cGMP pathway. (25). It is also possible that the relatively high concentrations of NO generated by SNP are sufficient to overcome mild acute oxidative inhibition of sGC. Future studies using chronic iron-loading models will be necessary to determine whether prolonged oxidative stress eventually impairs NO–cGMP signaling in this context.

The impaired vasorelaxation, inflammatory remodeling (increased CD68 and MMP9), diminished contractile reserve due to smooth muscle depletion (decreased amount of α SMA), and significant

elastin loss (EVG staining) are indicative of a maladaptive shift toward vascular stiffness and elevated tone, consistent with studies linking oxidative stress and impaired K_{ATP} channel function to hypertensive pathology (17, 26). Furthermore, vascular functional deterioration observed in our current study was accompanied by a rise in systolic and diastolic pressures in KO mice with high iron loading. In line with this, Sangartit et al. also reported hypertension and vascular dysfunction in iron-overloaded mice (27).

Patients with conditions such as hereditary hemochromatosis, transfusion-related iron overload, or those undergoing repeated intravenous iron therapy (e.g., in chronic kidney disease or thalassemia) often develop endothelial dysfunction and vascular complications due to excess free iron and oxidative stress (28). The acute iron surges modeled by tail-vein iron injections in our study parallel the vascular stress observed in these clinical settings. In KO mice, FPN levels failed to decrease as expected under iron loading, allowing iron to be exported from vascular tissues back into circulation rather than being sequestered as ferritin. This abnormal iron handling increases labile iron pools, exacerbating oxidative vascular damage. CSE-KO mice display a unique vascular phenotype after acute iron loading, characterized by iron deposition, oxidative remodeling, attenuated PE constriction and compromised H₂S vasorelaxation. These findings position H₂S deficiency as a mechanistically distinct pathway in vascular iron dysregulation, with direct clinical implications for conditions where impaired H₂S signaling may amplify iron-induced vascular injury, highlighting it as a potential therapeutic target.

In addition, our data may provide mechanistic insight into vascular complications observed in chronic kidney disease (CKD) and diabetes, where both H₂S levels and vascular function are often impaired (29, 30). Clinical studies have shown that weakened H₂S signaling in CKD patients

correlates with endothelial dysfunction and increased cardiovascular risk (31), which can be exacerbated by intravenous iron therapy (32). In diabetes, iron overload is increasingly recognized as a comorbid factor contributing to vascular damage and metabolic dysfunction, as supported by meta-analyses linking altered iron metabolism to type 2 diabetes (33). Similarly, in diabetes, low H₂S bioavailability has been linked to impaired vasorelaxation and oxidative stress in which STZ-induced diabetic rats exhibited both lowered physiological tissue H₂S levels and impaired endothelium-dependent relaxation (34). Our findings suggest that restoring H₂S signaling in such conditions could mitigate the vascular toxicity of iron therapy, offering a potential therapeutic strategy.

While the current study focuses on iron overload, the relationship between H₂S and anemia remains underexplored and warrants investigation. Since H₂S modulates key iron metabolism pathways (including ferritin and transferrin receptor regulation), its deficiency could impair both iron mobilization during anemia and influence erythropoietic processes. This potential dual role is supported by existing evidence: CSE deficiency has been shown to increase erythropoiesis through upregulation of coproporphyrinogen III oxidase and stimulation of heme biosynthesis - a compensatory mechanism to enhance hemoglobin production under low H₂S conditions (35). Furthermore, decreased endogenous H₂S production is linked to impaired renal erythropoietin synthesis (36), suggesting H₂S may regulate erythropoiesis at multiple levels. These findings raise the possibility that CSE/H₂S signaling serves as a critical regulator balancing iron availability in both deficiency and excess states.

The current study examines acute iron loading effects (24 hours post-injection), but chronic models will be essential to determine whether these early alterations progress to sustained vascular

dysfunction, fibrosis, or hypertension. Future investigations should explore whether H₂S's role in iron metabolism extends to influencing red blood cell quality and systemic iron homeostasis in anemia, potentially revealing novel therapeutic targets for iron-related disorders.

5.6. Conclusion and Perspectives

This study reveals that CSE/H₂S deficiency impairs iron homeostasis, leading to vascular iron accumulation, oxidative stress, and diminished H₂S-mediated vasorelaxation, ultimately contributing to early hypertensive changes under acute iron overload. These findings underscore the critical role of H₂S as a protective factor against iron-induced vascular injury. Future research should explore chronic iron-loading models, sex-specific responses, and the broader impact of H₂S in other organs, including the heart, brain, and erythropoietic system. Therapeutic strategies aimed at restoring H₂S signaling—such as exogenous donors (e.g. slow-release H₂S hydrogels (9)) or interventions boosting endogenous H₂S production (e.g., metformin or dietary restriction (37–40))—may mitigate oxidative stress, improve iron regulation, and prevent vascular dysfunction. Investigating these approaches, particularly in chronic settings, could offer translational insights for reducing cardiovascular risk in patients undergoing iron therapy or with H₂S metabolism disorders, while also informing therapeutic strategies for anemia-related iron dysregulation.

5.7. Author Contributions:

H.M.A.: conceptualization, methodology, data analysis, writing original draft. M.F.: methodology, data analysis. R.V.: methodology, R.W.: data analysis, writing, funding acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

5.8. Funding:

This study was supported by a Discovery grant to RW (RGPIN-2023-05099) from the Natural Sciences and Engineering Research Council of Canada.

5.9. Institutional Review Board Statement:

The animal study protocol was approved by The Animal Care Committee, York University (2019-11 R1)

5.10. Data Availability Statement:

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

5.11. Conflicts of Interest:

The authors declare no conflicts of interest.

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5.13. Supplemental Information

Supplementary Table S1. Summary of statistical analyses for all experimental outcomes presented in the main figures. The table lists the corresponding figure panels, measured variables, statistical tests performed, and the associated F-statistics (or equivalent test results) with degrees of freedom and p-values. Two-way ANOVA was used to assess the effects of genotype, treatment, and their interaction. In cases where one factor had no measurable values or changes (e.g., non-detectable expression or no visible production activity), one-way ANOVA was applied to evaluate treatment effects within the measurable group. Fgenotype = effect of genotype; Fconc = effect of iron concentration; Fint = genotype × concentration interaction.

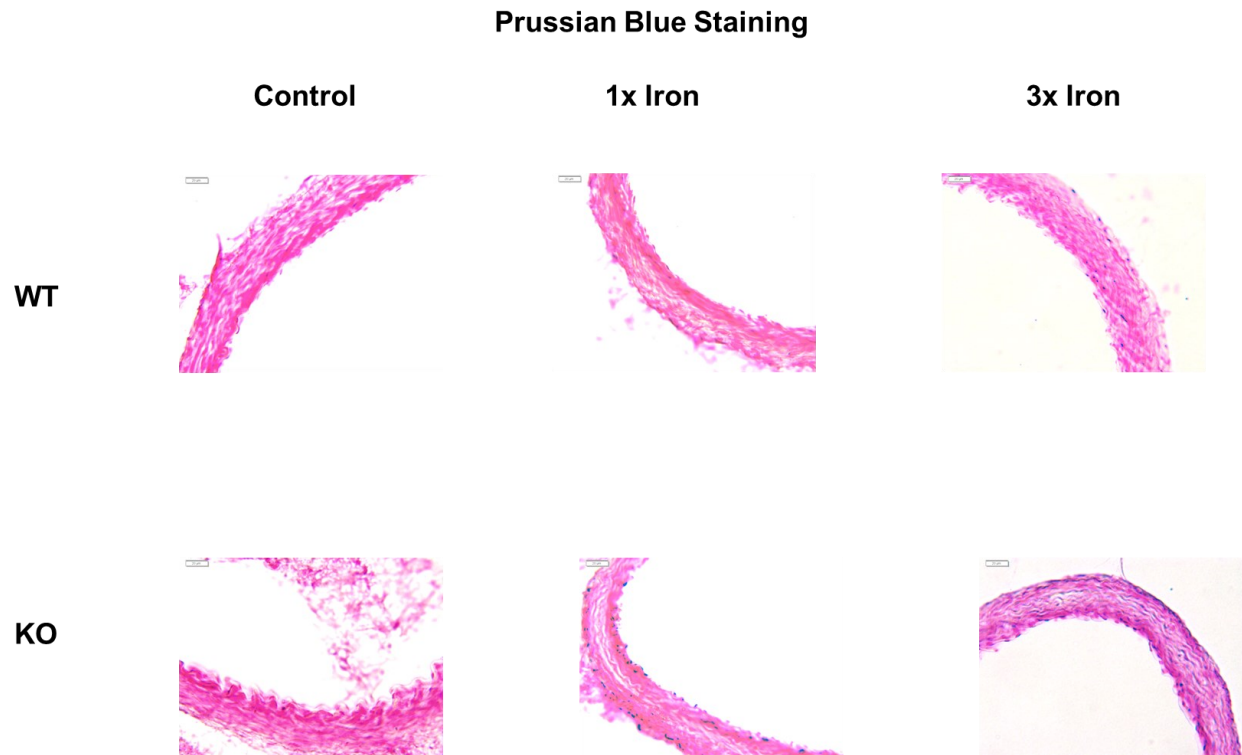
Figure	Measured Variable	Statistical Test	F-Statistics
Fig.1b,	Serum iron	Two-way ANOVA	(Fgenotype(1,42) = 182.3; Fconc(2,42) = 122.2; Fint(2,42) = 51.9; all p < 0.0001)
Fig.1c	Tstat	Two-way ANOVA	(Fgenotype(1,42) = 171.4; Fconc(2,42) = 128.5; Fint(2,42) = 62.3; all p < 0.0001)
Fig. 1d	Serum Ferritin	Two-way ANOVA	(Fgenotype(1,42) = 74.2; Fconc(2,42) = 141.5;

			$F_{int}(2,42) = 34.5$; all $p < 0.0001$)
Fig.1e	Plasma H ₂ S	Two-way ANOVA	$F_{genotype}(1,42) = 322.3$; $F_{conc}(2,42) = 4.28$; $F_{int}(2,42) = 11.3$; all $p < 0.05$
Fig.1h	Liver CSE/GAPDH	One-way ANOVA	$F(2,15) = 18.85$, $p < 0.0001$
Fig.1i	Liver Ferritin/GAPDH	Two-way ANOVA	$F_{genotype}(1,30) = 417.5$; $F_{conc}(2,30) = 385.0$; $F_{int}(2,30) = 382.5$; all $p < 0.0001$
Fig.1j	Liver H ₂ S production rate	One-way ANOVA	$F(2,15) = 9.66$, $p = 0.0020$
Fig.1k	Liver Hepcidin	Two-way ANOVA	$F_{genotype}(1,30) = 15.6$; $F_{conc}(2,30) = 35.1$; $F_{int}(2,30) = 8.5$; all $p < 0.01$
Fig.2b	Aorta CSE/GAPDH	One-way ANOVA	$F(2,15) = 27.56$, $p < 0.0001$

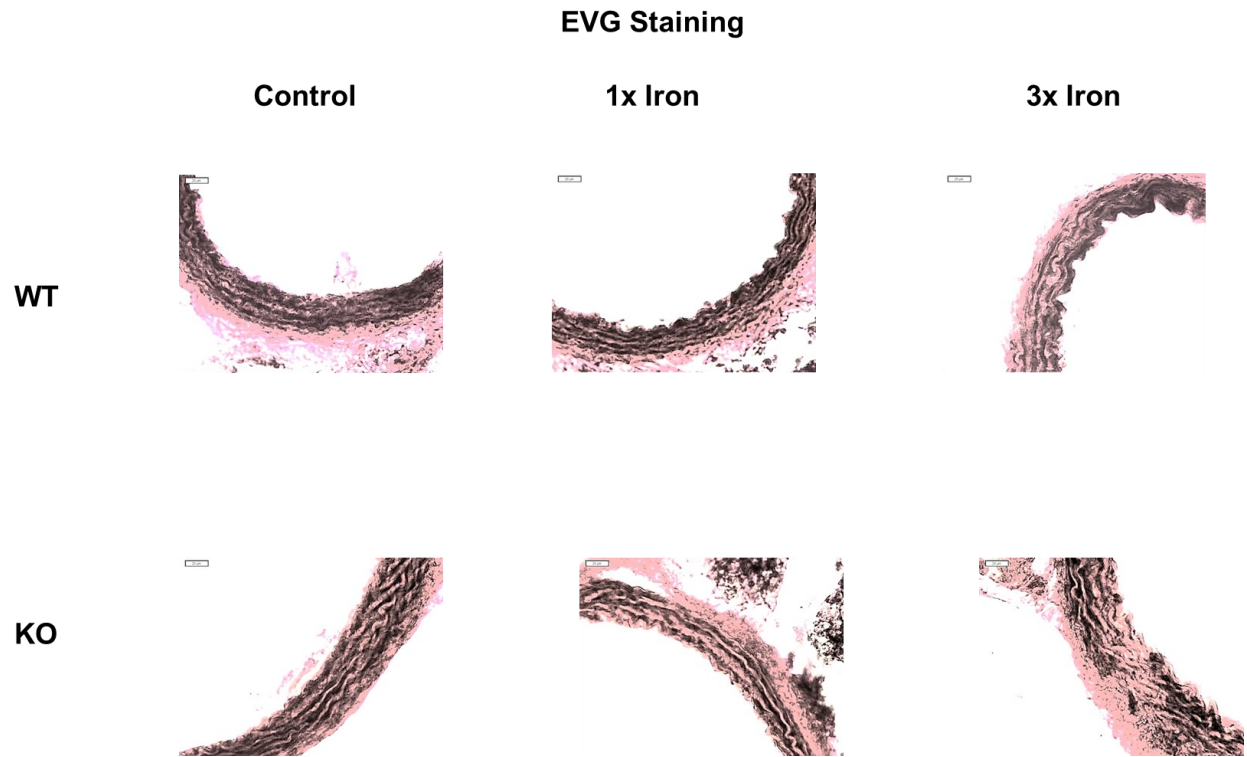
Fig.2c	Aorta Ferritin/GAPDH	Two-way ANOVA	Fgenotype(1,30) = 34.7; Fconc(2,30) = 22.7; Fint(2,30) = 37.9; all p < 0.0001
Fig.2e	Aorta DMT1/GAPDH	Two-way ANOVA	Fgenotype(1,30) = 98.5; Fconc(2,30) = 53.1; Fint(2,30) = 67.3; all p < 0.0001
Fig.2f	Aorta TfR1/GAPDH	Two-way ANOVA	Fgenotype(1,30) = 13.6; Fconc(2,30) = 26.8; Fint(2,30) = 6.7; all p < 0.01
Fig.2g	Aorta FPN/GAPDH	Two-way ANOVA	Fgenotype(1,30) = 15.6; Fconc(2,30) = 71.1; Fint(2,30) = 4.4; all p < 0.05
Fig.3b	Prussian Blue	Two-way ANOVA	Fgenotype(1,30) = 39.7; Fconc(2,30) = 39.7; Fint(2,30) = 39.7; all p < 0.0001

Fig.3c	CD68/DAPI	Two-way ANOVA	Fgenotype(1,30) = 59.9; Fconc(2,30) = 59.9; Fint(2,30) = 59.9; all p < 0.0001
Fig.3d	α -SMA/DAPI	Two-way ANOVA	Fgenotype(1,30) = 23.7; Fconc(2,30) = 11.6; Fint(2,30) = 4.6; p < 0.05
Fig.3e	MMP9/DAPI	Two-way ANOVA	Fgenotype(1,30) = 508.2; Fconc(2,30) = 508.2; Fint(2,30) = 508.2; all p < 0.0001
Fig.3f	EVG	Two-way ANOVA	Fgenotype(1,30) = 11.9; Fconc(2,30) = 45.2; Fint(2,30) = 7.1; all p < 0.01
Fig.4c	Optimal Pretension	Two-way ANOVA	Fgenotype(1,30) = 4.53, p < 0.05; Fconc(2,30) = 15.4, p < 0.0001; Fint(2,30) = 1.34, ns

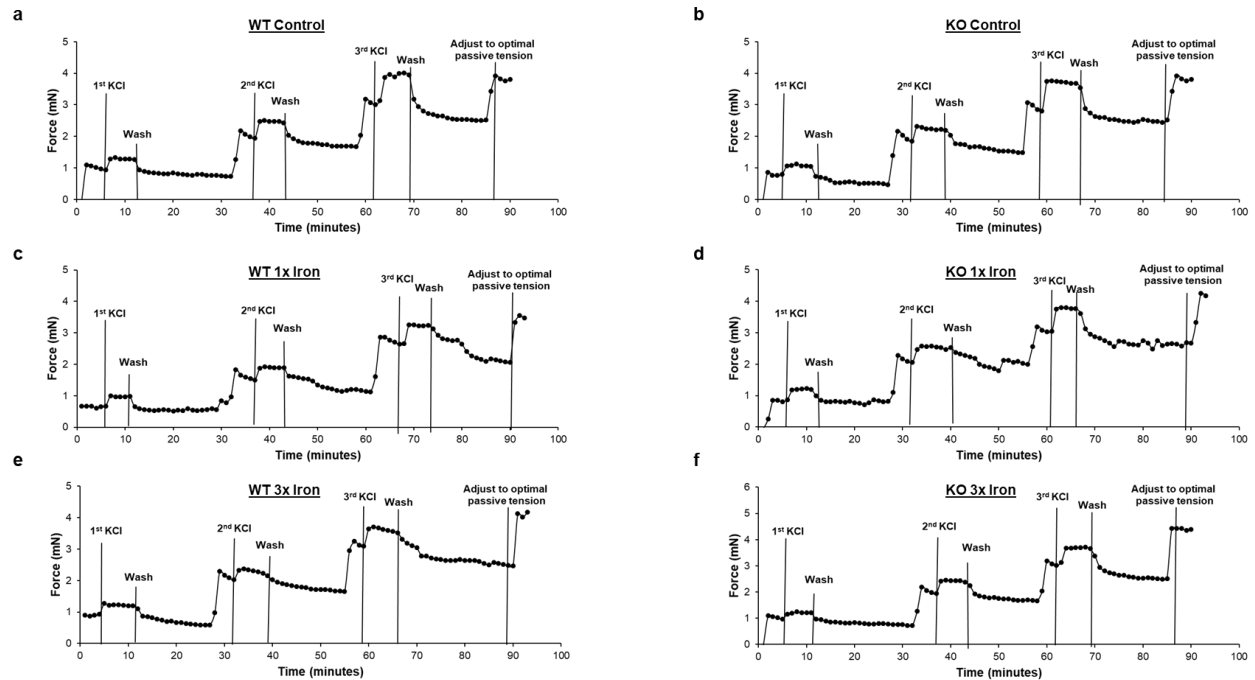
Fig.4d	Vasoconstriction response	Two-way ANOVA	Fgenotype(1,30) = 58.4; Fconc(2,30) = 60.9; Fint(2,30) = 11.6; all p < 0.001
Fig.4k	H ₂ S Vasodilation response	Two-way ANOVA	Fgenotype(1,30) = 18.1; Fconc(2,30) = 95.3; Fint(2,30) = 4.8; all p < 0.05
Fig.4l	Systolic	Two-way ANOVA	Fgenotype(1,42) = 2151.8; Fconc(2,42) = 416.8; Fint(2,42) = 147.3; all p < 0.0001
Fig.4l	Diastolic	Two-way ANOVA	Fgenotype(1,42) = 37.9, p < 0.0001; Fconc(2,42) = 3.2, p = 0.05; Fint(2,42) = 5.4, p < 0.01



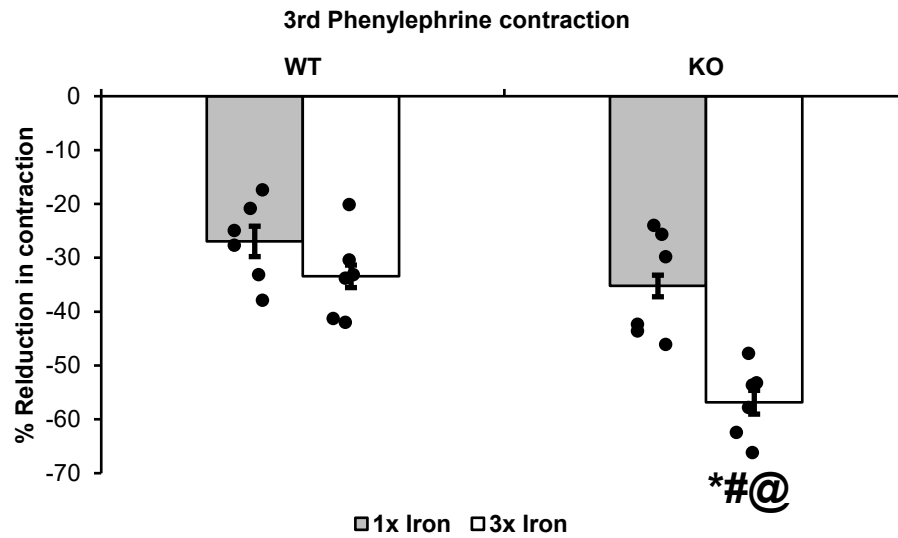
Supplementary Figure S1. Representative high-resolution images of Prussian Blue staining in aortic cross-sections from WT and CSE-KO mice following control, 1× iron, or 3× iron treatment. Scale bars = 20 μ m.



Supplementary Figure S2. High-resolution EVG-stained images of aortic cross-sections from WT and CSE-KO mice following acute iron loading (Control, 1× iron, 3× iron). Scale bars = 20 μ m.



Supplementary Figure S3. Representative original traces of vascular reactivity of WT and CSE-KO aortic rings. Time–force traces show DMT normalization for determining optimal passive tension. (a, b) Representative traces from WT and KO control groups, respectively. (c, d) Representative traces from WT and KO 1× iron groups, respectively. (e, f) Representative traces from WT and KO 3× iron groups, respectively.



Supplementary Figure S4. Reduction in third phenylephrine (PE)-induced contraction following iron loading in WT and CSE-KO mice. (a) Percent reduction in contraction in WT and KO aortic rings with 1× and 3× iron loading, calculated relative to the respective genotype controls. Bars represent mean ± SEM with individual data points overlaid (n = 6 per group). Effects of mouse genotypes, iron loading concentrations, and their interaction were assessed by two-way ANOVA ($F_{\text{genotype}(1,20)} = 17.86$, $p = 0.00041$; $F_{\text{concentration}(1,20)} = 22.70$, $p = 0.00012$; $F_{\text{interaction}(1,20)} = 5.17$, $p = 0.034$). Significance symbols represent Tukey's post-hoc comparisons. * $p < 0.05$ vs. WT 1× iron; @ $p < 0.05$ vs WT 3× iron; # $p < 0.05$ vs. KO 1× iron.

Chapter 6: Discussion and Future Directions

6.1. Key findings

Across the research presented in this thesis, a coherent and progressively developed mechanism emerges in which cystathionine γ -lyase (CSE)-derived hydrogen sulfide (H_2S) functions as an iron-responsive, redox-tuning signal. This signaling pathway coordinates multiple layers of cellular defense: iron export through ferroportin, iron sequestration via ferritin, antioxidant defense mediated by NRF2 activation, prevention of apoptosis through ATF4 signaling, and activation of autophagy via AMPK. Collectively, these actions position H_2S not merely as a secondary signaling molecule but as an integrative regulator of iron and redox homeostasis. Applying physiologically relevant ferric ammonium citrate (0.1 mM provides approximately 17 μM free iron complexed with 100 μM citrate) to vascular smooth muscle cells (VSMCs), we demonstrated that endogenous H_2S limits the accumulation of intracellular labile iron and suppresses ROS generation. In contrast, CSE deficiency permits labile iron to build up and shifts the mode of viability of VSMCs toward apoptosis at lower iron loads but toward ferroptosis at higher iron exposures.

Mechanistically, iron activates the integrated stress response (ISR), culminating in ATF4-driven upregulation of CSE. The resulting increase in H_2S promotes KEAP1 S-sulphydration and triggers NRF2 nuclear translocation, thereby reinforcing antioxidant defenses. In addition, H_2S facilitates the switch of IRP1 to its aconitase form, permitting ferritin translation, while simultaneously activating AMPK to induce autophagy. Together, these outcomes restore both iron buffering capacity and redox balance. Conversely, in the absence of CSE/ H_2S signaling, iron-mediated increases in ATF4 through the ISR converge on caspase-3 activation, ultimately triggering apoptosis. This duality emphasizes that while the ISR–ATF4 axis can be adaptive in the presence of H_2S , it becomes maladaptive and pro-apoptotic in the absence of H_2S .

The results from Chapter 3 define H₂S as a molecular fulcrum coupling iron metabolism to survival signaling. In CSE-KO cells, iron loading was associated with increased ROS generation through Fenton reaction and lipid peroxidation, as well as activation of apoptotic pathways marked by elevated caspase-3. These cellular vulnerabilities pointed to mitochondria as a critical site of stress integration, since mitochondria represent the principal source of ROS under physiological and pathological conditions, and can initiate caspase-3 activation through cytochrome c release. This recognition made it essential to assess mitochondrial health and bioenergetic parameters under conditions of iron loading in both WT and CSE-KO cells. Such analysis would not only clarify how cells attempt to maintain energy balance in the face of oxidative stress but also reveal whether H₂S contributes to mitochondrial resilience under these conditions.

Our results of cellular bioenergetics demonstrated that iron-exposed CSE-KO VSMCs lose maximal respiration, spare respiratory capacity, and mitochondrial membrane potential, but increased proton leak—hallmarks of mitochondrial dysfunction. In contrast, WT VSMCs preserved mitochondrial performance under the same iron load, maintaining higher basal and maximal respiration, stable membrane potential, and lower proton leak. Notably, CSE-KO cells operated near their bioenergetic ceiling even at baseline, which explained their fragility when challenged with oxidative stress. Exogenous H₂S partially restored respiration and membrane potential, and this observation highlights the importance of H₂S donors, as therapeutics for mitochondrial protection.

Mechanistically, the mitochondrial outcomes in Chapter 3 integrate with the findings from our results shown in Chapter 2: when ferritin upregulation and NRF2 antioxidant defenses fail to limit labile iron accumulation, mitochondria emerge as the primary site of vulnerability; conversely, H₂S-driven coordination of iron metabolism and antioxidant defenses, integrated with AMPK-linked

autophagy and mitophagy preserves bioenergetic resilience. Within this framework, the intrinsic apoptotic pathway becomes particularly relevant, since mitochondrial outer membrane permeabilization results in cytochrome c release and activation of downstream caspases, culminating in cell death (1). In contrast, the extrinsic (death receptor-mediated) pathway is triggered by the binding of extracellular ligands such as TNF- α or Fas ligand to their respective receptors, leading to caspase-8 activation and subsequent execution of apoptosis independent of mitochondrial involvement (1). Although cytochrome c release was not directly measured in these experiments, the mitochondrial membrane depolarization observed in iron-loaded CSE-KO cells, together with the mitochondrial dysfunction and caspase-3 activation identified in Chapter 2 under the same conditions, strongly supports the interpretation that apoptosis in KO cells proceeded predominantly through the intrinsic pathway. Mitochondrial integrity is particularly critical in VSMCs because their contractile and synthetic functions are tightly coupled to mitochondrial bioenergetics and calcium handling (2). Disruption of mitochondrial homeostasis therefore not only predisposes to cell death but also compromises vascular tone and remodeling capacity (2). These vulnerabilities highlight the translational implication that enhancing endogenous H₂S production or supplementing with sustained-release H₂S donors could stabilize mitochondrial function in iron-linked conditions such as diabetes and neurodegeneration, where low H₂S levels frequently coexist with iron dysregulation (3, 4).

A long-standing barrier to translational research on H₂S biology and therapeutics has been the reliable quantification of circulating H₂S. The classical methylene blue method, although simple and widely used, suffers from low sensitivity and specificity, and is prone to significant overestimation due to interference from thiols, proteins, and hemolysis-derived artifacts (5). The

HPLC-based monobromobimane (MBBR) method provides improved specificity by derivatizing H₂S and separating fluorescent products chromatographically, but it requires strict anoxic handling, specialized equipment, and rapid sample processing (6). These practical constraints have hindered its widespread adoption in routine translational or clinical workflows and created an unmet need for a more standardized, robust, and accessible method.

To address these limitations, we developed the HPLC-based methylene blue/methylene green (MBMG) method as described in Chapter 4. This assay incorporates stabilization endogenous H₂S with ascorbate and DTPA at the time of sample collection, the use of methylene green as an internal standard to correct for plasma matrix effects, organic phase clean-up, and chromatographic separation to avoid spectrophotometric overestimation. The MBMG method quantifies circulating H₂S in the physiologically relevant sub-micromolar range (~0.5 μ M in WT mice; ~0.15 μ M in CSE-KO mice), achieves six-month frozen stability of the derived methylene blue, and minimizes hemolysis-related artifacts that otherwise may inflate apparent H₂S via iron-catalyzed H₂S generation (7). This analytical foundation was essential for interpreting systemic changes of H₂S concentrations in our animal experiments, and more importantly, it provides a standardized, low-cost, and practical solution for multi-site or offline analyses in clinical studies where full anoxic workflows are not feasible.

To extend the mechanistic and methodological findings into a physiological context, we employed mouse models (WT and CSE-KO) of acute iron loading to investigate the impact of CSE deficiency on vascular iron handling and vascular function in vivo. This approach allowed direct assessment of vascular remodeling, vasomotor responses, and blood pressure as primary outcomes, while also incorporated systemic measures such as hepcidin expression and hepatic iron storage in

vascular tissues to provide a more comprehensive view of systemic iron regulation. In an acute iron-loading model, CSE-KO mice exhibited elevated serum iron and transferrin saturation, a blunted hepcidin response, and critically impaired ferritin upregulation in both liver and aorta. This dysregulation resulted in vascular iron deposition, elastin degradation, α SMA loss, and heightened CD68/MMP9 expression signaling inflammation and vascular remodeling. Functionally, KO vessels displayed reduced phenylephrine-induced contraction and attenuated H₂S-mediated relaxation, accompanied by increased blood pressure in the acute iron loading window.

In contrast, WT mice responded to acute iron loading with adaptive upregulation of CSE and elevated H₂S levels in both aorta and liver. These changes were accompanied by increased ferritin expression and appropriate downregulation of transferrin receptor 1 (TfR1), thereby buffering labile iron accumulation and limiting oxidative stress. Structural integrity of the vasculature was largely preserved, with only minimal α SMA and elastin loss and no evidence of inflammatory cell infiltration. Functionally, WT vessels showed a modest reduction in phenylephrine-induced contraction and H₂S-mediated relaxation compared with controls, but these changes were far less pronounced than in KO mice. Importantly, blood pressure remained unchanged, highlighting the protective capacity of intact H₂S signaling under acute iron stress.

Taken together, the *in vivo* findings presented in Chapter 5 demonstrate that defective iron handling in the absence of H₂S drives vascular remodeling, impaired vasomotor responses, and elevated blood pressure, whereas intact H₂S signaling preserves vascular integrity and function. This experimental framework also reflects clinical contexts in which iron surges coincide with low H₂S—such as hereditary hemochromatosis, chronic kidney disease with intravenous iron therapy, and type 2 diabetes—highlighting the therapeutic potential of restoring H₂S to mitigate iron-driven

vascular injury (3, 8–11) . Collectively, this thesis outlines a continuum from cytosolic iron handling and redox control to mitochondrial resilience and whole-vessel physiology, firmly establishing H₂S as a key determinant of systemic adaptation and vascular health.

6.2. Methodological Considerations and Experimental Design Rationale

This thesis was designed to interrogate how iron overload intersects with endogenous CSE–derived H₂S signaling to shape vascular stress responses and functional outcomes. To achieve mechanistic clarity from cellular level up to whole animals, experimental models and conditions were selected to prioritize physiological relevance while minimizing confounding sources of variability that could obscure iron–H₂S–dependent effects.

VSMCs were used as the primary cellular model because they constitute the principal effector cell population responsible for regulating vascular tone, contractility, and structural remodeling. Alterations in VSMC stress adaptation, mitochondrial function, and survival directly translate into changes in arterial stiffness, vasomotor responsiveness, and blood pressure regulation. Accordingly, cellular studies were structured to define VSMC-intrinsic responses to iron burden and H₂S signaling, while complementary *in vivo* and *ex vivo* experiments captured integrated vascular function at the level of systemic blood pressure regulation and aortic tissues, where additional cell types contribute to the integrated regulation of vascular functions.

While VSMCs represent the principal effector cells governing vascular tone and contractility, vascular function *in vivo* reflects coordinated interactions among multiple cell types within the vessel wall. Endothelial cells play a central regulatory role by modulating smooth muscle contraction, vascular inflammation, and redox balance through the release of endogenous

vasoactive mediators, including nitric oxide and H₂S, which directly influence VSMC signaling, mitochondrial function, and stress adaptation (5, 12, 13). Endothelial dysfunction under iron overload conditions can therefore amplify oxidative stress, alter paracrine signaling, and indirectly shape smooth muscle–dependent functional outcomes (10).

In addition, fibroblasts and extracellular matrix (ECM) components critically determine vascular stiffness, mechano-transduction, and load-bearing properties of the vessel wall, thereby influencing how cellular stress responses translate into changes in systemic blood pressure regulation (14). Iron-induced remodeling of the ECM or altered matrix–cell interactions may further modify vascular compliance independent of VSMC-intrinsic effects. Resident immune cells and vascular progenitor populations also contribute to inflammatory signaling, repair processes, and remodeling responses during chronic stress, and may modulate disease progression under conditions of iron overload (10, 15).

Although the present thesis focused on VSMC-intrinsic mechanisms to establish a clear mechanistic link between iron stress and CSE/H₂S signaling, these additional vascular cell types likely participate in shaping the integrated physiological phenotype observed *in vivo*. Importantly, the systemic blood pressure measurements used in this study inherently reflect the combined influence of smooth muscle, endothelium, ECM, and inflammatory components within the intact vasculature. Future studies will be required to dissect cell-type–specific contributions and intercellular signaling networks, particularly under chronic iron loading or in female models, to fully resolve how iron and H₂S signaling coordinate vascular adaptation and dysfunction.

To preserve VSMC viability and functional integrity during isolation, a commercially defined Type II collagenase preparation (Worthington Biochemical; CLS-2, LS004174) was used for dissociation of thoracic aorta. Although the vascular tissue is enriched with type I collagen (16), collagenase selection in our study is guided by overall enzymatic activity and tissue dissociation behavior rather than collagen subtype specificity. The CLS-2 formulation contains a balance of collagenase and associated protease activities that is routinely applied for dissociation of structurally robust tissues, including cardiac, skeletal muscle, and vascular preparations, and differs from Type I collagenase formulations (e.g., CLS-1, LS004194) that are more commonly used for softer tissues such as liver, lung, or adipose. Accordingly, Type II collagenase was selected following manufacturer-recommended use and established vascular isolation protocols to ensure effective liberation of vascular smooth muscle cells while preserving cell viability and phenotype. Consistent with this selection, Ray *et al.* demonstrated that Type II collagenase yields superior recovery and viability of vascular smooth muscle cells from murine aorta, whereas substitution with other collagenase preparations resulted in inferior isolation outcomes (17). Type II collagenase has been used extensively and successfully to isolate VSMCs from mouse or rat aortic tissues in our own laboratory (12, 18, 19) and by other teams (20–22).

VSMCs were isolated from eight-week-old mice to ensure analysis of fully developed yet non-aged vasculature. At this stage, vascular structure and contractile machinery are mature, while age-associated remodeling, fibrosis, and baseline oxidative stress remain minimal. This design choice minimized confounding influences arising from developmental signaling or vascular aging, allowing iron- and H₂S-dependent effects to be examined under stable baseline conditions (14).

Male mice were used in our study during acute mechanistic interrogation of iron–H₂S signaling. This sex choice was for maintaining a stable hormonal background, as fluctuations in estrogen across the estrous cycle are known to modulate CSE expression and endogenous H₂S production. Limiting hormonal variability enabled clearer attribution of observed cellular, mitochondrial, and vascular responses to iron burden and CSE/H₂S status under defined experimental conditions (23).

Sex-specific differences in iron homeostasis have been consistently documented in clinical and population-based studies and vary substantially with age and reproductive status. In women of reproductive age, iron stores are generally lower due to ongoing menstrual iron loss, whereas iron indices increase progressively following the menopausal transition. Longitudinal analysis from the Study of Women's Health Across the Nation (SWAN) demonstrated that serum ferritin increased from a mean of approximately 70 ng/mL in premenopausal women (mean age ~48 years) to approximately 130 ng/mL after menopause (mean age ~53 years), consistent with a significant rise in iron stores across the menopausal transition (24). Comprehensive clinical reviews further indicate that postmenopausal iron accumulation approaches levels commonly observed in males, coinciding with increased cardiometabolic and vascular risk (8).

Accumulating evidence links elevated iron burden with endothelial dysfunction, oxidative stress, arterial stiffening, and impaired vascular reactivity, highlighting iron as a contributor to vascular pathology in both sexes (10). Importantly, these findings indicate that iron-driven vascular stress emerges in a sex- and age-dependent manner rather than uniformly across the lifespan. Together, these observations underscore that biological sex introduces clinically relevant heterogeneity in iron handling and vascular risk, complicating direct comparison across experimental groups and reinforcing the need for sex-stratified investigation in future studies.

6.3. Limitations and Future Work

While this thesis establishes a coherent mechanistic framework linking hydrogen sulfide (H₂S) to iron regulation, redox control, and vascular adaptation, several experimental boundaries must be acknowledged. These limitations primarily relate to model scope, duration of exposure, tissue specificity, and representation of biological sex. Accordingly, the following section outlines future research directions designed to address these boundaries and further advance the mechanistic, methodological, and translational insights presented herein.

Building on these considerations, future work should expand and deepen the mechanistic framework established in this thesis. One important direction is the further investigation of mitochondrial iron handling and bioenergetics. While our work demonstrated a role for IRP1–cytosolic aconitase in coordinating ferritin translation and redox balance the contributions of mitochondrial aconitase and iron–sulfur (Fe–S) cluster biogenesis remain largely uncharacterized in this context. Exploring how H₂S signaling intersects with Fe–S cluster formation and stabilization could provide insight into mitochondrial iron trafficking pathways that remain incompletely defined in vascular tissues. Complementary approaches could include live-cell or ex vivo imaging of mitochondrial health in intact vessels using mitochondrial membrane potential–sensitive dyes or genetically encoded biosensors, alongside functional readouts such as mitochondrial respiration in freshly isolated vascular rings. These experiments would help bridge molecular regulation with tissue-level mitochondrial physiology.

Our findings that H₂S enhances ferritin expression and curbs labile iron accumulation were validated in VSMCs, aorta, and liver, but these molecular interactions should be extended to other tissues of physiological and clinical importance. The heart represents an especially relevant target, as the CSE/H₂S system is absent there (25). The endothelium is another promising focus, where MST is an important H₂S-producing enzyme, raising the possibility of tissue-specific regulatory mechanisms (13). Functional outcomes beyond ferritin regulation should also be explored—for example, whether H₂S-mediated iron control supports angiogenesis in endothelial cells, or whether it modulates cardiomyocyte contractility and survival under iron overload. Such studies would determine the breadth of H₂S–iron crosstalk across organ systems and its potential relevance to systemic iron disorders.

The MBMG method developed in this thesis provides a practical tool for quantifying circulating H₂S and should be leveraged in future work to chart circulatory H₂S levels across diverse disease models. Mapping circulating H₂S concentrations alongside systemic and tissue-specific iron parameters (e.g., ferritin, transferrin saturation, labile iron pool) would clarify whether correlations exist between disrupted H₂S metabolism and iron imbalance in pathophysiological states. Such cross-sectional datasets would provide valuable baselines for translational studies and might identify biomarker profiles predictive of iron-mediated vascular injury.

The acute iron-loading model used in this thesis generated marked phenotypes in CSE-KO mice, including impaired ferritin induction, vascular iron deposition, inflammatory remodeling, αSMA loss, blunted contractility, reduced H₂S-mediated vasorelaxation, and elevated blood pressure. Nevertheless, chronic iron-loading model remains essential for

understanding long-term disease processes and the durability of H₂S-mediated protection. Chronic iron exposure could reveal whether various signaling pathways that were preserved under acute iron stress, such as NO–sGC–mediated relaxation and acetylcholine-dependent vasodilation, become impaired with prolonged iron overload. Parallel studies in cultured cells and in vivo models would allow molecular dissection of adaptive versus maladaptive responses, thereby mapping the progression from acute injury to chronic vascular pathology.

Sex as a biological determinant also demands careful consideration in future studies. The present work focused on male mice to minimize confounding, but accumulating evidence suggests that estrogen regulates H₂S production. Indeed, our previous work demonstrated that estrogen enhances the generation of CSE-derived H₂S in liver and vascular tissues (23). Incorporating both sexes into chronic iron-loading models would clarify whether female mice, particularly WT animals, display greater resilience to iron-induced vascular damage compared to males, while CSE-KO mice remain equally vulnerable regardless of sex. MBMG-based plasma H₂S measurement, combined with vascular iron indices such as ferritin, labile iron, DMT1, TFR1, and FPN, would provide a robust dataset for dissecting sex- and hormone-dependent influences. Importantly, chronic rather than acute iron loading protocols will be required to account for age- estrous cycle–related fluctuations in estrogen and other reproductive hormones. Insights from these experiments could extend to clinically relevant conditions such as diabetes, Alzheimer’s disease, or Parkinson’s disease, where sex differences in disease burden are well documented (15, 26, 27).

Finally, once circulating H₂S has been charted across disease models, interventional studies are warranted to determine whether restoring H₂S improves outcomes. Both direct H₂S donors and indirect approaches, such as metformin administration—which increases tissue H₂S via CSE (28)—should be tested. Atherosclerosis represents a particularly informative context, as iron overload accelerates lesion progression. Studies in iron-loaded mice fed an atherogenic diet, with or without metformin, could determine whether boosting H₂S attenuates vascular iron deposition and atherogenesis. Such experiments would not only test the therapeutic potential of H₂S but also clarify whether restoring H₂S can mitigate iron-mediated aggravation of chronic vascular disease.

Taken together, these future directions emphasize the importance of extending the present findings beyond acute vascular models to encompass mitochondrial iron handling, tissue- and sex-specific regulation, chronic disease contexts, and interventional strategies. By integrating mechanistic insight with methodological innovation, the field is now positioned to determine whether restoring or augmenting H₂S can provide durable protection against iron-driven vascular injury. Such efforts will not only clarify the breadth of H₂S–iron interactions across organ systems but also define their translational potential in complex disease settings, ultimately bridging basic vascular biology with clinically relevant therapies.

6.4. Concluding perspective

In summary, the research presented in this thesis establishes cystathionine γ-lyase–derived H₂S as a central regulator of vascular iron handling, redox balance, mitochondrial resilience, and systemic vascular adaptation. Through integrated studies spanning molecular, cellular,

methodological, and in vivo approaches, we demonstrate that intact H₂S signaling preserves iron buffering, limits oxidative stress, sustains mitochondrial function, and protects vascular integrity, whereas its deficiency precipitates iron deposition, mitochondrial failure, vascular remodeling, and blood pressure elevation. By developing a robust method for quantifying circulating H₂S, we also provide a practical foundation for translational investigation. While limitations remain—including the focus on acute iron loading, exclusion of sex as a variable, and the need for broader tissue and disease contexts—the mechanistic and methodological groundwork outlined here sets the stage for future exploration of chronic models, sex-specific effects, and therapeutic interventions. Collectively, these findings position H₂S not merely as a modulator of redox biology but as a fundamental determinant of vascular health, offering a clear framework for translating basic insights into strategies that address iron-driven cardiovascular and systemic disease.

6.5. References

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