H_2S *S*-sulfhydration of pyruvate carboxylase in gluconeogenesis and its regulation by Trx1

A Thesis Presented to the Faculty of Graduate Studies of

Lakehead University

By

YoungJun Ju

Submitted in partial fulfillment of requirements

for the degree of Doctor of Philosophy in Biotechnology

September 04, 2015

PERMISSION TO USE STATEMENT

I agree that the libraries of Lakehead University may make this thesis freely available for inspection. I further agree that permission for copying of this thesis in any manner for scholarly purpose may be granted by the professors who supervised this thesis or the Head of Graduate studies of Lakehead University in their absence. It is understood that use of this thesis in any manner for financial gain shall not be allowed without my expressed written permission. It is also understood that due recognition shall be given to me and Lakehead University in any scholarly use which may be made of any material of this thesis.

Requests for permission to copy or make other use of material in this thesis in whole or in part should be addressed to:

Head of Graduate studies,

Lakehead University,

955 Oliver Road,

Thunder Bay, Ontario,

Canada P7B 5E1

ABSTRACT

Hydrogen sulfide (H_2S), regarded as the third gasotransmitter, plays diverse physiological and pathological roles in the body along with another two gasotransmitters, including nitric oxide (NO) and carbon monoxide (CO). In the liver, cystathionine gamma-lyase (CSE) is the main enzyme to generate H_2S. The functions of H_2S in the liver are largely unknown even though many studies showed H_2S and CSE are important in redox control of liver. *S*-sulfhydration, a novel post-translational modification of proteins, is now considered as an important mechanism of H_2S effects under various physiological and pathological conditions.

Pyruvate carboxylase (PC), a mitochondrial enzyme, is an enzyme in the first step of gluconeogenesis in the liver. PC plays a critical role in tricarboxylic acid (TCA) cycle in mitochondria and in gluconeogenesis in the liver. PC is also involved in diverse metabolic pathways, such as lipogenesis and the biosynthesis of neurotransmitters. Here we found that H_2S regulates gluconeogenesis through PC *S*-sulfhydration. First, PC activity and glucose production were decreased in liver of CSE-knockout (KO) mice comparing to the liver of wild type (WT) littermates. PC *S*-sulfhydration was lower in the liver tissue of CSE-KO mice than WT mice. NaHS which is commonly used for the H_2S study as a H_2S donor treatment induces glucose production and PC activity in the primary liver cells from mice. PC *S*-sulfhydration was increased by NaHS treatment in a time–dependent manner. Through mutation study with site direct mutagenesis, we further confirmed that the cysteine residue 265 is responsible for H_2S *S*-sulfhdyration of PC. In cysteine 265 mutant-transfected HEK293 cells, PC activity was significantly decreased and PC *S*-sulfhydration was abolished.

Thioredoxin 1 (Trx1), a well-known redox protein, is reported to be involved in transnitrosylation and/or denitrosylation of proteins. Here we first demonstrated that Trx1 acts as a *S*-desulfhydrase. Overexpressed Trx1 in HEK293 and HepG2 cells diminished H_2S

induced *S*-sulfhydration of both glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and PC proteins. The *S*-desulfhydration activity of Trx1 was inhibited by PX12, an inhibitor of Trx1, and DNCB, an inhibitor of thioredoxin reductase. Furthermore, the cysteine 32 in Trx1 is required for the direct interaction of Trx1 with GAPDH or PC, and mutation of cysteine 32 abolished the *S*-desulfhydration activity of Trx1.

Taken together, our studies demonstrated that H_2S induces the gluconeogenesis through PC *S*-sulfhydration, and Trx1 acts as a *S*-desulfhydrase. Our findings will help to understand the mechanisms of H_2S regulation of metabolic pathways via *S*-sulfhydration. These understandings will also extend the knowledge of physiological and pathological roles of H_2S in both health and diseases.

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisors, Drs. Guangdong Yang and Rui Wang, who provided me encouragement and support over the course of my thesis and gave me a wonderful opportunity to work in this world class laboratory. I am proud of involving in this laboratory.

I learned the passion for science from Dr. Guangdong Yang and the keen scientific vision from Dr. Rui Wang during my PhD program. I appreciate the patient guidance, encouragement, and advice from Dr. Guangdong Yang for my thesis projects. Special thanks to Dr. Rui Wang, his vision and motivation for science showed me the scientist who I want to be. Both supervisors encouraged me every moment to improve myself and get the confidence in science as a real scientist. Drs. Neelam Khaper and Wensheng Qin as my graduate supervisory committee members supported me with great helps for my research. I also would like to thank Dr. Heidi Schraft, the former Graduate Coordinator of the program, for her helpful and considerate guidance program. Dr. Kam Leung showed me the model of friendship in science and academy.

I also wish to thank all members of the Cardiovascular & Metabolic Research Unit, past and present, for sharing the knowledge for research and creating such a good atmosphere in the lab. It has been a great pleasure to work with them.

I would like to acknowledge the financial support provided by Graduate Assistantship at Lakehead University and Faculty Research scholarship.

Finally, I would like to thank to my family, grandparents, parents, brother, and sister to support me wholeheartedly. Without their supports, I cannot successfully finish my thesis. I appreciate my wife, MiSung′s support and patience during my PhD program. Her unwavering support is what brought me here to do my work pleasantly and successfully. I cannot imagine

winning this out without her. Thank you, lady.

What a wonderful day! I would like to thank all my family, my friends, and whoever supports me, again. I hope world peace and happiness to everyone!

DEDICATION

The thesis is dedicated to my parents, wife, brother, sister and grandparents who support and love me all the way.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3MST, 3-mercaptopyruvate sulfurtransferase;

ADT-OH, 5-(4-hydroxyphenyl)-3*H*-1,2-dithiol-3-thione;

AOA, Aminoxyacetic acid;

BCA, Beta-cyanoalanine;

BSA, Biotin switch assay;

BMT, Bisulfide methyltransferase;

CBS, Cystathionine beta-synthase;

CREB, cAMP-responsive element binding protein;

CSE, Cystathionine gamma-lyase;

DMEM, Dulbecco's Modified Eagle's medium;

DNCB, 2,4-dinitrochlorobenzene;

DTT, Dithiothreitol;

ETHE1, Mitochondrial persulfide dioxygenase enzyme;

FBP, Fructose 1,6-bisphosphatase;

Foxa2/HNF3beta, Forkhead transcription factor boxA2;

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase;

G6Pase, Glucose 6-phosphatase;

GSNO, *S*-nitrosoglutathione;

GSSH, Glutathione persulfide;

GSSG, Oxidized glutathione;

H_2O_2, Hydrogen peroxide;

H_2S, Hydrogen sulfide;

HAT, Histone acetyltransferase;

HDAC, Histone deacetylase;

HFD, High fat diet;

KO, Knockout;

MDH, Malate dehydrogenase;

MMTS, Methyl methanethiosulfonate;

NADH, Reduced nicotinamide adenine dinucleotide;

NAD^+, Oxidized nicotinamide adenine dinucleotide;

NMDA, N-methyl-D-aspartate;

PBS, Phosphate buffered saline;

PC, Pyruvate carboxylase;

PEPCK, Phosphoenolpyruvate carboxykinase;

PPG, Propargylglycine;

PRMTs, Peptidylarginine methyltransferases;

ROS, Reactive oxygen species;

SQR, Sulfide-quinone oxidoreductase;

SQRDL, Sulfide-quinone reductase-like protein;

Trx1, Thioredoxin 1;

TrxR, Thioredoxin reductase;

Txnip, Trx-interacing protein;

TSR, Thiosulfate reductase;

TSST, Thiosulfate sulfurtransferase;

WT, Wild type.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Hydrogen Sulfide (H_2S)

1. 1 Gasotransmitters

Gasotransmitter refers to a gaseous messenger molecule which is synthesized and released into the extracellular spaces to transit a series of signals and affect various cellular functions (1). The characterization of gasotransmitters has been clearly stated by Dr. Rui Wang (2) as: 1) small molecules of gas; 2) freely permeable to membranes; 3) endogenously and enzymetically generated and the generation is regulated; 4) well-defined specific functions at physiologically relevant concentrations; 5) mandatory to have specific cellular and molecular targets. The classification for gasotransmitters recently was updated including one more criteria as described below (3). 1) small molecule of gas, dissolved in biological milieu or not; 2) freely permeable to membrane not rely on cognate membrane receptors or other transportation machineries; 3) endogenously generated in mammalian cells with specific substrates and enzymes; 4) well-defined specific functions at physiologically relevant concentrations; 5) functions of endogenous gases can be mimicked by their exogenously applied counterparts; 6) specific cellular and molecular targets, and involved in signal transduction.

Gasotransmitters play important roles in various physiological and pathological condition, such as blood pressure regulation, neurotransmitter release, inflammation, cell growth and differentiation, and longevity, as reviewed in the article (4). Their unique roles have been received attentions since these simple inorganic compounds, such as hydrogen sulfide (H_2S), nitric oxide (NO), and carbon monoxide (CO), act as important biological messengers in various cellular metabolisms and functions of vertebrates. After the identification of NO and CO as the first two gasotransmitters, the effects of H_2S in physiological processes has been demonstrated and now H_2S is well recognized as the third gasotransmitter (1, 3).

1. 2 H_2S

H_2S is a colorless, flammable, water-soluble gas with a strong odor of rotten eggs. This gas is generally produced by bacteria breakdown of waste material, and is found in volcanic gases and hot springs in nature, or contaminant from petroleum industry (5). Its toxicity has been known for more than 300 years in humans and animals (6). It is lethal in mammals above 1,000 ppm when inhaled, and it causes severe malfunctions when inhaled above safe levels, such as anxiety, irritation to eyes and respiratory tract, fatigue, and headache (2).

In spite of its toxicity, H_2S has received attention as a physiological molecule since it is found in the body and plays a significant role in mammals. In cardiovascular system, H_2S induced vasorelaxation was found in numerous types of blood vessels, such as aorta, portal vein, mesenteric artery, cerebral arteries, and vas deferens, from different species (rats, mice, cows, guinea pigs, sheep, and humans) (5). The vasorelaxant effect of H_2S results from the modulation of K_{ATP} channel (7-10). In central nervous system, H_2S also exerts multifaceted and important effects through the modulation of neurotransmitters, as reviewed in the article (5). Glutamate is an important neurotransmitter which is well known for its role in learning and memory in mammalian brain (11). It was found that H_2S selectively enhances N-methyl-D-aspartate (NMDA) receptor-mediated currents and expedites the induction of hippocampal long-term potentiation (LTP) in rats (12,13). In endocrine system, H_2S-induced inhibition of insulin release was shown in INS-1E cells from a pancreatic insulinoma cell line (14) and in another insulin-secreting cell line, HIT-T15 (15) through the stimulation of K_{ATP} channels. Furthermore, diverse roles of H_2S were found in various conditions of body, such as inflammation, cardiac protection, hypoxic damage, and etc (16). However, many things about the regulatory mechanisms of H_2S on physiological roles in various tissues still remain unclear even though diverse roles of H_2S were reported as described above.

1. 3 Biosynthesis of H_2S

The biosynthesis of H_2S in mammals occurs via enzymatic and non-enzymatic pathways (2). In enzymatic biosynthesis of H_2S, it can be synthesized endogenously in mammalian tissues from L-cysteine by cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CSE), and/or 3-mercaptosulfurtransferase (3-MST) (17). The expression of these enzymes is tissue specific. In some tissues, CBS, CSE and 3-MST are all needed for the generation of H_2S, whereas in others, one enzyme suffices. H_2S is synthesized mainly by CBS in the brain, whereas H_2S is mostly generated by CSE in the liver, kidney, and vascular smooth muscles (2). In liver and kidney, 3-MST expression was higher than other tissues and 3-MST is mainly localized in mitochondria (18). However, CSE is responsible for H_2S production more than 3-MST in liver and kidney and 3-MST is expressed relatively more than CSE in other tissues (19). Based on its localization, 3-MST is responsible for the H_2S production in mitochondria (20,21). Other sources of H_2S is generated via non-enzymatic reduction of elemental sulfur, which is derived from the reducing equivalents of the oxidized glucose during glycolysis (22). H_2S production through non-enzymatic reduction of elemental sulfur is minor in the cells compared to enzymatic production. The equation of H_2S production in glycolysis is (22): $\mathbf{2C_6H_{12}O_6 + 6S^0 + 3H_2O \rightarrow 3C_3H_6O_6 + 6H_2S + 3CO_2}$

H_2S production via non-enzymatic pathways is further described in 1. 3. 4 Non-enzymatic synthesis of H_2S. In gastrointestine, H_2S can be uptaken from bacteria which can produce H_2S. In the mucosa of the gut, cells and tissues are continuously exposed to H_2S produced by sulfate-reducing bacteria, such as *Desulfovibrio, Desulfomicro-bium, Desulfobulbus, Desulfobacter, Desulfomonas,* and *Desulfotomaculum* genera (23). Patients with chronic fatigue syndrome (CFS) are shown with higher levels of H_2S in intestine and higher concentrations of intestinal bacteria than normal (24). Abnormal chronic exposure of high concentration of H_2S may be involved in the pathological effects in CFS. The healthy

microbiota is an important factor to regulate normal concentration of H_2S in gastrointestine.

1. 3. 1 CBS

CBS (EC 4.2.1.22) is a member of the beta family of pyridoxal phosphate-dependent enzyme to catalyze homocysteine to cystathionine in the first irreversible step of the transsulfuration pathway. Cystathionine is subsequently converted to cysteine, alpha-ketobutryate, and H_2S by CSE (Fig. 1. 1). CBS is composed with 551 amino acids and molecular weight of 63 kDa. The gene of CBS is located on chromosome 21 in humans or 17 in mouse (25,26). The active form of CBS is a homotetramer and the enzyme activity is dependent on the heme group (protoporphyrim 1X). CBS is an essential enzyme for homocysteine metabolism in the cells (27). Human CBS deficiency, which is caused by inherited rare disorder, influences the homocysteine catabolism, and causes hyperhomocystinuria (28). Furthermore, CBS deficiency is associated with a wide range of clinical symptoms such as mental retardation, ectopia lentis, osteoporosis, skeletal abnormalities and hepatic steatosis (29).

1. 3. 2 CSE

CSE (EC 4.4.1.1) is composed of 398 amino acids and the molecular weight is 43 kDa, and CSE gene is located on chromosome 1 (2). CSE catalyzes cystathionine to cysteine, alpha-ketobutyrate, and ammonia in the second step of transsulfuration pathway (Fig. 1. 1). CSE then catalyses cysteine to H_2S, ammonia, and pyruvate (30). CSE plays a critical role for supplying cysteine in various cellular functions (31,32). Cysteine is a necessary component for the synthesis of glutathione (GSH), a major intracellular antioxidant. CSE is critically involved in the biogenesis and regulation of GSH in physiological conditions (33-35). CSE is expressed in many tissues such as the cardiovascular system, respiratory system, liver, kidney,

placenta, and pancreatic islets (7,36,37). Its expression is tissue-specific (2,5,12,38). In human, CSE deficiency is caused by autosomal recessive inheritance, which leads to cystathioninuria (39). The hypercystathioninemia triggers the development of atherosclerosis and cancer (40,41).

1. 3. 3 3-MST

Recently, it was found that MST (EC 2.8.1.2) is involved in the H_2S synthesis in the cells. 3-MST is located in both mitochondria and cytosol, while it mainly contributes to generate H_2S in mitochondria (20,21). 3-MST catalyzes the sulfur transfer reactions from 3-mercaptopyruvate (3-MP) to sulfite or other sulfur acceptors or forming elemental sulfur as shown in Fig 1. 1. Many studies on the physiological roles of 3-MST have been done in brain and neuron system (20,42,43). 3-MST was found in vascular endothelium of thoracic aorta and it plays an important role for the cellular bioenergenetic response in mitochondria (21,44). However, human deficiency of 3-MST is rare and it is not life-threatening (45).

1. 3. 4 Non-enzymatic synthesis of H_2S

Non-enzymatic synthesis of H_2S is a minor source of total endogenous H_2S generation in mammalian systems (2). H_2S is generated from glucose, thiocystine, glutathione and thiosulfate in non-enzymatic synthesis as shown in Fig 1. 1 (46). Glucose and phosphogluconate can produce H_2S via glycolysis and NADPH oxidase, respectively. In glycolysis, reducing equivalents of the glucose oxidation pathway mediates to reduce elemental sulfur to H_2S. H_2S is also generated through direct reduction of glutathione and thiocystine. Reductive reaction of thiosulfate can generate H_2S from thiosulfate through the reaction with pyruvate, which is a hydrogen donor in this reaction.

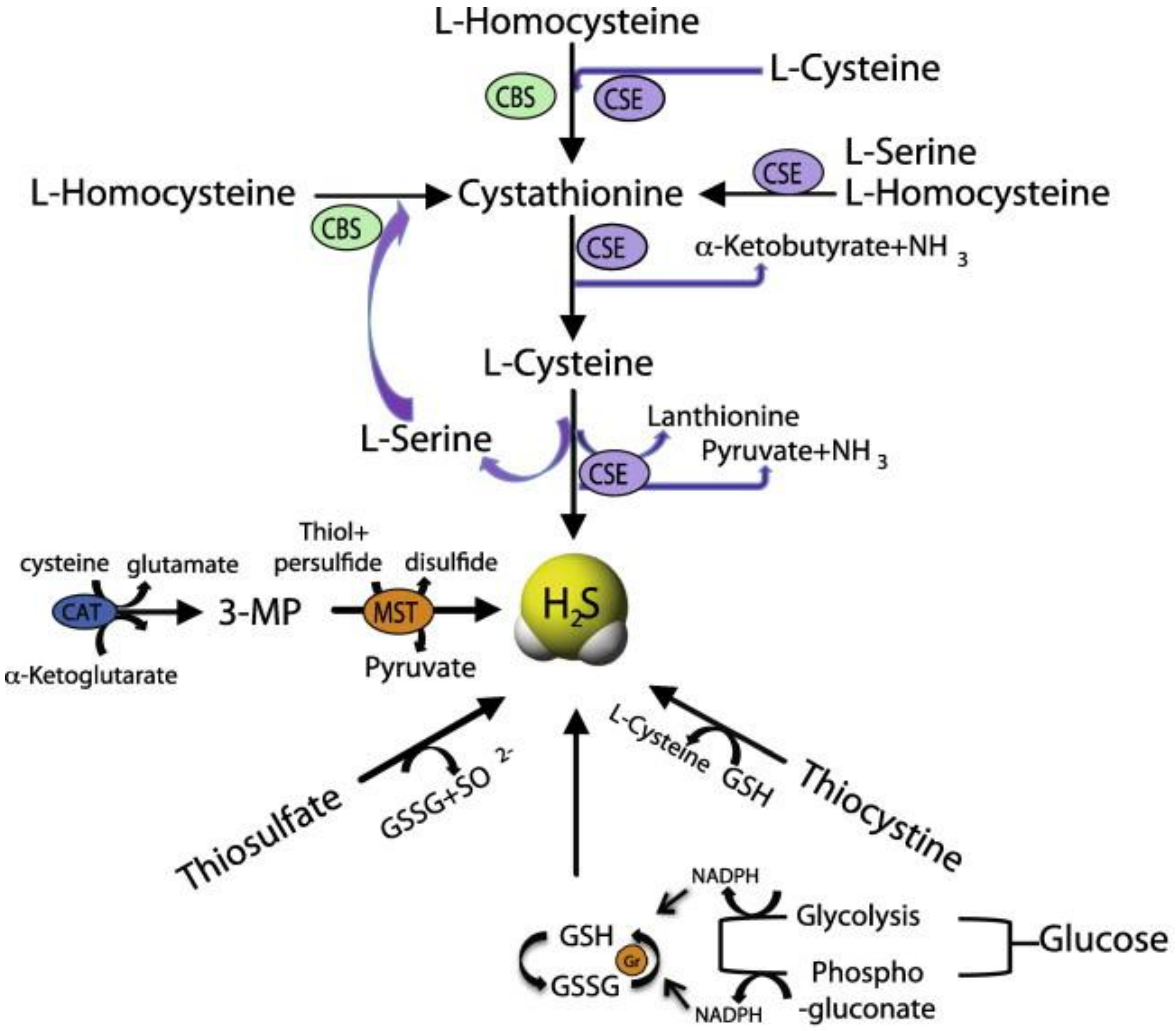


Figure 1. 1 Enzymatic and non-enzymatic synthesis of H_2S (46). Cystathionine β-synthase (CBS), Cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (MST) are responsible for generating H_2S in the mammalian systems. Through glucose, glutathione and polysulfides, non-enzymatic synthesis of H_2S occurs under physiological condition.

1. 4 H_2S metabolism

Endogenous H_2S in the cells exists in various forms, such as free sulfide, acid-labile, and bound sulfide as cellular bioavailable pools. Cellular redox status or acidic pH may regulate the mobility of H_2S from these pools for physiological responses (46). H_2S is metabolized through expiration and excretion, methylation, scavenging, and oxidation in the body (Fig. 1. 2). Firstly, H_2S can be metabolized into methanethiol and thiosulfate by bisulfide methyltransferase (BMT) and thiosulfate reductase (TSR) enzymes respectively as shown in Fig 1. 2. Produced thiosulfate is further oxidized to sulfite by thiosulfate sulfurtransferase (TSST) and subsequently to sulfate (46). Methanethiol can be methylated to dimethylsulfide. Alternately, H_2S can be scavenged by methemoglobin or metallo- or disulfide-containing molecules such as oxidized glutathione (GSSG) (2,6,47). BMT and TSR are located in cytosol and periplasm, respectively. TSST is a mitochondrial enzyme.

It was recently found that sulfide-quinone reductase-like protein (SQRDL), the verterbrate homolog of sulfide-quinone oxidoreductase (SQR), contributes to H_2S metabolism in mammalian cells (48). SQR catalyses bisulfide (SH^-) and quinine to polysulfide and quinol. SQR mediates in regulation of H_2S levels in yeast (49). In various tissues in rats, it was found that SQRDL plays the same role as SQR in mitochondria (48). These researches showed the possibility that SQRDL can be responsible for the regulation of H_2S metabolism in mitochondria, along with TSST.

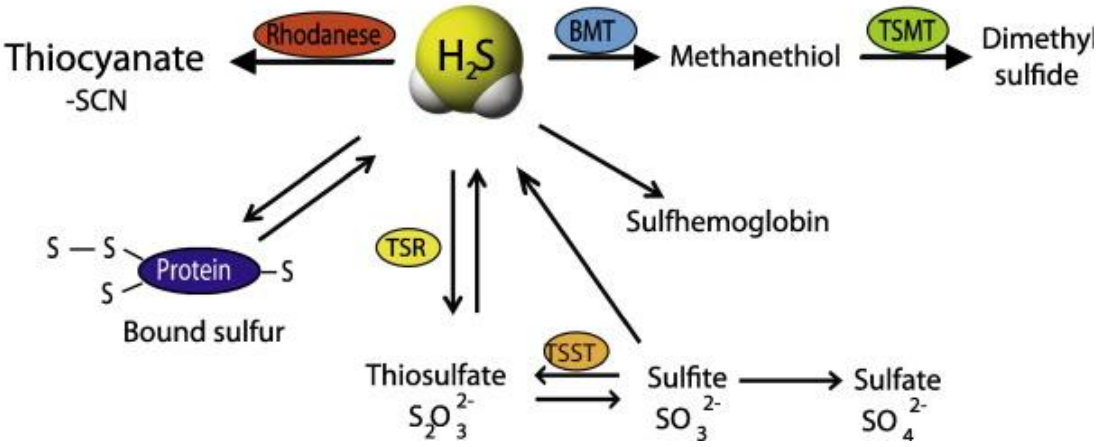


Figure 1. 2 Metabolism of H_2S (46). H_2S is metabolized into thiocyanate, methanethiol and thiosulfate catalyzed by rhodanese, bisulfide methyltransferase (BMT) and thiosulfate reductase (TSR) enzymes respectively. Thiosulfate can be further oxidized to sulfite through thiosulfate sulfurtransferase (TSST) and subsequently to sulfate. H_2S reacts with hemoglobin to form sulfhemoglobin and with proteins in the tissues in the form of bound sulfur pool.

1. 5 Pharmacological inhibitors of H_2S producing enzymes

The development of pharmacological inhibitors of H_2S producing enzymes and H_2S releasing drugs are critical for the study of H_2S effects on various physiological and pathophysiological events. Inhibitors for each H_2S-generaing enzyme are shown in Table 1. 1. Propargylglycine (PPG), commonly used for a specific inhibitor of CSE, targets the pyridoxal phosphate binding site of CSE and irreversibly inhibits CSE activity. Aminoxyacetic acid (AOA) is an inhibitor for aminotransferase and is proposed as an inhibitor of CBS (50). To date, AOA and beta-cyanoalanine (BCA) are claimed as a selective inhibitor of CBS. However, it was shown that AOA and BCA can inhibit effectively CSE as well (51). Furthermore, it was found that BCA more selectively inhibits CSE than CBS and is a more potent inhibitor for CSE than PPG with a reversible inhibition (51). 3-mercaptopropionic acid is a non-competitive inhibitor of 3-MST. The specificity of these inhibitors are still questionable (5).

These pharmacological inhibitors of H_2S-generating enzymes, including PPG, BCA, and AOA, have been employed to test the biological effect of inhibition of endogenous H_2S production in several studies. PPG or BCA (50 mg per kg) in rats subjected to haemorrhagic shock accelerated the recovery of arterial blood pressure (52). In rodent models of local inflammation, PPG treatment (25-75 mg per kg) dose-dependently decreased paw oedema and paw neutrophil infiltration in a carrageenan-induced inflammation model of rat (53), and PPG (100 mg per kg) attenuated pancreatic necrosis and reduced the degree of acute lung injury in a cerulean model of pancreatitis (54). Furthermore, it was recently found that AOA treatment (0.2 mM, daily) inhibits colon cancer growth in patient-derived tumor xenografts and breast cancer growth *in vivo* (55).

1. 6 H_2S releasing drugs (donors)

NaHS and Na_2S are two well-used H_2S donor. NaHS and Na_2S are convenient to handle, however they are not appropriate for the study of long term exposure since H_2S are easily evaporated and H_2S is released immediately from NaHS and Na_2S in biological media. Within 2 hours, H_2S is not able to be detected in biological media treated by NaHS and Na_2S (56,57). Nowadays, many H_2S releasing drugs are developed for investigating the physiological effects of H_2S. Those are convenient for controlling the concentration of H_2S in biological media and H_2S releasing can be lasted longer compared with NaHS and Na_2S (57-62). Sustaining H_2S concentration in culture medium is observed when incubation of the cells with GYY4137 (400 μM) from 2 hours to 7 days (57). Other slow-releasing H_2S drugs similar to GYY4137 include *S*-diclofenac (ACS15 and ACS32), *S*-sildenafil (ACS6), *S*-latanoprost (ACS67), and *S*-mesalamine. These drugs are produced from combination of parent compound (drug chemical) and 5-(4-hydroxyphenyl)-3*H*-1,2-dithiol-3-thione (ADT-OH), which in turn break down to H_2S (38). The moiety of ADT-OH is the most widely used for synthesizing slow-releasing H_2S drugs and H_2S releasing drugs are included the moieties of H_2S donor similar to ADT-OH.

GYY4137 and ATB346 (the derivate of naproxen) as well as ACS32 (derivate of *S*-diclofenac) induced cell death of several cancer cell lines but not in normal cells (57,63,64). GYY4137 and ATB346 further exhibit anti-inflammation activity along with ACS85 and ACS86, which are derivates of levadopa (65-71). Cardiovascular protection effects of Thioamide, perthiols, and GYY4137 were further found (72-74). Rhodanine derivates showed anti-virus and anti-microbial activity (75-77). Furthermore, GYY4137 regulates ion channels similar to H_2S roles as an opener of ion channel proteins (78,79). The list and functions of H_2S releasing drugs are shown in Table 1. 2.

Recently, functional H_2S releasing donors are under investigation. General H_2S

donors are lacked to mimic the slow and continuous H_2S generation process *in vivo*. Cysteine activated H_2S donors, as a controllable H_2S donor, which can produce H_2S in presence of excess cysteine were developed (73, 74, 80). H_2S releasing from donors can be regulated by the cysteine concentration. However, further biological data will be required to evaluate the pharmacological functions of donors. For the study of H_2S releasing donors, cytotoxicity of donors is critical in the cell. H_2S releasing donors based on ADT-OH induced cellular trafficking of ADT-OH and then toxic to the cells. To prevent the cytotoxicity of H_2S releasing donors based on ADT-OH, a poly (ethylene glycol)-ADT was developed (81). It was successfully to release H_2S in macrophages and was not shown obvious cytotoxicity compared to H_2S releasing donors based on ADT-OH. Furthermore, mitochondrially-targeted H_2S releasing donor, AP39, [(10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl) triphenylphosphonium bromide], was developed and investigated the effects of AP39 on bioenergetics, viability, and mitochondrial DNA integrity (82). The control of H_2S releasing location in the cells is a great benefit to study of pharmacological and physiological effects of H_2S.

Table 1. 1 Inhibitors of H_2S producing enzyme

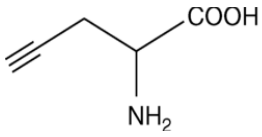
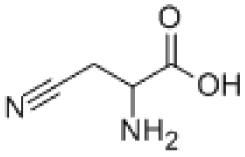
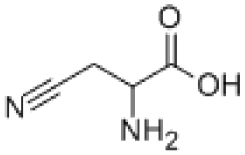
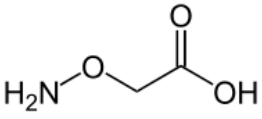
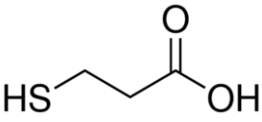
Enzyme	Inhibitor	Structure	Reference
CSE	Propargylglycine (PPG)		(83)
	Beta-cyanoalanine (BCA)		(51)
CBS	Beta-cyanoalanine (BCA)		(84)
	Aminoxyacetic acid (AOA)		(85)
3-MST	3-mercaptopropionic acid		(86)

Table 1. 2 H_2S releasing drugs

Donor	Effect	Reference
GYY4137	Ion channel regulation	(78,79)
	Cardiovascular protection	(72)
	Anti-cancer	(57)
	Anti-inflammation	(70,71,87,88)
Rhodanine derivatives	Anti-HIV	(75)
	Anti-microbial	(76,77)
ATB346	Anti-cancer	(64)
	Reduce toxicity and anti-inflammation	(65,66)
	Anti-nociceptive and anti-inflammation	(67)
ACS32	Anti-cancer	(63)
ACS85 and ACS86	Nervous protection and anti-inflammation	(68,69)
Thioamide	Cardiovascular protection	(73)
Perthiols	Cardiovascular protection	(74)

1. 7 Liver

1. 7. 1 Gluconeogenesis in liver

Glucose metabolism is a critical biochemical process in liver since glucose is an energy source in mammalian systems. Especially, liver regulates glucose level through gluconeogenesis. Insulin inhibits gluconeogenesis in the liver, while glucagon and glucocorticoid induce gluconeogensis to produce glucose.

Gluconeogenesis is a metabolic pathway that generates glucose from non-carbohydrate carbon substrates such as pyruvate, lactate, glycerol, and glucogenic amino acids. When the glucose level drops (hypoglycemia) in blood, there are two ways for supplying glucose including degradation of glycogen via glycogenolysis and gluconeogenesis from non-carbohydrate carbon source. In animals, gluconeogenesis takes place mainly in the liver and cortex of kidneys. Targeting gluconeogenesis is an efficient strategy for the therapy of type 2 diabetes. Metformin, an antidiabetic drug, is well known for the inhibition of glucose formation and stimulation of glucose uptake by cells (89).

Gluconeogenesis is regulated by a series of enzymes (Fig. 1. 3). Depending on the substrates, gluconeogensis occurs in the mitochondria from pyruvate or from glycerol in the cytoplasm. In the mitochondria, pyruvate carboxylase (PC) catalyzes pyruvate into oxaloacetate. Oxaloacetate is reduced to malate for its transportation to cytosol. Malate is oxidized to oxaloacete in the cystosol for remaining steps of gluconeogenesis. In gluconeogenesis, oxaloacetate is decarboxylated and then phosphorylated to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PEPCK). The next steps are the reverse direction of glycolysis from phosphoenolpyruvate to glucose.

PC is a key enzyme in the control of gluconeogenesis. Most factors which are involved in the regulation of gluconeogenesis act through modification of activity or

expression of PC. Aacetyl CoA and citrate activate PC, respectively (90). Global control of gluconeogenesis is mediated by hormones. Those hormones are actually regulated by blood glucose level. When blood glucose is low, glucagon and glucocorticoid are released from pancreas and zona fasciculate of the adrenal cortex, respectively. Once glucagon is released from pancreas, glucagon binds to the glucagon receptors in liver cells. Liver cells then produce glucose through promotion of gluconeogenesis and glycogenolysis. Glucagon turns off glycolysis and provides the glycolytic intermediates to gluconeogenesis in order to synthesize glucose. Also, glucocorticoid stimulates gluconeogenesis in the liver. Glucocorticoid binds to glucocorticoid receptors in the liver and then induces the synthesis of glucose from non-hexose substrates, such as amino acids and glycerol, in gluconeogenesis. Insulin is produced by pancreas when blood glucose is high. Insulin increases glucose uptake in muscle, adipose and several tissues and glycogen synthesis for storage of glucose in liver and muscle cells. In addition, insulin decreases gluconeogenesis for preventing further glucose production from non-hexose (91,92).

Gluconeogenesis is associated with metabolic syndromes, such as diabetes, obesity, etc. Metabolic syndrome is clinically defined by the National Cholesterol Education Program (NCEP) as any three of the following five traits: abdominal obesity, impaired fasting glucose (reflecting insulin resistance), hypertension, hypertriglyceridemia, and low HDL cholesterol (93). Obesity initiates metabolic syndrome through increase of lipogenesis and gluconeogenesis with insulin resistance (93). Increased abdominal fat mass induces the accumulation of intracellular fatty acids and its metabolites (fatty acyl CoA, diacylglyceride), causing insulin resistance. Insulin resistance induces gluconeogenesis to stimulate plasma glucose content. Increased glucose and insulin raises lipogenesis which enhances adipocyte insulin resistance. Gluconeogenesis is critically involved in the development of metabolic syndrome and can therefore be a therapeutic target.

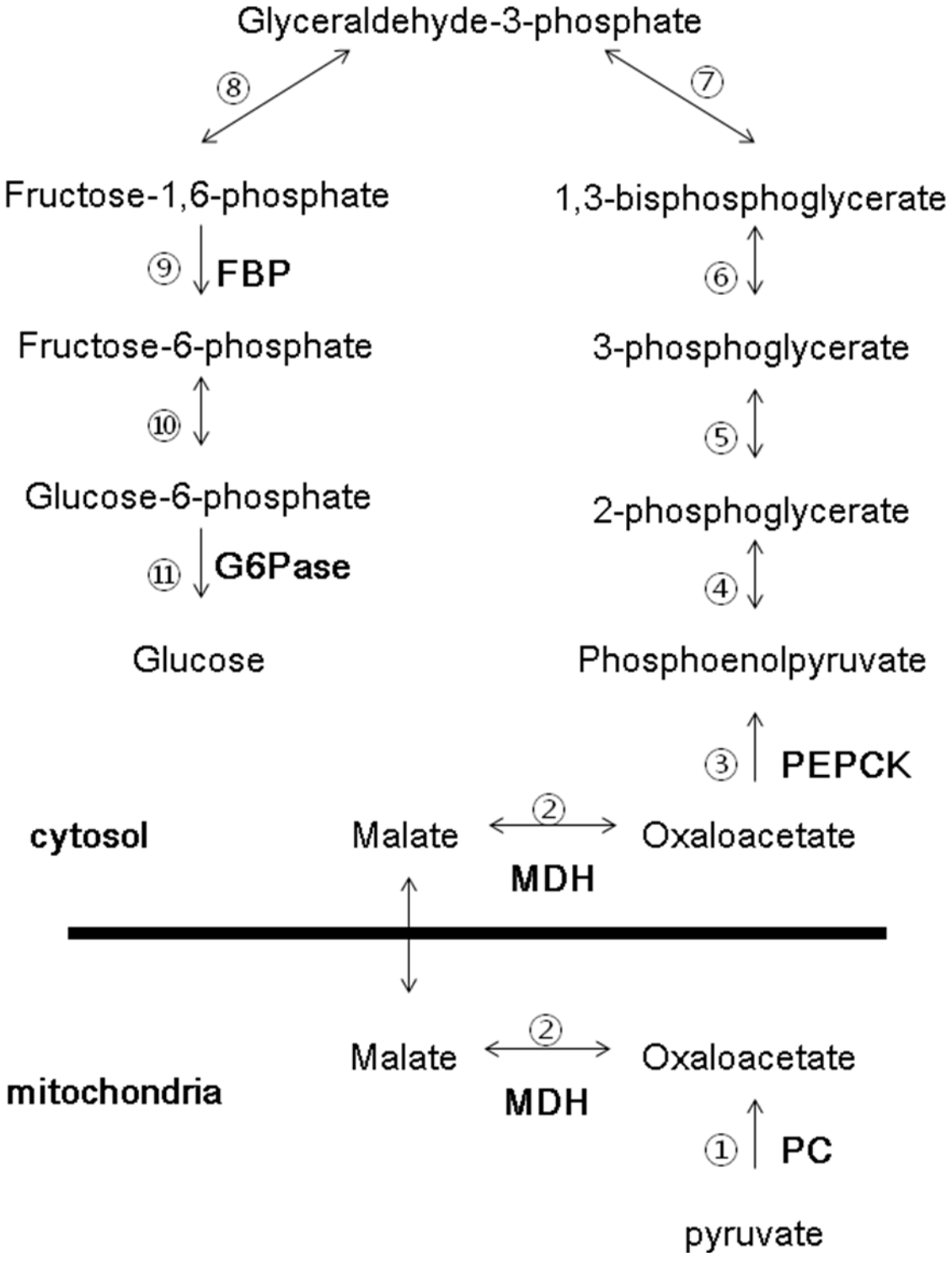


Figure 1. 3 Gluconeogenesis. PC, pyruvate carboxylase; MDH, malate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; FBP, fructose 1,6-bisphosphatase; G6Pase, glucose 6-phosphatase; Number is the sequence of gluconeogenic step in liver.

1. 7. 2 The roles of H_2S in liver

CSE is a main enzyme to generate H_2S in the liver. Deficiency of CSE gene diminished H_2S production by more than 90% in mouse liver (37,94). H_2S affects diverse metabolisms which take place in the liver, such as lipid, glucose, and bioenegetic metabolism (95). Lipid metabolism is critically associated to cholesterol and low density lipoprotein (LDL) cholesterol. In previous study, it has been found that CSE knockout (KO) mice on atherogenic diet showed increased plasma total cholesterol and LDL cholesterol levels and decreased HDL cholesterol compared to wild type (WT) mice (96). It was also reported that H_2S is directly involved in the lipid metabolism (97). H_2S significantly impaired basal and insulin-stimulated glucose uptake and glycogen storage, and enhanced gluconeogenesis and glycogenolysis (98). H_2S induced by MST in the mitochondria of murine hepatoma cell line Hepa1c1c7 enhanced mitochondrial electron transport and cellular bioenergetics (99). Altered hepatic H_2S generation and metabolism have been demonstrated to be involved in the pathogenesis of many liver diseases, such as ischemia/reperfusion injury, hepatic fibrosis and cirrhosis (100-103). Furthermore, H_2S is involved in hepatoprotection. H_2S attenuates hepatic ischemia-reperfusion injury through preservation of intracellular redox balance and by inhibition of apoptosis in the ischemia-reperfusion injury (104).

2. Pyruvate Carboxylase (PC)

PC (EC6.4.1.1) is a nuclear encoded mitochondrial enzyme that catalyses pyruvate into oxaloacetate (105). PC was first discovered in 1959 and isolated from chicken liver (106). In mammals, PC plays a crucial role in gluconeogenesis and lipogenesis, in the biosynthesis of neurotransmitters, and in glucose-induced insulin secretion by pancreatic islets (107). PC is highly expressed in the liver, kidney, adipose, and pancreatic islets (108). The pyruvate carboxylation reaction of PC is shown in Fig 1. 4 (109).

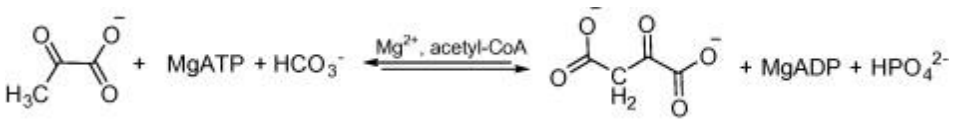


Figure 1. 4 The overall pyruvate carboxylation reaction by PC (109).

PC is an important metabolic enzyme, and its expression is regulated in different pathphysiological conditions, including nutritional alterations, diabetes, hormonal changes, and neonatal development (110). PC activity and expression have been shown to be induced in fasting animals (91,111-113). Hormonal alterations affect PC activity and expression. Hepatic PC activity was increased in hyperthyroid rats, whereas in hypothyroid rats PC was decreased (114). Furthermore, hormonal regulation is involved in the increase of PC activity and expression in fasting through glucagon and glucocorticoids (92). It has been recently found that PC plays an important role in insulin release of pancreatic β-cells in obesity and type II diabetes (115,116), and is involved in the development of insulin resistance in mice (117). PC expression was elevated in genetically obese Zucker fatty rats (118). In humans, PC deficiency is an autosomal, recessively inherited disease (110). PC deficiency leads to congenital lactic acidosis and deterioration of the central nervous system (119,120). In mild PC deficiency, patients suffer from mild-to-moderate lactic acidaemia, delayed development and psychomotor retardation. Patients in severe PC deficiency exhibit severe lactic acidaemia with hyperammonaemia, citrullinaemia, and hyperlysinaemia, and they rarely survive longer than 3 months after birth (110).

PC activity was reduced in the islets of animal models of type 2 diabetes and is a critical factor for the beta cell adaptation to insulin resistance in the rat (121). Furthermore, it was found that decreased PC activity in islet is related to the development of type 2 diabetes in Agouti-K mice (122). In pancreas, reduced PC activity is a pathological factor for the development of type 2 diabetes. However, recent study showed that tissue-specific inhibition

of PC is a potential therapeutic approach for hepatic insulin resistance and type 2 diabetes (116). Specific antisenese oligonucleotide (ASO) for PC decreases PC expression in liver and adipose tissue in rats. Decreased PC activity by ASO reduces gluconeogenesis in liver and improved heaptic insulin sensitivity. Therefore, PC will be a promising therapeutic target for type 2 diabetes and insulin resistance through the regulation of PC activity in tissue-specific manner, for example, PC activity is induced in pancreas and reduced in liver.

2. 1 Regulation and modification of PC

Fasting or starvation enhances glucose production in liver sustained by an increased pyruvate flux through increases of PC activity and expression (123). Transcriptional regulation of PC gene is extensively studied. The distal promoter of the rat PC gene is active in pancreatic islets but the proximal promoter is active in gluconegenic and lipogenic tissue (Fig. 1.5) (107). The basal transcription activity of PC is regulated by Sp1/Sp3 and NF-Y in the distal promoter. Pancreatic specific promoters, such as forkhead transcription factor boxA2 (Foxa2/HNF3beta), upstream stimulatory factors (USF1 and USF2), PDX1, and v-Mafa, are found in pancreatic cells and tissues through analysis of PC gene promoters and identification of its functions in PC regulation (107). In the proximal promoter, Peroxisome proliferator-activated receptor (PPAR) gamma1 and PPARgamma2 are involved in the transcriptional regulation of PC in the adipose tissues. cAMP-responsive element binding protein (CREB) is found to be involved in the regulation of proximal promoter of PC in the liver. PC expression is increased in rodent models of type 1 diabetes which is induced by streptozotocin (124,125) and obese Zucker diabetic fatty rats (126). The proximal promoter in the liver and adipocytes is involved in the increase of PC expression in diabetes, not the distal promoter. Islet PC expression is decreased in severe type 2 diabetic Agouti-K mice and human patients with type 2 diabetes (122,127). The different PC expression among liver,

adipocytes, and pancreas in diabetic conditions results from the different transcriptional regulation of PC by proximal and distal promoter. The detail regulatory mechanisms of PC expression in diabetic conditions are not clearly determined yet. However, these regulations may be related to the development of diabetes and dysfunction of glucose homeostasis in the body**.** For the regulation of PC activity, the allosteric regulation is one of the important factors. Acetyl CoA is a well known allosteric activator of PC. In some bacteria, such as *Mycobacterium smegmatis* and *Rhodobacter capsulatus*, PC activity exhibits a nearly absolute requirement for acetyl CoA while mammalian PC activity is retained between 2 % and 30 % of maximal activity in the absence of acetyl CoA (109). PC activity is dependent on the concentration of Acetyl CoA. Acetyl CoA cooperates with Mg^{2+}, which is an allosteric activator. In yeast PC, it was shown that acetyl CoA and K^+ are the enzyme activator and cysteine 249 in yeast PC is involved in the regulation of binding and action of acetyl CoA and K^+ to active site of PC (128).

Catalytic activity of PC is dependent on the presence of pyruvate, HCO_3^-, and MgATP along with acetyl Co-A and Mg^{2+}, as described above (106). Pyuvate is related to the PC activity, as a main substrate. PC catalytic carboxylation of pyruvate is composed of two steps in distinct active sites (110,129). These two partial reactions occurs in biotin carboxylase (BC) domain and carboxyl transferase (CT) domain. HCO_3^-, and MgATP, except for pyruvate and Acetyl-CoA, are mainly involved in the catalytic reaction of BC domain which is the first partial step of catalytic reaction of PC. HCO_3^- is the substrate in the first partial step of pyruvate carboxylation. MgATP is necessary for the pyruvate carboxylation and acts as a cofactor. Especially, the activation of HCO_3^- is dependent on MgATP. The use of MgATP is required for the diminished electrophilicity of HCO_3^- to form the highly labile carboxylphosphate intermediate via the nucleophilic attack of HCO_3^- on the gamma-phosphate of ATP. However, MgATP-dependent carboxylation did not occur through the

formation of a phophorylated-enzyme intermediate (130). Every intermediate is prepared in the first partial step of pyruvate carboxylation. In CT domain, the second step is occurred that pyruvate reacts with CO_2 to form oxaloacetate.

The post-translational modification on PC has not been well-studied as compared to the PC transcription regulation by hormone. *S*-nitrosylation of PC has been observed in yeast and HeLa cells (131,132), however, the functions and roles of PC *S*-nitrosylation were not determined**.**

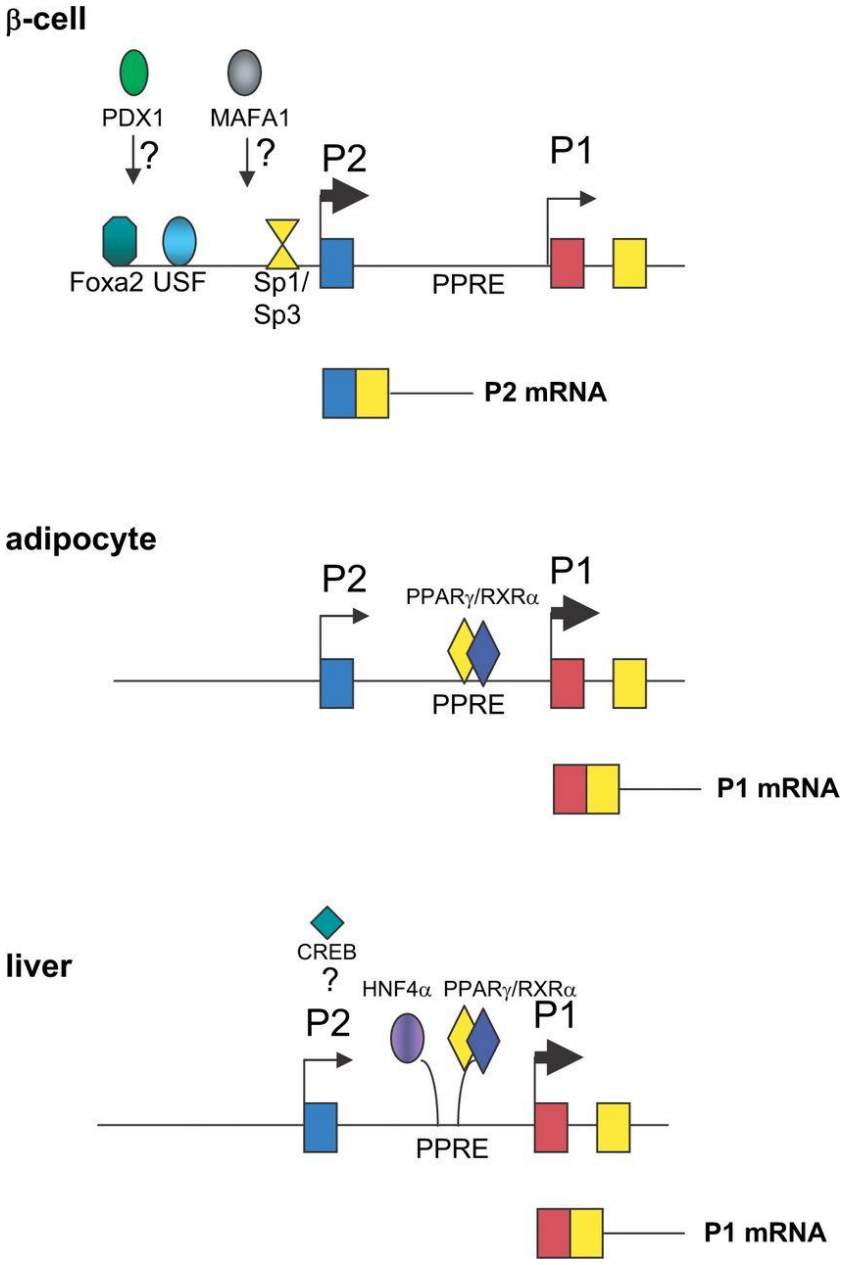


Figure 1. 5 The promoter of PC in mammalian tissues (107). The PC gene is regulated by two alternative promoters, the proximal (P1) and the distal (P2). P1 promoter mainly is active and responsible for the expression of PC in adipose and gluconeogenic tissue. P2 is a main promoter for regulation of PC expression in pancreatic tissue.

3. Post-translational modification of proteins (PTM)

Post-translational modification is a common mechanism for regulating the activity of proteins. There are many factors involved in protein post-translational modification, such as phosphate, acetate, methane, various lipids and carbohydrates.

3. 1 Phosphorylation

Phosphorylation is the addition of a phosphate group to a protein. Phosphorylation plays a significant role in a wide range of cellular processes, and a large number of proteins can be regulated by phosphorylation. Phosphorylation occurs with the aid of kinases, which transfers phosphate from ATP to the target proteins. The specificity of kinases ranges broadly. The commonly occurring sites of phosphorylation in proteins are on the serine, threonine, and/or tyrosine residue (133,134). Phosphorylation is regulated by kinases, which phosphorylate proteins. In many enzymes and receptors, phosphorylation and dephosphorylation play a regulator of enzymes and receptors, as a switch-on and off of enzyme activity (135). Those regulatory mechanisms resulted from a conformational change in the structure of enzymes and receptors. ATP is required for the phosphorylation as a donor of phosphate. Kinases transfer one of phosphoryl groups of ATP to a specific amino acid as described above. Phosphatases reverse the effects of kinases by catalyzing the removal of phosphate from phosphorylated protein. Antibodies for recognizing phosphorylated proteins are powerful tools for the detection of protein phosphorylation. These antibodies can detect phosphorylation-induced conformational changes in proteins based on the addition of phosphate in target site, called phospho-specific antibodies. Hundreds of phospho-specific antibodies are commercially available for basic research and clinical diagnosis.

3. 2 Acetylation

Protein acetylation is an important modification of proteins which consists of N-terminal acetylation and lysine acetylation. N-terminal acetylation is a common co-translational covalent modification of proteins which occurrs concurrently with its mRNA translation by N-terminal acetyltransferases. N-terminal acetylation plays a critical role in the synthesis, stability and localization of proteins (136,137). Lysine acetylation occurs in lystine residues in proteins as a post-translational modification. Acetyl-coenzyme A is an important factor for lysine acetylation, as the acetyl group donor. Lysine acetylation is important for the histone acetylation and deacetylation to regulate gene expression. These reactions are regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC). The regulatory mechanism of lysine acetylation is analogous to phosphorylation and dephosphorylation by kinases and phosphatases.

Lysine acetylation is an important regulation of transcription factors, such as effector proteins and molecular chaperones for the gene regulation. Furthermore, histone acetylation and deacetylation are critical targets for the transcriptional regulation in the epigenetics. The detection of protein acetylation relies on acetylation antibodies. These antibodies can detect a conformational change by acetyl group on the specific amino acid residue from the acetylated proteins.

3. 3 Methylation

Protein methylation typically occurs in arginine or lysine amino acid residues in proteins (137). Arginine can be methylated for one or two times on terminal nitrogens by peptidylarginine methyltransferases (PRMTs) and lysine can be methylated up to three times by lysine methylatransferases. Protein methylation is commonly studied in the histones which methylation is occurred by histone methyltransferases. Histone methyltransferases transfer

methyl groups from S-adenosyl methionine to histones. Methylated histone by histone methyltransferases act epigenetically to repress or activate gene expression (138). The detection methods for protein methylation also rely on the specific methylated antibodies similar to protein acetylation.

3. 4 Cysteine modifications: *S*-nirosylation, *S*-sulfhydration, *S*-glutathionylation, and sulfenylation

Cysteine and methionine are two sulfur-containing amino acids in protein. Methionine does not have a catalytic role because sulfur in methionine possesses high stability compared to sulfhydryl (thiol) group in cysteine (139). Cysteine possesses a free thiol side chain which is nucleophilic, acidic (pKa ~8) and redox active (140). Thiol side chain in cysteine plays important roles for regulating protein structure and function through the formation of disulfide bond or interactions with metal ions, such as zinc, copper, and iron in proteins (141,142). High reactivity of cysteine in proteins is essential for the function of proteins. Cysteine modification in proteins is critical for regulation of protein activity, including *S*-nitrosylation, *S*-sulfhydration, *S*-glutathionylation, and sulfenylation (143).

3. 4. 1 *S*-nitrosylation

S-nitrosylation is a well known mechanism for NO-based signaling, which is the covalent incorporation of a NO moiety into thiol group of cysteine to form *S*-nitrosothiol (*S*-NO) in the targeted protein. Many factors are essential for the formation of *S*-nitrosylation, including i) the acid-base motif in 3D structure of proteins i.e, cysteine residue is located between aspartic acid (acid) and histidine (base); ii) nucleo-philicity (pKa) of target cysteine residue; and iii) presence of different metal ions, such as Mg^{2+} or Ca^{2+} (144).

It is predicted that *S*-nitrosylation influences protein functions through

conformational change (144). A large number of studies have reported that *S*-nitrosylation is involved in diverse regulations, such as epigenetics, transcription, metabolism, and the activities of membrane-associated proteins, and so on (145). During neuronal development, nNOS induced *S*-nitrosylation of histone deacetylase 2 (HDAC2) leading to its dissociation from chromatin (146). *S*-nitrosylation of HDAC2 promotes chromatin remodeling and the activation of genes which are associated with neuronal development. *S*-nitrosylation of HDAC2 is essential for the neuronal development. *S*-nitrosylation-induced activation of glucokinase and inhibition of insulin receptor contribute to metabolic changes in pancreatic beta-cells (147,148). The activities of membrane-associated proteins, such as ion channels, are also regulated by *S*-nitrosylation (145). L-type Ca^{2+} channels are inhibited by *S*-nitrosylation in smooth muscle cells and K^{+} and Ca^{2+} channels are modified by *S*-nitrosylation in the heart (145,149). *S*-nitrosylation influences NMDA receptor which are associated with ion channels(150). In mutated NMDA receptor in cysteine 399 on the NR2A subunit, *S*-nitrosylation was deleted and the regulation of ion channels by NMDA receptor was not modulated by endogenous NO.

The studies for the forming mechanism of protein *S*-nitrosylation are commonly focusing on searching the consensus motifs from the linear sequence of amino acids. Based on the analysis of NO transfer in hemoglobin, the acid-base motif was suggested as a concept for *S*-nitrosylation in linear sequence of amino acids (151). In the report by Ascenzi and colleagues (152), the acid-base motif has been advanced in the three-dimensional (3D) structure basis in order to elucidate the concept for *S*-nitrosylation and denitrosylation. It was suggested that the mechanism of protein *S*-nitrosylation is dependent on its 3D structure, but not on linear sequence of amino acids in the protein. Therefore, acid-base motif may be a docking site of NO donor for protein *S*-nitrosylation and donor molecules of NO are necessary to form protein *S*-nitrosylation in acid-base motif. The proposed forms of NO

donor molecules for protein *S*-nitrosylation are nitrosothiols (RSNO). Nitrosoglutathione (GSNO) is a well known endogenous RSNO which is critically involved in NO signaling and acts as a pool of NO in the cells. Endogenous GSNO is regulated by GSNO reductase (GSNOR) and GSNOR is involved in the controlling protein *S*-nitrosylation (153,154). However, GSNOR is not directly related to the regulation of protein *S*-nitrosylation. The regulation of protein *S*-nitrosylation by GSNOR is in the indirect regulation of protein *S*-nitrosylation through the controlling of GSNO levels in the cells. Along with GSNO, another bioavailable source of NO is a nitrosocysteine (CSNO). CSNO is often employed as a donor of NO for protein *S*-nitrosylation (155-157). Since some cases of protein *S*-nitrosylation were found not followed with the acid-base motif, the acid-base motif for the forming mechanism of *S*-nitrosylation is lacked to explain all the cases of protein *S*-nitrosylation (158). Marino and Gladyshev suggested that these exceptional cases were explained with protein-protein interaction and direct interaction by NO to form protein *S*-nitrosylation. In these cases, *S*-nitrosylated cysteine residues in 3D structure of protein are available to directly bind with other proteins and NO. Diverse forming mechanisms of protein *S*-nitrosylation may be existed (158).

For the detection of *S*-nitrosylation, NO-based strategies are mainly considered for either the direct NO detection or NO switched manner. The sulfur-nitrogen bond in SNO is particularly labile, and the lability of NO in SNO is an important factor for the detection of *S*-nitrosylation. Based on this concept, many methods were developed for detection of *S*-nitrosylation. Biotin switch assay (BSA) is often used for the detection of protein *S*-nitrosylation (159). The overview of BSA procedure is as follow. First, free thiol groups in proteins are blocked with thiol-specific methylthiolating agent, methyl methanethiosulfonate (MMTS). NO in SNO is decomposed and reduced to free thiol with ascorbate, and then labeled with a thiol-specific biotinylating reagent. Total *S*-nitrosylated proteins can be

detected with anti-biotin antibody by immunoblotting. To detect specific protein which is *S*-nitrosylated, biotinylated proteins were purified with avidin conjugated beads and then immunoblotted with specific antibody to visualize specific *S*-nitrosylated protein for analysis.

3. 4. 2 *S*-sulfhydration

S-sulfhydration, which is a novel post-translational modification of cysteine residues in proteins, is similar to *S*-nitrosylation. H_2S interacts with the free thiol group and forms a hydropersulfide group (-SSH) in the target proteins. It is predicted that H_2S posttranslational modification of protein through *S*-sulfhdyration mediates most of H_2S bioactivity in the body. H_2S acts as an endothelium-derived hyperpolarizing factor through the *S*-sulfhydration of potassium channels (160). *S*-sulfhydration of Keap1 activates Nrf2 and protects against cellular senescence (34). *S*-sulfhydration MEK1 leads to PARP activation and improves DNA damage repair (161).

The regulatory mechanisms of protein *S*-sulfhydration are relatively unknown compared to the regulatory mechanisms of protein *S*-nitrosylation. The regulation of protein *S*-sulfhydration by GSSH was suggested (162). It was found that the abundant quantities of GSSH and H_2S are related to protein *S*-sulfhydration and transsulfuration reactions when compared in plasma, brain, and liver. The concentration of GSSH in tissues was in direct proportion to the concentration of H_2S. Furthermore, the concentration of GSSH was related to the increase of protein *S*-sulfhydration in the tissues, like a GSNO in protein *S*-nitrosylation. Similar to GSNOR in degradation of GSNO in the cells, a mitochondrial persulfide dioxygenase enzyme, ETHE1, mediates degradation of GSSH (162,163). ETHE1 indirectly governs protein *S*-sulfhydration through the controlling of GSSH, like a GSNOR in protein *S*-nitrosylation. Therefore, acid-base motif might be a potential concept for the formation of protein *S*-sulfhydration with GSSH, as a donor of H_2S for protein S-

sulfhydration.

At present, BSA is employed as a main technique to determine *S*-sulfhydrated proteins. The difference between BSA detection of *S*-sulfhydration and *S*-nitrosylation is in the first step, where deferoxamine is used to reduce SNO to free thiol and then all free thiols are blocked by MMTS. The procedures of BSA for protein *S*-sulfhydration and *S*-nitrosylation are shown in Fig 1. 6.

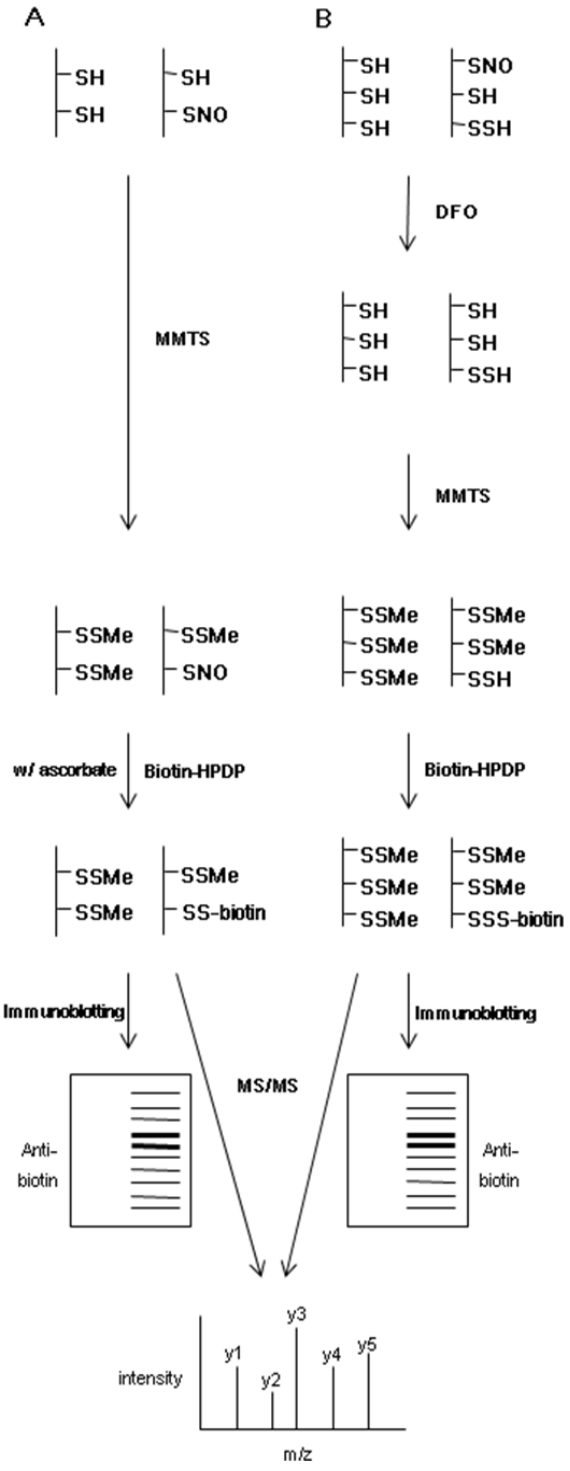


Figure 1. 6 The overview of BSA for *S*-nitrosylation (A) and *S*-sulfhydration (B).

3. 4. 3 *S*-glutathionylation

S-glutathionylation, a reversible modification of cysteine residues, is formed by a reaction of oxidized glutathione (GSSG) with free thiol. *S*-nitrosylated thiol is one of the possible intermediates for *S*-glutathionylation (143). *S*-glutathionylation of proteins are involved in the various diseases such as diabetes, cardiovascular disease, and neurodegenerative diseases (164). Aldose reductase (AR) plays an important role in glucose metabolism to provide sorbitol for the synthesis of fructose, which is associated with diabetes. *S*-glutathionylation of AR at cysteine 298 inhibits its activity with normal glucose concentration (165). Ryanodine receptors (RyR) regulate calcium release from the endoplasmic reticulum into the cystosol which is an important subject for pathological implications, including diabetes, cardiovascular complications, and Alzheimer's disease (164). *S*-glutathionylation of RyR1 at cysteine 3635 is involved in calcium release and RyR1 is critically regulated by *S*-glutathionylation along with *S*-nitrosylation at same cysteine residue (166). Furthermore, *S*-glutathionylation was found in various proteins which are related to signal transduction in the cells, such as Ras, MAPK/ERK kinase kinase (MEKK), c-Jun, Akt, IkB kinase (IKK), and protein kinase C (PKC) (164).

3. 4. 4 Sulfenylation

Sulfenylation (SOH) is an oxidation in cysteine residue of proteins induced by reactive oxygen species **(**ROS), such as hydrogen peroxide (H_2O_2) and superoxide ($^{\cdot}O^{2-}$). Sulfenylation is considered to be the intermediate for redox-signaling as the process of deleterious oxidative damage (143). Sulfenic acid is reactive and unstable with short life span in the cells. Sulfenylation of proteins were identified in a relatively small number of proteins because the identification of this modification remains difficult (142). Forty seven sulfenylated-proteins have been identified by crystal structure analysis (167). The regulation

and mechanism of sulfenylation in proteins are not well studied because crystal structure analysis and direct mass analysis are problematic for the identification of sulfenylation (143).

4. The regulation of *S*-nitrosylation and *S*-sulfhydration

So far, there is no report about the regulatory mechanisms of protein *S*-sulfhydration, whereas many studies have been conducted to analyze the regulation of protein *S*-nitrosylation. The knowledge on the regulatory mechanism for *S*-nitrosylation will definitely provide clues for further exploring protein *S*-sulfhydration.

4. 1 The regulation of *S*-nitrosylation

The regulation of *S*-nitrosylation by Thioredoxin 1 (Trx1) has been reported (168-171). It was suggested that Trx1 acts a denitrosylase and/or transnitrosylase. Trx1 mediates denitrosylation of caspase3 and TrxR is also involved in the denitrosylation of caspase3 (170). Denitrosylation of Trx1 is dependent on the cysteine 32 and 35 in Trx1 (132). However, the transnitrosylation activity of Trx1 relies on cysteine 69 and 73 in Trx1. *S*-nitrosylation of nuclear factor κB (NF- κB) inhibits its activity and Trx1 increases cytokine-induced NF-κB activation through the denitrosylation of NF- κB (169). Cysteine 69 in Trx1 plays a critical role for the transnitrosylation of proteins in redox regulation and anti-apoptotic functions of Trx1 (168). Trx1 is *S*-nitrosylated itself and *S*-nitrosylation of Trx1 transnitrosylates proteins which are related to apoptosis, such as caspase-3 and apoptosis signal-regulating kinase 1 (ASK1) (172,173). Cyteine 73 of Trx1 also plays an important role for the transnitrosylation of target proteins which are interacted with Trx1 (132). The target proteins of denitrosylation or transnitrosylation by Trx1 are shown in Table 1. 3 and 1.4. *S*-nitrosylation of diverse proteins is regulated by Trx1 and Trx1 acts differently in target proteins. Detail regulatory mechanism of protein *S*-nitrosylation by Trx1 still is not clear.

Table 1. 3 Target proteins and peptides denitrosylated by Trx1 (174).

Protein name	Swiss-Prot Identifier	Peptide
40S ribosomal protein S4, X isoform	RS4X	K.FDTGNLCMVTGGANLGR.I
40S ribosomal protein SA	RSSA	R.ADHQPLTEASYVNLPTIALCNTDSPLR.Y
40S ribosomal protein SA	RSSA	R.YVDIAIPCNNK.G
60S acidic ribosomal protein P1	RLA1	K.ALANVNIGSLICNVGAGGPAPAAGAAPAGGPAPST AAAPAEEK.K
ATP-dependent RNA helicase A	DHX9	K.SSVNCPFSSQDMK.Y
Cofilin-1	COF1	K.AVLFCLSEDKK.N
Cytoplasmic dynein 1 heavy chain 1	DYHC1	K.TSAPITCELLNK.Q
Dihydropyrimidinase-related protein 2	DPYL2	R.GLYDGPVCEVSVTPK.T
Dopamine beta-hydroxylase	DOPO	K.VISTLEEPTPQCPTSQGR.S
Elongation factor 1-alpha 1	EF1A1	K.SGDAAIVDMVPGKPMCVESFSDYPPLGR.F
Elongation factor 2	EF2	R.ETVSEESNVLCLSK.S
Eukaryotic translation initiation factor 5A-1	IF5A1	K.KYEDICPSTHNMDVPNIK.R
Far upstream element-binding protein 1	FUBP1	R.SCMLTGTPESVQSAK.R
Fascin	FSCN1	R.LSCFAQTVSPAEK.W
Filamin-A	FLNA	K.IVGPSGAAVPCK.V
Galectin-1	LEG1	K.DSNNLCLHFNPR.F
Glyceraldehyde-3-phosphate dehydrogenase	G3P	R.VPTANVSVVDLTCR.L
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GBB1	R.LFVSGACDASAK.L
Heterogeneous nuclear ribonucleoprotein H	HNRH1	R.DLNYCFSGMSDHR.Y
Heterogeneous nuclear ribonucleoprotein H	HNRH1	R.YGDGGSTFQSTTGHCVHMR.G
Heterogeneous nuclear ribonucleoprotein K	HNRPK	K.GSDFDCELR.L
Heterogeneous nuclear ribonucleoprotein L	HNRPL	R.VFNVFCLYGNVEK.V
Heterogeneous nuclear ribonucleoproteins A2/B1	ROA2	K.LTDCVVMR.D
High mobility group protein B1	HMGB1	K.MSSYAFFVQTCR.E
High mobility group protein B1	HMGB1	K.RPPSAFFLFCSEYRPK.I
L-lactate dehydrogenase A chain	LDHA	R.VIGSGCNLDSAR.F
Malate dehydrogenase, cytoplasmic	MDHC	K.VIVVGNPANTNCLTASK.S
Myosin light chain 6B	MYL6B	K.ILYSQCGDVMR.A
Nucleoside diphosphate kinase B	NDKB	R.GDFCIQVGR.N
Phosphoglycerate kinase 1	PGK1	K.ACANPAAGSVILLENLR.F
Poly(rC)-binding protein 1	PCBP1	R.INISEGNCPER.I
Prostaglandin E synthase 4	TEBP	K.LTFSCLGGSDNFK.H

Pyruvate kinase isozymes M1/M3	KPYM	R.NTGIICTIGPASR.S
Serine/arginine–rich splicing factor 1	SRSF1	R.EAGDVCYADVYR.D
Small glutamine-rich tetratricopeptide repeat-containing protein alpha	SGTA	K.AIELNPANAVYFCNR.A
T-complex protein 1 subunit gamma	TCPG	R.TLIQNCGASTIR.L
Triosephosphate isomerise	TPIS	R.IIYGGSVTGATCK.E
Tubulin alpha-1A chain	TBA1A	R.TIQFVDWCPTGFK.V
Tubulin alpha-1B chain	TBA1B	R.SIQFVDWCPTGFK.V
Tubulin beta-2A chain	TBB2A	K.LTTPTYGDLNHLVSATMSGVTTCLR.F
Tubulin beta-2A chain	TBB2A	R.EIVHIQAGQCGNQIGAK.F
Ubiquitin carboxyl-terminal hydrolase isozyme L1	UCHL1	K.NEAIQAAHDAVAQEGQCR.V
Voltage-dependent anion-selective channel protein 1	VDAC1	K.YQIDPDACFSAK.V

Table 1. 4 Target proteins and peptides transnitrosylated by Trx1 (174).

Protein name	Swiss-Prot Identifier	Peptide
60S ribosomal protein L12	RL12	R.CTGGEVGATSALAPK.I
60S ribosomal protein L12	RL12	K.EILGTAQSVGCNVDGR.H
60S acidic ribosomal protein P0-like	RLA0L	R.AGAIAPCEVTVPAQNTGLGPEK.T
Alpha-enolase	ENOA	K.VNQIGSVTESIQACK.L
Heat shock cognate 71 kDa protein	HSP7C	K.GPAVGIDLGTTYSCVGVFQHGK.V
Peptidyl-prolyl cis-trans isomerase A	PPIA	K.KITIADCGQLE.-
Peptidyl-prolyl cis-trans isomerase A	PPIA	R.IIPGFMCQGGDFTR.H
Peroxiredoxin-1	PRDX1	K.HGEVCPAGWKPGSDTIKPDVQK

4. 2 The regulation of *S*-sulfhydration

Protein *S*-sulfhydration shares many similar properties with *S*-nitrosylation. Firstly, both of them occur in the cysteine residues on the proteins. Secondly, formation of *S*-sulfhydration may depend on a chemical reaction similar to other cysteine modifications, such as *S*-nitrosylation, *S*-glutathionylation, and sulfenylation, but not phosphorylation. Thirdly, they all belong to redox modifications on cysteine residues. The detail mechanisms on the regulation of protein *S*-sulfhydration has not been determined yet. In forming mechanism of *S*-nitrosylation, *S*-nitrosogluathione (GSNO) is a candidate as a *S*-nitrosylating agent. NO itself can induce *S*-nitrosylation but it was shown that GSNO may be an intermediate for the forming of *S*-nitrosylation in proteins (175,176). Especially, GSNO/GSH ratio is responsible for the *S*-nitrosylation of GAPDH in *Arabidopsis thaliana* (175). Glutathione persulfide **(**GSSH) is suggested as a *S*-sulfhydrating agent (162). Similar to the formation of *S*-nitrosylation, GSSH/GSH ratio may influence on the formation of *S*-sulfhydration on proteins. Compared to regulation of protein *S*-nitrosylation by other proteins, such as Trx1, the understanding of the regulation of protein *S*-sulfhydration is still in its early stages. However, the regulation of protein *S*-sulfhydration by other proteins may be similar to the regulation of protein *S*-nitrosylation as described above if the similarity of protein *S*-nitrosylation and *S*-sulfhydration is considered. Therefore, Trx1 may be a candidate for the regulation of protein *S*-sulfhydration as a trans/de-sulfhydrase. Further study on the regulation of *S*-sulfhydration will be helpful for understanding the physiological roles of H_2S.

HYPOTHESIS & OBJECTIVES OF THE STUDY

S-sulfhydration is considered as a novel post-translational modification of protein, which mediates most of the activities of H_2S in biological system. H_2S is required for maintenance of normal liver function. Here, we hypothesized that H_2S induces gluconeogenesis in liver through PC *S*-sulfhydration, and Trx1 is involved in the regulation of protein *S*-sulfhydration. The objectives of this study are to:

1. Determine the effects of exogenous and endogenous H_2S on gluconeogenesis in liver through *S*-sulfhydration of PC.
2. Evaluate the difference of PC *S*-sulfhydration in pathological conditions, such as obesity.
3. Investigate the regulatory mechanism of protein *S*-sulfhydration by Trx1.

CHAPTER 2

H_2S-induced *S*-sulfhydration of pyruvate carboxylase contributes to gluconeogenesis in liver cells

YoungJun Ju[1,2], Ashley Untereiner[1,3], Lingyun Wu[1,3], Guangdong Yang[1,2]*

[1]Cardiovascular and Metabolic Research Unit, [2]The school of Kinesiology, [3]Department of Health Science, Lakehead University, Ontario, Canada

Running title: H_2S *S*-sulfhydration of PC induces gluconeogenesis

This chapter currently is accepted in Biochimica et Biophysica Acta (BBA-GEN).

2. 1 Abstract

Cystathionine gamma-lyase (CSE)-derived hydrogen sulfide (H_2S) possesses diverse roles in the liver, affecting lipoprotein synthesis, insulin sensitivity, mitochondrial bioenergetics and biogenesis. H_2S *S*-sulfhydration is now proposed as a major mechanism for H_2S-mediated signaling. Pyruvate carboxylase (PC) is an important enzyme for gluconeogenesis. *S*-sulfhydration regulation of PC by H_2S and its implication in gluconeogenesis in liver have been unknown. In the present study, we demonstrated that exogenously applied H_2S stimulates PC activity and gluconeogenesis in both HepG2 cells (a human hepatocellular liver carcinoma cell line) and mouse primary liver cells. CSE overexpression enhanced but CSE knockout reduced PC activity and gluconeogenesis in liver cells, and blockage of PC activity abolished H_2S-induced gluconeogenesis. We further found that H_2S has little effect on the expressions of PC mRNA and protein, while H_2S *S*-sulfhydrates PC in a dithiothretiol-sensitive way. PC *S*-sulfhydration was significantly strengthened by CSE overexpression but attenuated by CSE knockout, suggesting that H_2S enhances glucose production through *S*-sulfhydrating PC. Mutation of cysteine 265 in human PC diminished H_2S-induced PC *S*-sulfhydration and activity. In addition, high fat diet feeding of mice decreased both CSE expression and PC *S*-sulfhydration in liver, while glucose deprivation of HepG2 cells stimulated CSE expression. The finding displays a novel physiological signaling for H_2S regulation of gluconeogenesis via *S*-sulfhydrating PC in liver.

Key words: H_2S, CSE, PC, *S*-sulfhydrarion, Gluconeogenesis

2. 2 Introduction

Hydrogen sulfide (H_2S) is considered as a novel gasotransmitter that plays a critical role in liver functions, including lipoprotein synthesis, insulin sensitivity, mitochondrial bioenergetics and biogenesis, and detoxification of various metabolites etc. (95,96,99,104,177). In analogy with protein *S*-nitrosylation, protein *S*-sulfhydration has been proposed as a major mechanism for H_2S-mediated signaling (94,178,179). H_2S can be endogenously produced in a variety of cells, tissues, organs, and systems by cystathionine beta-synthase, cystathionine gamma-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase. The expressions of these genes are tissue-specific (2,5). Compared with all other tissues in the body, all these three genes are expressed in liver with a large amount of H_2S production (37,94,95,180). Deficiency of CSE gene diminished H_2S production by more than 90% in mouse liver, suggesting CSE acts as a major H_2S-generating enzyme in liver (37,94). Altered hepatic H_2S generation and metabolism have been demonstrated to be involved in the pathogenesis of many liver diseases, such as ischemia/reperfusion injury, hepatic fibrosis and cirrhosis (100-103).

Pyruvate carboxylase (PC; EC6.4.1.1) is a nuclear encoded mitochondrial enzyme that catalyses pyruvate to form oxaloacetate (105). PC serves two biosynthetic purposes: it sustains the level of oxaloacetate in the tricarboxylic acid cycle, and it provides oxaloacetate for phosphoenolpyruvate carboxykinase to convert to phosphoenolpyruvate (129). Phosphoenolpyruvate can be converted into glucose, therefore, PC is considered as an enzyme that is crucial for intermediary metabolism, controlling fuel partitioning toward gluconeogenesis (129). Gluconeogenesis is a ubiquitous process, present in animals, plant, fungi, and other microorganisms. In animals, gluconeogenesis takes place mainly in the liver (181). In the fed state, the liver stores energy as glycogen from glucose. Conversely, when plasma glucose concentration is decreased during fasting or under nutrition, the liver

produces glucose through glycogenolytic and gluconeogenic pathways (182). PC is positively regulated by glucagon and glucocorticoids while negatively regulated by insulin (183). H_2S regulation of PC expression and/or activity as well as its involvement in liver gluconeogenesis is not clearly understood.

In the present study, we performed detailed investigation on H_2S modification of PC protein by *S*-sulfhydration and the actual *S*-sulfhydration site(s), and explored the functional relevance of PC *S*-sulfhydration in liver glucose production. By using human hepatocellular liver carcinoma cell line (HepG2) and mouse primary hepatocyte isolated from both wild-type (WT) mice and CSE knockout (CSE-KO) mice, we found that H_2S induces PC activity directly by *S*-sulfhydrating PC protein at cysteine 265, and increased PC activity contributes to H_2S-stimulated gluconeogenesis. We further demonstrated that high fat diet (HFD) feeding decreases both CSE expression and PC *S*-sulfhydration in mouse liver. This study advances our understanding of H_2S signal in liver gluconeogenesis by targeting PC.

2. 3 Materials and methods

Cell culture and animal preparation

HepG2 and HEK293 cells (American Type Culture Collection, Manassas, VA**)** were maintained in Dulbecco's Modified Eagle's medium (DMEM, Sigma, Oakville, ON) supplemented with 10 % fetal bovine serum (FBS, Clontech, Mountain View, CA) and 1 % penicillin-streptomycin solution (Sigma). For overexpression of PC and CSE, recombinant hPC, or PC mutant plasmids, or CSE cDNA plasmid (184) was transfected into HEK293 or HepG2 cells using Lipofectamine^{TM} 2000 reagent as described by the manufacturer's protocol (Invitrogen, Burlington, ON). Before treatment with high glucose, HepG2 cells and primary liver cells were pre-incubated overnight in DMEM containing 1 % FBS and 1 mM glucose and then subjected to 25 mM glucose for additional 24 hours.

CSE-KO mice were generated as previously described (37). All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and approved by the Animal Care Committee of Lakehead University, Canada. Animals were maintained on standard rodent chow and had free access to food and water. For high-fat diet (HFD, paigen-type) feeding, mice were initially fed with a standard rodent chow diet (Rodent RQ 22–5, Zelgler Bros Inc, PA) until 6 weeks of age and then switched to either a HFD (TD.02028, Harlan Tekald, Madison, WI) or control diet (TD.05230, Harlan Tekald, Madison, WI) for 12 weeks. In all HFD feeding experiments, we followed the procedure according to previous study (96).

Isolation of primary liver cells

Hepatocytes were isolated from 12-week-old male WT and CSE-KO mice as described previously with modification (185). Briefly, liver organ were perfused through the

inferior vena cava with a buffer (140 mM NaCl, 2.6 mM KCl, 0.28 mM Na_2HPO_4, 5 mM glucose, and 10 mM HEPES (pH 7.4)). The perfusion was first for 5 min with buffer A (0.5 mM EGTA) and then for 10 min with buffer B (5 mM $CaCl_2$ and 100 U/ml collagenase type IV) (Worthington, Lakewood, NJ). All solutions were pre-warmed at 37 ^oC incubator. The isolated hepatocytes were filtered on nylon mesh (100 μm pore size), and selected by centrifugation in a 36 % Percolliso density gradient. Selected cells were seeded in collagen-coated plates with DMEM containing 10 % fetal bovine serum and 5.5 mM glucose.

PC activity

For the measurement of PC activity, coupled enzyme assay was employed as described previously (186). Briefly, cells or liver tissues were sonicated in a buffer containing 10 mM HEPES (pH 7.4), 250 mM sucrose, 2.5 mM EDTA, 2 mM cysteine, 0.02 % bovine serum ablumin, and then centrifuged at 13,000 g for 30 min at 4 ^oC. Collected extract was then added to reaction buffer containing 80 mM Tris/HCl (pH 8.0), 2 mM ATP, 8 mM potassium pyruvate, 21 mM $KHCO_3$, 9 mM $MgSO_4$, 0.16 mM acetyl CoA, 0.16 mM reduced nicotinamide adenine dinucleotide (NADH), and 5 U/ml malate dehydrogenase. The activity of PC was calculated by the conversion of NADH to NAD^+ with the measurement of the change in absorbance at 340 nm over time. Absorbance at 340 nm was measured in a multicell spectrophotometer (Fisher Scientific, Ottawa, ON) and the PC activity was expressed as nmol/min/mg of total protein. Data was normalized by protein concentration determined by the Bradford method.

Biotin switch assay of *S*-sulfhydration

BSA was carried-out as described previously with some modifications (94). Briefly, cells or mouse liver tissues were homogenized in HEN buffer (250 mM HEPES (pH 7.7), 1

mM EDTA, and 0.1 mM Neocuproine) supplemented with 100 μM deferoxamine and centrifuged at 13,000 x *g* for 30 min at 4°C. The lysates were added to blocking buffer (HEN buffer adjust to 2.5% SDS and 20 mM methyl methanethiosulfonate (MMTS)) at 50°C for 20 min with frequent vortexing. The MMTS was then removed by acetone and the proteins were precipitated at -20°C for 20 min. Proteins were resuspended in HENS buffer (HEN buffer containing 1 % SDS) and 4 mM biotin-N-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio) propinamide (HPDP) in DMSO without ascorbic acid. After incubation for 2 h at 25°C, biotinylated proteins were purified by streptavidin-agarose beads, which were then washed with HENS buffer. The biotinylated proteins were eluted by SD*S*-PAGE sample buffer and subjected to Western blotting analysis with anti-PC antibody or anti-His6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Western blotting

The cells or mouse liver tissues were harvested and lysed in a cell lysis buffer (Cell signaling, Danvers, MA) including protease inhibitor cocktail (Sigma). The extracts were separated by centrifugation at 14,000 g for 15 min at 4°C. Equal amount of proteins were boiled in 1 × SDS sample buffer (62.5 mM Tri*s*-Cl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue) and run in a 10% SD*S*-PAGE gel, and then transferred onto pure nitrocellulose blotting membranes (Pall Corporation, Ville St. Laurent, QC). The dilutions of primary antibodies were 1:500 for PC (Santa Cruz Biotechnology), 1:500 for His6 (Santa Cruz biotechnology), 1:1,000 for CSE (Proteintech, Chicago, USA), 1:10,000 for β-actin (Sigma), and HRP-conjugated second antibody was diluted as 1:5,000 (Sigma). The blots were developed using chemiluminescence (GE Healthcare life sciences, Baie d'Urfe, QC).

Glucose production in HepG2 and primary hepatocytes

HepG2 cells or primary hepatocytes were planted in 6-well plates at a density of 1.5 × 10^5/ well at 37 ^oC in a humidified atmosphere (5% CO_2) for 24 h. Cells were then washed twice with cold PBS (phosphate buffered saline) to remove excess glucose from the media and then incubated for another 3 h in glucose and phenol red-free DMEM containing 2 mM sodium pyruvate and 20 mM sodium lactate (pH 7.4). Medium (300 μl) were collected for glucose measurement using glucose assay kit (Sigma). Glucose concentration was normalized with cellular protein content.

Site-directed mutagenesis

Human PC cDNA construct was purchased from Origene (Rockville, MD). PC cDNA was cloned into pcDNA3.1/Myc-His tag (Invitrogen). Single mutation at cyteine-265 (hPC-C265) or cysteine-739 (hPC-C739) in PC was conducted using the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) (34). The oligonucleotides using for mutagenesis were 5'- TGTACGAGCGAGACTCCTCCATCCA-GCGGCG-3' (forward) and 5'-GCCGCTGGATGGAGGAGTCTCGCTCGTACAG-3' (reverse) for cysteine-265, and 5'-CGAGCTGGCACCCACATCCTGTCCATCAAGGACAT-3' (forward) and 5'-ATGTCCTTGA-TGGACAGGATGTGGGTGCCAGCTCG-3' for cysteine-739. The site-directed mutants were confirmed by DNA sequencing at the Paleo-DNA Laboratory of Lakehead University, ON, Canada.

Immunohistology

Mouse livers were dissected out and fixed in 4% paraformaldehyde for 18 h and then cryoprotected in 30% sucrose/phosphate-buffered saline at 4 °C for 3 days. Samples were embedded in optimal cutting temperature compound (Triangle Biomedical Sciences, Durham,

NC). Sections were then cut into 10-μm with Leica CM1850 UV microtome-cryostat (Leica Biosystems, Concord, ON) and picked up on poly-L-lysine-coated slides. Slides were incubated with 2% bovine serum albumin at room temperature for 30 min. Primary antibody against PC and CSE was added at 4°C for overnight. Slides were then washed twice with PBS following incubation with fluorescent secondary antibody for PC (Goat anti-rabbit IgG, Sigma) and for CSE (Rabbit anti-mouse IgG, Sigma) for 1h. The slides were washed twice with PBS and mounted with media containing 4',6-diamidino-2-phenylindole. Stained tissue sections were observed under a fluorescent microscope (Olympus, Richmond Hill, ON).

Statistical analysis

Data were presented as means ± SEM, representing at least three independent experiments. Student's *t-test* was used to evaluate the difference between two groups. For multiple groups, Two ANOVA was employed followed by post-hoc Turkey test using SigmaPlot 12.0 (Systat Software Inc. San Jose, CA). The significance level was set at $p<0.05$.

2. 4 Results

H_2S increases PC activity

We first observed that incubation of HepG2 cells with NaHS (a well known H_2S donor) at as lower as 10 μM significantly increases PC activity by 78.0% (Fig. 2. 1A). The stimulatory role of NaHS on PC activity was time-dependent, as NaHS treatment (50 μM) of HepG2 cells for 30 min, 2 hrs and 12 hrs induced PC activity by 35.4 %, 69.3 %, and 105.6 %, respectively (Fig. 2. 1B). We further validated that CSE overexpression in HEK293 cells enhances PC activity in comparison with the control or vehicle transfected-cells (Fig. 2. 1C and 1D). CSE is a major source of H_2S production in the liver with cysteine as substrate. Here we found that PC activity in liver tissues from CSE-KO mice is only 58.5 % of that from WT mice (Fig. 2. 1E). NaHS (50 μM) incubation with both WT and CSE-KO liver lysates significantly increased PC activity, while cysteine (1 mM) only stimulated PC activity in liver lysates from WT mice but not CSE-KO mice (Fig. 2. 1E and 1F).

PC mediates H_2S-stimulated glucose production

To see the functional implication of H_2S-stimualted PC activity in liver cells, we measured glucose production in HepG2 cells and primary hepatocytes. Incubation of HepG2 cells with 50 μM NaHS induced glucose production by 29.3 % compared with the control cells (Fig. 2. 2A). siRNA-mediated PC knockdown completely reversed NaH*S*-stimulated glucose production (Fig. 2. 2A and 2B), indicating the involvement of PC in H_2S-initiated glucose production. PC knockdown also significantly decreased the basal level of glucose production, while PC overexpession increased glucose production in HepG2 cells (Fig. 2. 2A, 2C, 2D). We further confirmed that incubation of HepG2 cells with phenylacetic acid (PAA), a PC inhibitor, evidently blocks H_2S-induced PC activity and glucose production (Fig. 2. 2E and 2F). By using primary hepatocytes isolated from both WT and CSE-KO mice, we

demonstrated that glucose production is decreased by 55.6 % in CSE-KO hepatocytes in comparison with WT hepatocytes (Fig. 2. 2G). Similarly, PAA also significantly blocked NaH*S*-stimulated glucose production in both WT and CSE-KO hepatocytes (Fig. 2. 2G).

H_2S *S*-sulfhyrates PC at cysteine 265

To investigate how H_2S stimulates PC activity, we studied the effects of H_2S on PC protein and mRNA expression. Interestingly, treatment of HepG2 cells with different concentration of NaHS (10-100 μM) for 0.5-12 h did not affect PC protein and mRNA expression (Fig. 2. 3A-3C). CSE deficiency also had no effect on PC protein and mRNA expressions in mouse liver tissues (Fig. 2. 3D and 3E), suggesting H_2S may induce PC activity at post-translational level. So next we explored whether H_2S induces PC activity directly by *S*-sulfhydrating PC protein with modified BSA. Treatment of HepG2 cells with NaHS (10, 50, 100 μM) for 2 h significantly increased PC *S*-sulfhydration compared to the untreated cells, and PC was basically *S*-sulfhydrated even in the absence of exogenous H_2S (Fig. 2. 4A). We further demonstrated that NaHS (50 μM) induces PC *S*-sulfhydration in a time-dependement manner. In comparison with the control cells, the level of *S*-sulfhydrated PC was increased by 14.1, 38.0, and 76.9 % after 30 min, 2 h, and 12 h of NaHS incubation, respectively (Fig. 2. 4B). PC *S*-sulfhydration was DTT sensitive, and DTT significantly reduced the basal and H_2S-induced PC *S*-sulfhydration (Fig. 2. 4C). In line with these data, DTT also markedly attenuated H_2S-induced PC activity in HepG2 cells (Fig. 2. 4D). The basal level of PC *S*-sulfhydration was significantly higher in WT liver than that in CSE-KO liver, and the supplement of cysteine further strengthened PC *S*-sulfhydration in liver lysates from WT mice but not CSE-KO mice (Fig. 2. 4E). In addition, CSE overexpression in HEK293 cells stimulated PC *S*-sulfhydration by 40.2 % compared with the control and vehicle-transfected cells (Fig. 2. 4F).

There are total 13 cysteine residues in human PC protein, and two of them are highly conserved among mammalian and microorganism (Fig. 2. 5A) (131,171,187). Through bioinformtic analysis, we found only cysteine 265 in human PC (hPC), one of the highly conserved cysteine residues, is located in the surface of PC protein, which forms acid-base motif and is easily targeted by external stimuli (Fig 2. 5B) (152,158). To validate whether cysteine 265 is responsible for PC *S*-sulfhydration, we mutated cysteine 265 to serine in hPC. We also randomly mutated cysteine 739, which is located inside PC protein and could not form acid-base. NaHS significantly induced PC *S*-sulfhydration in hPC-transfected HEK293 cells, and mutation of Cysteine 265 but not cystiene 739 abolished the basal level of PC *S*-sulfhydration. NaHS further strengthened PC *S*-sulfhydration in cysteine 739 mutant transfected cells but failed in Cysteine 265 mutant transfected cells, pointing to the critical role of cystiene 265 in PC *S*-sulfhydration (Fig. 2. 5C). Consistently, NaHS induced more PC activity in both hPC and cysteine 739 mutant transfected cells when compared with Cysteine 265 mutant transfected cells (Fig. 2. 5D).

Reduced liver CSE expressioin and PC *S*-sulfhydration in high fat diet (HFD)-feeding mice

We first found that HFD feeding of WT mice for 12 weeks decreases liver PC *S*-sulfhydration by 37.5 % compared with the control diet-feeding WT mice (Fig. 2. 6A). Liver PC *S*-sulfhydration was hardly detected in control diet-feeding CSE-KO mice, which was not changed by HFD feeding (Fig. 2. 6A). Similar to the change of PC *S*-sulfhydration, liver PC activity was decreased by 22.1 % in HFD-feeding WT mice comparing to control diet-feeding WT mice (Fig. 2. 6B), and PC activity was not altered in CSE-KO liver under either control diet or HFD feeding (Fig. 2. 6B). With immunohistology, we observed that HFD feeding significantly decreases CSE expression in WT mice (Fig. 2. 6C). As expected, CSE

expression was undetectable in CSE-KO mice with control diet or HFD feeding (Fig. 2. 6C). We next determined that high glucose (25 mM) treatment of both HepG2 cells and primay hepatocytes reduced CSE expression by 76.9 % and 86.0 %, respectively (Fig. 2. 6D and 6E). In parallel of lower CSE expression, PC *S*-sulfhydration was also significantly reduced by high glucose incubation (Fig. 2. 6D). Differently, compared with the control cells, CSE expression was increased by 42.2 % and 49.0 % in glucose deprived-HepG2 and primary hepatocytes , respectively (Fig. 2. 6F and 6G).

2. 5 Discussion

PC is an important enzyme in gluconeogenesis along with fructose-1,6-bisphosphatase, glucose-6-phosphatase, and phosphoenolpyruvate carboxylase (106,188,189). PC expression and activity in human is greatest in hepatic cells, where PC catalyzes the first committed step in gluconeogenesis and is well poised to regulate hepatic glucose balance (190). PC provides the oxaloacetate for both gluconeogenesis and the replenishment of TCA cycle intermediates (90). Altered liver PC activity has been directly correlated with the pathophysiology of Type 2 diabetes, obesity and other metabolic syndrome (116,126,183). Given the importance of PC in gluconeogenesis, it is essential to fully understand the fundamental catalytic and regulatory mechanism of PC activity.

In the present study, we demonstrated that H_2S, a novel gasotransmitter, stimulates PC activity in liver cells, while deficiency of CSE in mouse liver tissues attenuates PC activity. We previously reported that H_2S plays a critical role in regulating gluconeogensis in liver (98). Gluconeogenesis is a metabolic pathway that results in the generation of glucose from non-carbohydrate carbon substrates, which mainly occurs in liver tissue. To investigate whether increased PC activity is involved in H_2S-induced gluconeogenesis, we used a loss-of-function approach. Consistent with our previous discoveries, exogenously applied H_2S or CSE overexpression significantly induced gluconeogenesis in liver cells. siRNA-mediated knockdown of PC mRNA or inhibition of PC activity by PAA blocked H_2S-strenthenghed gluconeogenesis, clearly indicating the mediation of PC in H_2S-induced glucose generation. Gluconeogenesis has been the target of therapy for diabetes, and inhibition of CSE/H_2S pathway would decrease PC activity and lower glucose formation (90,98). Besides with PC, many other enzymes are involved in the process of gluconeogenesis (90). It has been reported that H_2S enhances renal and liver gluconeogenesis by activating peroxisome proliferation-activated receptor-γ-1α, fructose-1,6-bisphoaphatase and PEPCK (98,191). Nevertheless, it is

not clear how H_2S regulates the activities of these gluconeogenesis-related enzymes.

Our next focus is to study how H_2S affects PC activity. H_2S-induced activation of PC is not caused by increased PC transcription and translation, because both PC mRNA and protein expressions are not changed by exogenously applied NaHS, CSE overexpression or CSE knockout in liver cells. H_2S has been recently demonstrated to post-translational modification of proteins by formation of a persulfide (-SSH) bond to reactive cysteine residues of target proteins, termed as *S*-sulfhydration (94). Interestedly, we observed that PC is basically *S*-sulfhydrated and PC *S*-sulfhydration further enhanced by H_2S. Lack of CSE significantly reduced but overexpression of CSE stimulated PC *S*-sulfhydration. To locate the responsible cysteine residue for H_2S *S*-sulfhydration of PC, we considered three aspects: the conservativeness of cysteine residue among species, the accessibility by H_2S, and the potential acid-base motif (152,158,192,193). The conserved amino acid residue(s) among various species can be often targeted by external stimuli (192,194,195). Accessibility of H_2S to the target cysteine residue is critical for forming *S*-sulfhydration (193). *S*-sulfhydration is also dependent on the acid dissociation constant (pKa) of cysteine residues, and the cysteine residue in acid-base motif can be easily targeted by the intermediate which can induce protein *S*-sulfhydration (152,158,179). In consideration of these factors, we found that cysteine-265 in human PC is highly potential for forming *S*-sulfhydration. Cysteine-265 in human PC is conserved with cysteine-249 in yeast, the latter has been reported to be the target for nitric oxide to form *S*-nitrosylation (128,131). Cysteine-249 in yeast was also involved in the binding with K^+ and acetyl-CoA (128). With mutation study, we validated that cysteine-265 in BCdomain of PC protein is responsible for *S*-sulfhydration, because mutation of cysteine-265 to serine completely abrogated *S*-sulfhydration of PC. Cysteine-739 in CTdomain appears not responsible for PC *S*-sulfhydration.

PC consist of four functional domains, the N-terminal BC domain, the CT domain, the

allosteric domain and the C-terminal biotin carboxyl carrier protein domain (129). The regulation of PC activity is extensively reliant on the multifarious interactions of various factors with the functional domains (107). The crystal structure of PC protein demonstrated the active sites for several critical factors, including MgADP, HCO_3^-, Mg^{2+} and free biotin, are located in BC domain (107). Acetyl CoA is well known as an allosteric activator of PC, and the main binding site of acetyl CoA is located in the BC domain, where acetyl CoA stabilizes the tetrameric structure of the enzyme, enhances the binding of some substrates, and stimulates the reaction rates (107). It is predicted that, by change in electrostatic environment, hydrophobicity, contiguity and orientation of aromatic side chains, and proximity of target thiols to transition metals or redox centers, *S*-sulfhydration can alter protein conformation and the final function and activity of target proteins (161,162). In line with acetyl CoA, we deduced that the interaction of H_2S and PC through cysteine-265 in BC domain may cause a conformational change in PC protein and alter the binding of MgADP, HCO_3^-, Mg^{2+} or free biotin with BC domain, which finally trigger higher pyruvate flux and gluconeogenesis (107,183). Further kinetic and structural analysis of *S*-sulfhydrated PC is needed to fully define the roles of the interactions of H_2S with cysteine-265 and understand the mechanism by which it induces enzyme activation.

Fasting or starvation usually lower endogenous glucose level, in this case, PC activity is induced leading to increased pyruvate flux and higher gluconeogenesis (183). In our study, we found that glucose deprivation stimulates CSE expression, which would induce H_2S generation and enhance glucose generation by *S*-sulfhydrating PC activity. In contrast, HFD feeding of mice reduced CSE expression and PC activity, which may inhibit gluconeogenesis but induces glycolysis in liver (196,197). Similarly, we also proved that high blood glucose decreases CSE expression in the liver cells. In the presence of higher glucose, lower level of H_2S due to reduced CSE expression would diminish PC *S*-sulfhydration and eliminate PC

activity. Lower PC activity will subsequently attenuate the process of gluconeogenesis and decrease glucose production in liver. Following the change of glucose level, CSE expression and H_2S production can be restored leading to higher PC activity by *S*-sulfhydration. Increased PC activity by *S*-sulfhydration then activates gluconeogenesis. Therefore, CSE/H_2S pathway would act as a critical factor for regulating gluconeogenesis and maintaining glucose balance in the liver (Fig. 2. 7).

Taken together, our results indicated that CSE/H_2S pathway plays an important role in the regulation of glucose production through *S*-sulfhydrating PC in liver. Tissue-specific regulation of CSE/H_2S pathway might be a promising therapeutic target of diabetes and other components of metabolic syndromes.

Acknowledgments: This study was supported by a grant-in-aid from the Heart and Stroke Foundation of Canada. G.Y. was supported by a New Investigator award from the Heart and Stroke Foundation of Canada.

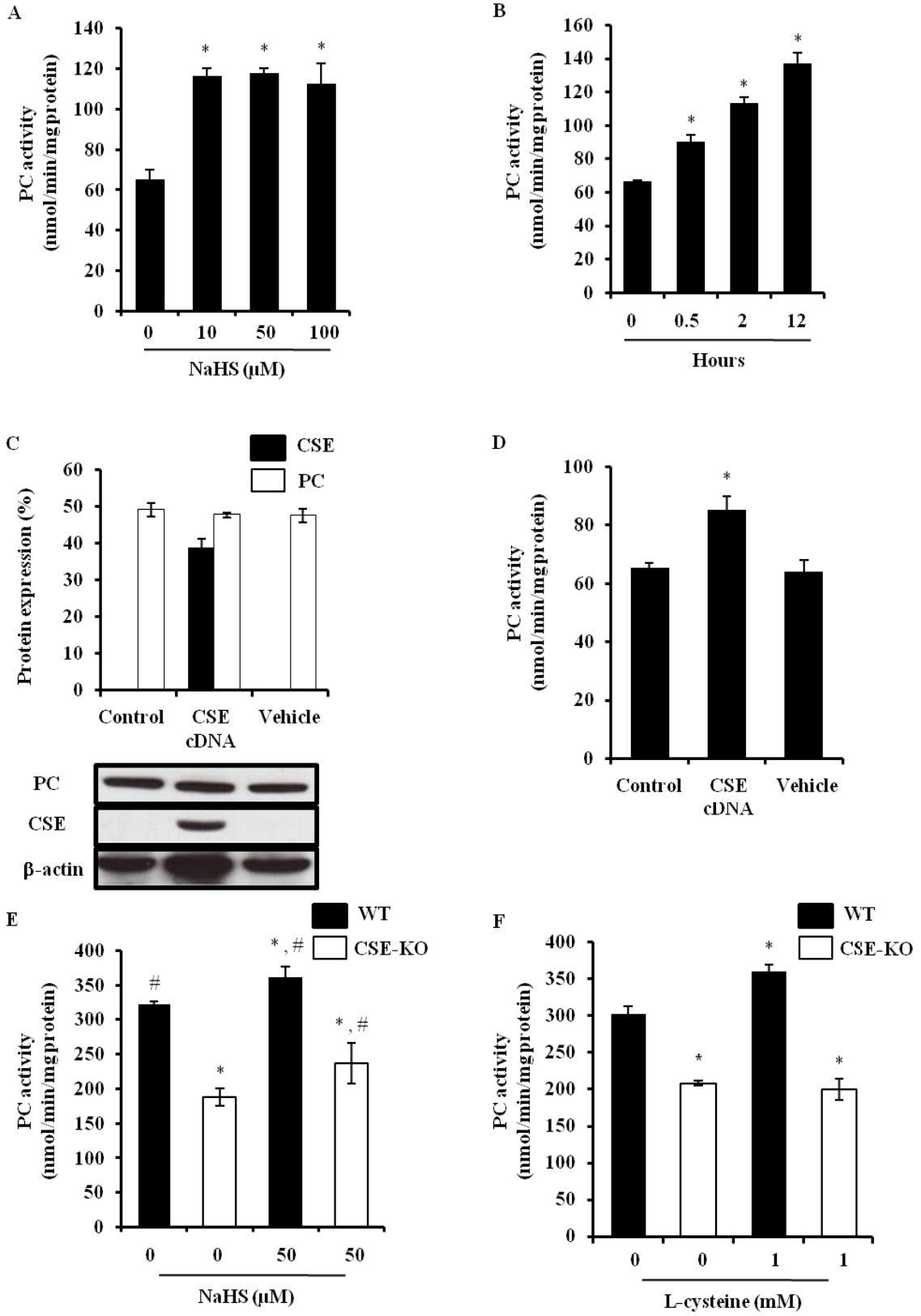


Figure 2. 1 **H_2S stimulates PC activity**. (A) H_2S induced PC activity. HepG2 cells were incubated with NaHS at the indicated concentrations (10-100 μM) for 2 h. * $p<0.05$ versus

the control. n=4. (B) H_2S induced PC activity in a time-dependent manner. HepG2 cells were incubated with NaHS (50 μM) for 0.5, 2 and 12 h, respectively. For long term treatment of NaHS (12 h), HepG2 cells were treated once in every 3 h with replacement of old medium. * $p<0.05$ versus the control. n=4. (C) and (D) CSE overexpression stimulated PC activity in HEK293 cells. HEK293 cells were transfected with CSE cDNA (184) for 24 h to overexpress CSE, the cells were then collected to analyze CSE expression and PC activity, respectively. * $p<0.05$ versus all other groups. n=4. (E) H_2S induced PC activity in liver tissues. Liver lysates from both WT and CSE-KO mice were incubated with 50 μM NaHS for 30 min. * $p<0.05$ versus WT without NaHS treatment; # $p<0.05$ versus CSE-KO without NaHS treatment. (F) Cysteine strengthened PC activity in liver lysates from WT mice but not CSE-KO mice. Liver lysates from both WT and CSE-KO mice were incubated with 1 mM cysteine for 30 min. * $p<0.05$ versus WT without cysteine treatment. Eight 12-week-old male WT mice and CSE-KO mice were used for these studies.

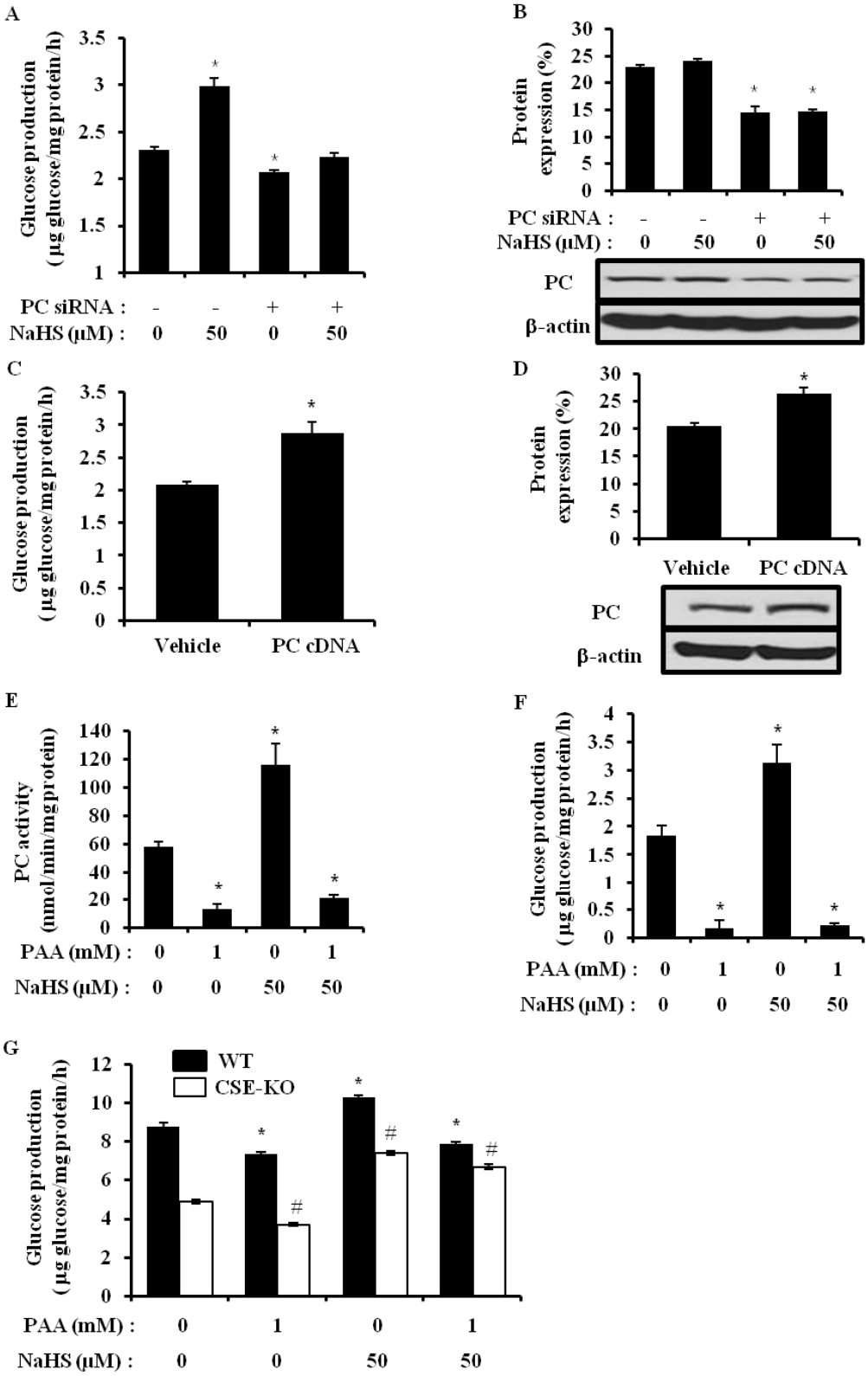


Figure 2. 2 PC mediates H_2S-stimulated glucose production. (A) and (B) knockdown

of PC reversed H_2S-induced glucose production. HepG2 cells were transfected with PC siRNA for 24 h in the presence or absence of NaHS (50 μM), and the cells were then collected for analyzing glucose production and PC protein expression. *, $p<0.05$ versus the cells transfected with control siRNA. n=3. (C) and (D) PC overexpession increased glucose production in HepG2 cells. HepG2 cells were transfected with PC cDNA for 24 h, the cells were then collected for analyzing glucose production and PC protein expression. *, $p<0.05$. n=4. (E) and (F) PAA lowered down H_2S-induced PC activity and glucose production. HepG2 cells were incubated with PAA (1 mM) for 30 min and/or NaHS (50 μM) for 2 h, the cells were collected for measuring PC activity and glucose production. *, $p<0.05$ versus control. n=4. (G) glucose production was decreased in CSE-KO hepatocytes. Hepatocytes isolated from WT and CSE-KO mice were incubated with PAA (1 mM) for 30 min and/or NaHS (50 μM) for 2 h, the cells were collected for measuring glucose production. * $p<0.05$ versus WT control; # $p<0.05$ versus CSE-KO control. n=4.

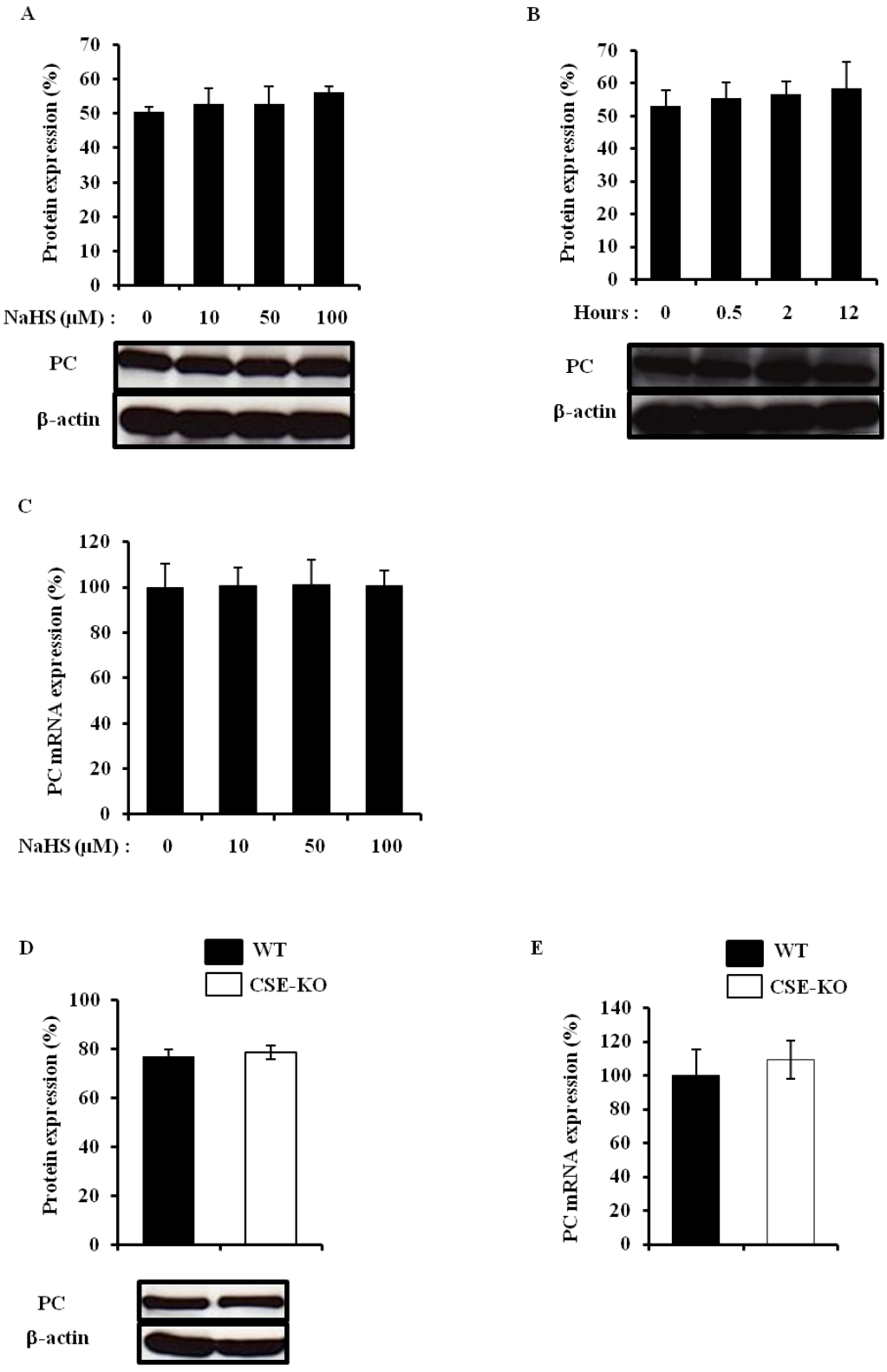


Figure 2. 3 H_2S has no effect on PC mRNA and protein expression. (A), (B) and (C) PC protein and mRNA expression was not changed by NaHS treatment. HepG2 cells were incubated with different concentration of NaHS (10-100 μM) for 2 h (A) and 50 μM of NaHS

for the indicated time (0.5-12 h) (B), the cells were then collected and lysed for western blotting analysis of PC protein expression (A and B). (C) HepG2 cells were incubated with different concentration of NaHS (10-100 μM) for 2 h and real-time PCR analysis of PC mRNA expressioin was then performed. n=4 (D) and (E) CSE deficiency did not affect PC protein and mRNA expressions in mouse liver tissues. four WT and four CSE-KO mice were used to prepare liver tissues.

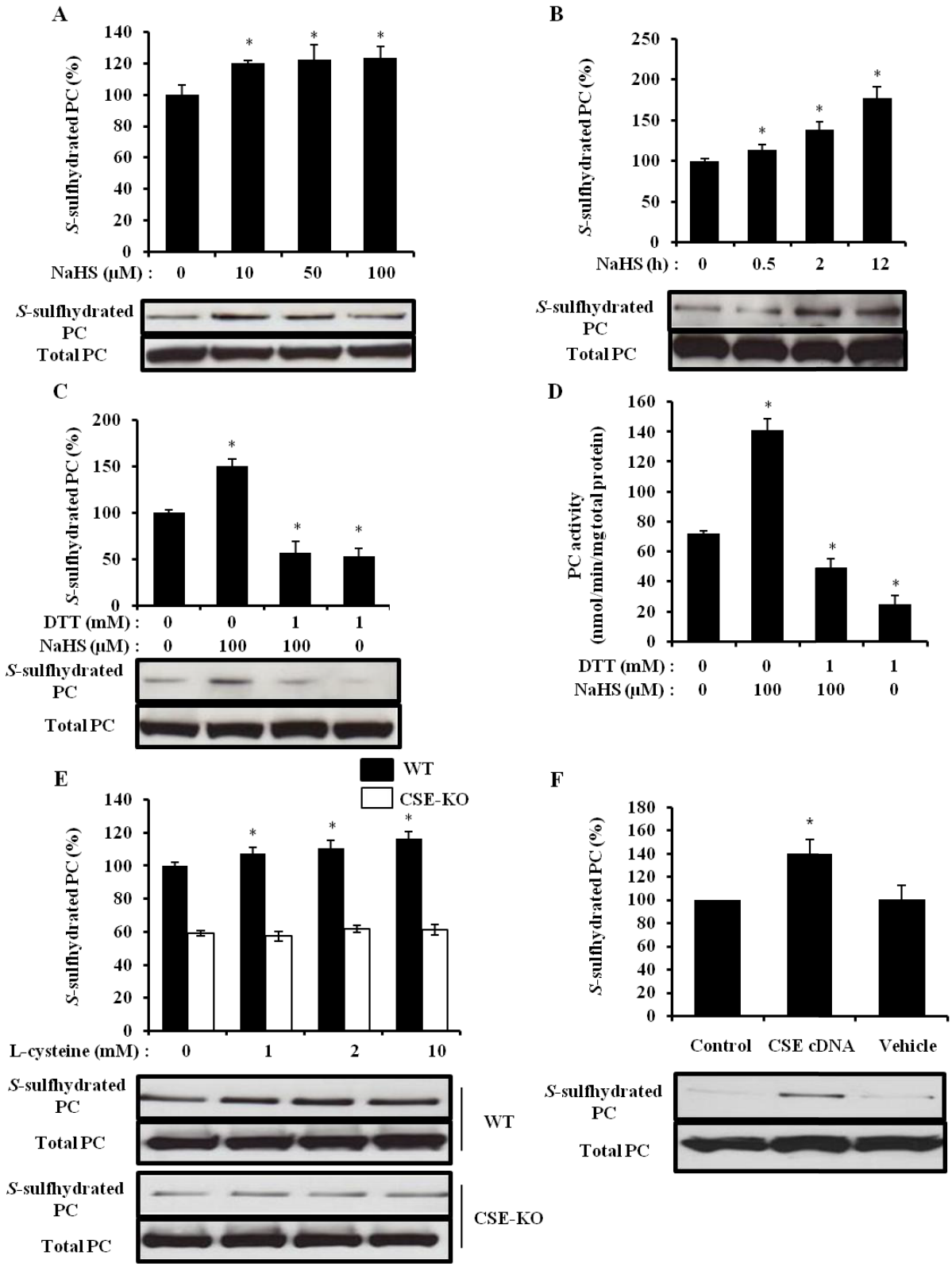


Figure 2. 4 **H_2S *S*-sulfhydrates PC protein**. (A) H_2S induced PC *S*-sulfhydration. HepG2 cells were incubated with NaHS at the indicated concentrations (10-100 μM) for 2 h,

and PC *S*-sulfhydration was then analyzed by BSA with anti-PC antibody. * $p<0.05$ versus the control. n=4. (B) H_2S induced PC *S*-sulfhydration in a time-dependent manner. HepG2 cells were incubated with NaHS (50 μM) for 0.5, 2 and 12 h, respectively. * $p<0.05$ versus the control. n=4. (C) and (D) DTT reversed H_2S-induced PC *S*-sulfhydration and PC activity. HepG2 cell lysates were incubated with NaHS (100 μM) and/or DTT (1 mM) for 30 min, the cells were then collected for analyzing PC *S*-sulfhydration and PC activity, respectively. * $p<0.05$ versus control. n=4. (E) Cysteine strengthened PC *S*-sulfhydration. Both WT and CSE-KO liver lysates were incubated with L-cysteine (1-10 mM) for 30 min. * $p<0.05$ versus WT control. n=4. (F) CSE overexpression stimulated PC *S*-sulfhydration. HEK293 cells were transfected with CSE cDNA (184) for 24 h following detection of PC *S*-sulfhydration. * $p<0.05$ versus all other groups. n=4.

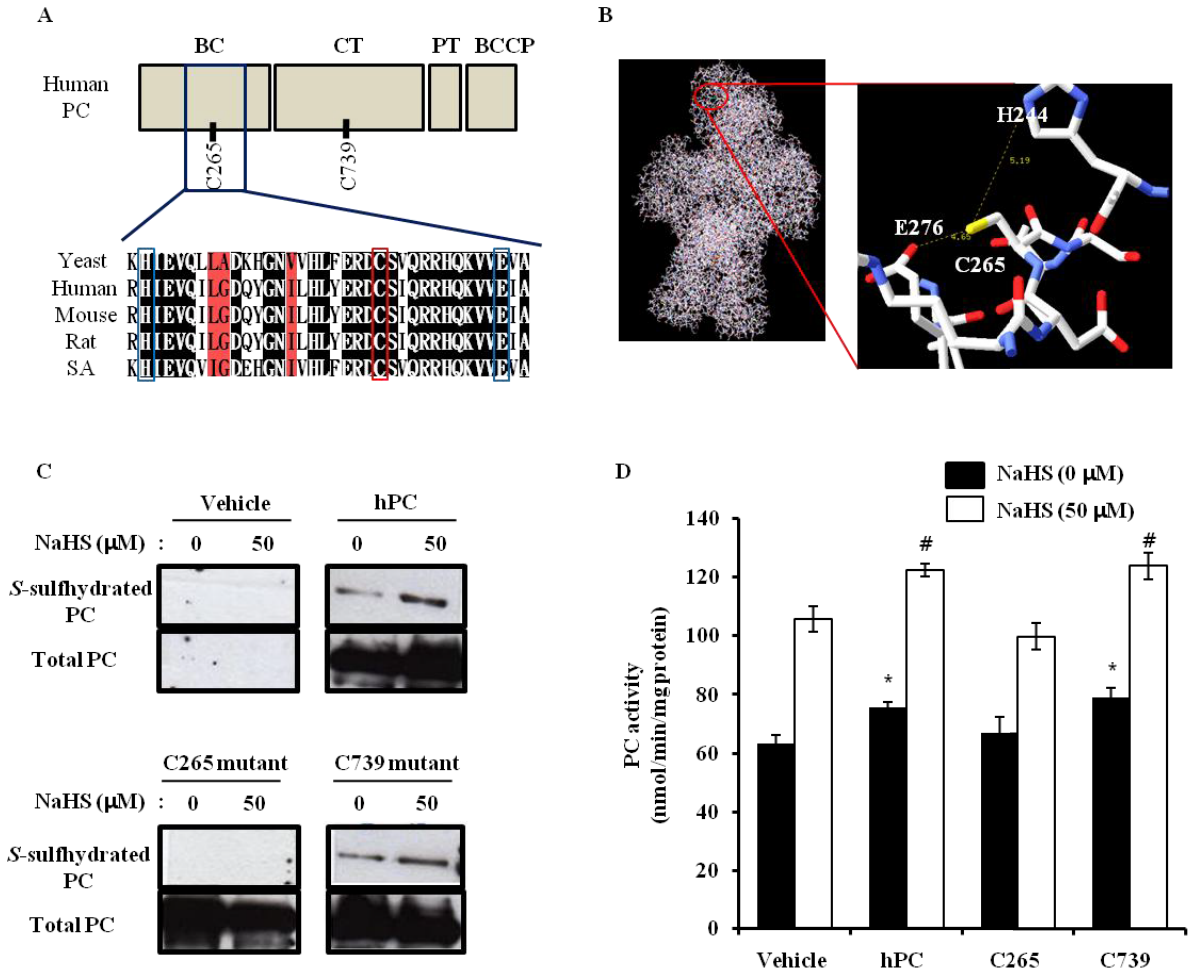


Figure 2. 5 Cysteine 265 is responsible for H_2S *S*-sulfhydration of PC. (A) The domains of human PC and the sites of highly conserved cysteine residues. SA, *Staphylococcus aureus*. (B) The location of cysteine 265 in crystallographic structure of PC. The crystallographic structure of PC was determined in Swiss-PDB viewer 4.0.1. PDB #, 3BG5 (crystal structure of PC in *Staphylococcus aureus*). (C) and (D) Mutation of Cysteine 265 attenuated H_2S-induced PC *S*-sulfhydration and PC activity. HEK-293 cells were transfected with vehicle, hPC, cysteine 265 mutant, or cysteine 739 mutant for 24 h following incubation of NaHS (50 μM) for additional 2 hours. After that, the cells were collected for BSA with anti-His6 antibody to differentiate recombinant PC from endogenous PC in HEK293 cells (C) and measuring PC activity (D), respectively. * $p<0.05$ versus the control without NaHS treatment; # $p<0.05$ versus the control with NaHS treatment. n=4.

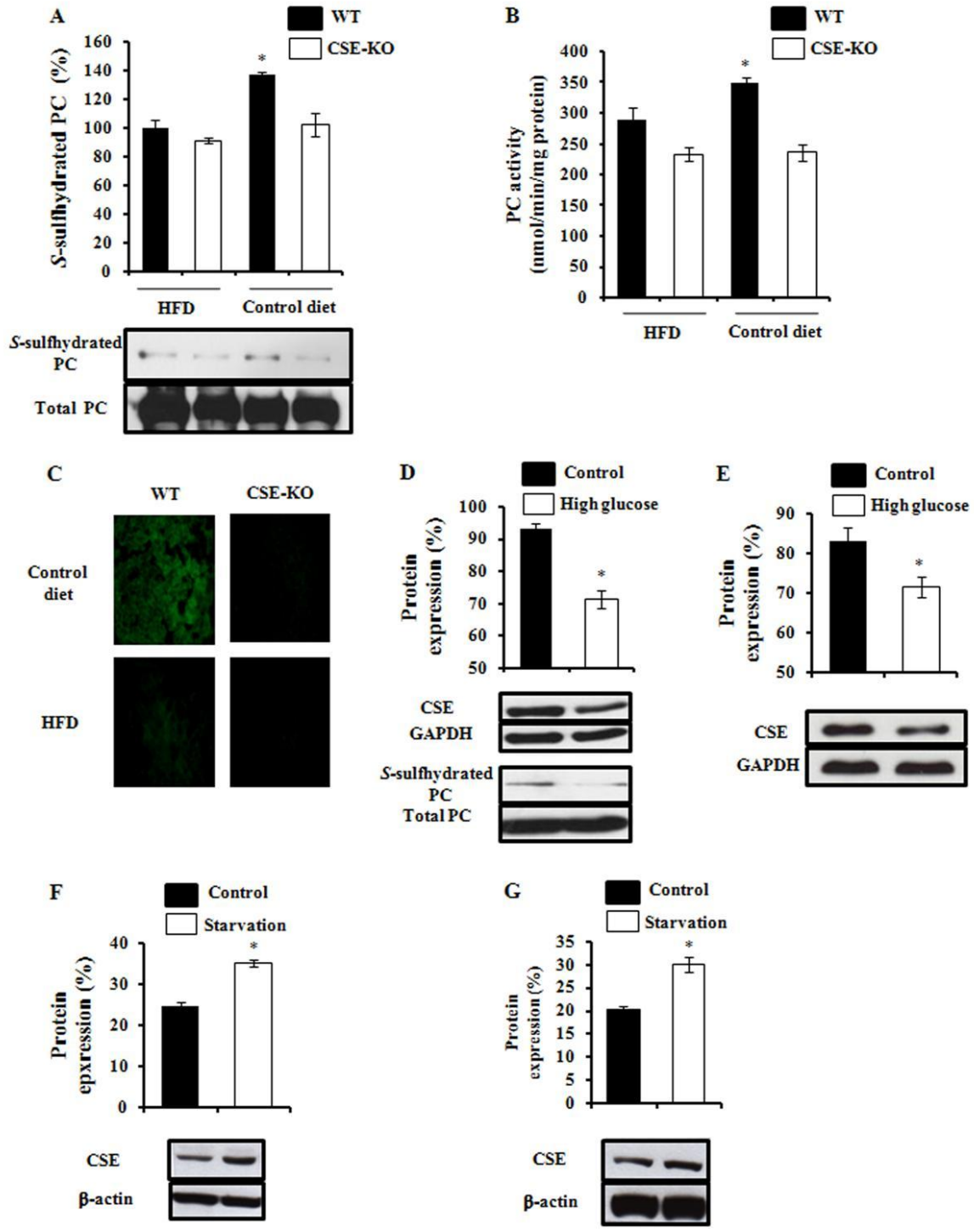


Figure 2. 6 Reduced liver CSE expression and PC *S*-sulfhydration in HFD-feeding mice. (A) and (B) HFD feeding of WT mice decreased liver PC *S*-sulfhydration and activity. Both WT and CSE-KO mice were feed with HFD for 12 weeks following detection of PC*S*-sulfhydration (A) and activity (B). * $p<0.05$ versus all other groups. n=4. (C) HFD feeding

significantly decreased CSE expression in WT mice. Both WT and CSE-KO mice were feed with HFD for 12 weeks following detection liver CSE expression by immunohistology. Bar: 20 μm. (D) and (E) High glucose inhibited CSE expression and PC *S*-sulfhydration in both HepG2 cells and primay hepatocytes. The cells were incubated with different concentration of glucose (5.5 and 25 mM) for 24 hours following detection of CSE expression and PC *S*-sulfhydration. * $p<0.05$. n=3. (F) and (G) Glucose deprivation enhanced CSE expression in both HepG2 cells and primay hepatocytes. The cells were deprived of glucose for 24 hours following detection of CSE expression. * $p<0.05$. n=3**.**

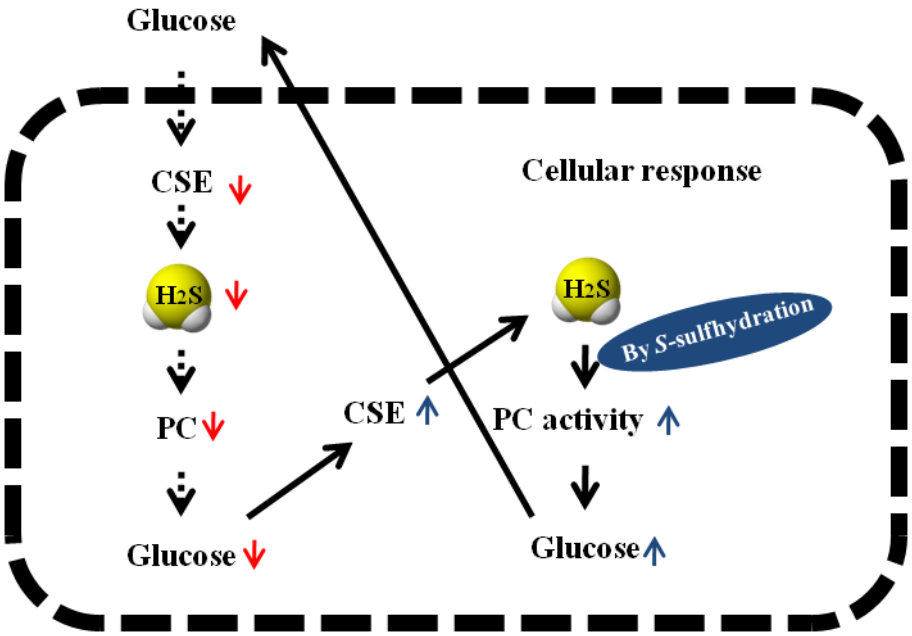


Figure 2. 7 The proposed signaling pathway underlying H_2S regulation of PC activity by *S*-sulfhydration in liver gluconeogenesis.

CHAPTER 3

Thioredoxin 1 is essential for protein *S*-desulfhydration

Youngjun Ju[1,2], Guangdong Yang[1,2]*

[1]Cardiovascular and Metabolic Research Unit, [2]The School of Kinesiology, Lakehead University, Ontario, Canada

Running title: Trx1 acts as an *S*-desulfhydrase

This chapter currently is under revision in Biochemistry and Biophysics Reports (BBREP).

3. 1 Abstract

The importance of H_2S in biology and medicine has been widely recognized in recent years, and protein *S*-sulfhydration is proposed to mediate most of H_2S bioactivity in the body. Thioredoxin 1 (Trx1) is an important reducing enzyme that cleaves disulfides in proteins and acts as an *S*-denitrosylase. The regulation of Trx1 on protein *S*-sulfhydration is unclear. Here we showed that Trx1 is essential for protein *S*-desulfhydration. Overexpression of Trx1 attenuated the basal level and H_2S-induced protein *S*-sulfhydration by direct interacting with *S*-sulfhydated proteins, i.e., glyceraldehyde 3-phosphate dehydrogenase and pyruvate carboxylase. Mutation of cysteine-32 but not cysteine-35 in the Trp-Cys^{32}-Gly-Pro-Cys^{35} motif eliminated the binding of Trx1 with *S*-sulfhydated proteins and abolished the *S*-desulfhydrating effect of Trx1. In contrast, blockage of Trx1 redox activity with PX12 or 2,4-dinitrochlorobenzene enhanced protein *S*-sulfhydration. All these data suggest that Trx1 acts as an *S*-desulfhydrase.

Key works: *S*-sulfhydration, *S*-desulfhydration, hydrogen sulfide, thioredoxin 1

3. 2 Introduction

Hydrogen sulfide (H_2S) can *S*-sulfhydrate proteins by yielding a hydropersulfide moiety (–SSH) in the active cysteine residues (94,198,199). It is predicted that *S*-sulfhydration can alter protein conformation and the final function and activity of target proteins (160,198). This particular redox modification of cysteine by *S*-sulfhydration has been achieved using proteomics techniques by coupling a specific enrichment strategy (biotin-switch assay and/or tag-switch assay) with high-throughput mass spectrometry analysis (94,179,200). So far, there have been a dozen proteins observed to be modified by H_2S through *S*-sulfhydration. K_{ATP} channel is a direct target for H_2S in regulating vasodilation and cardioprotection (7). In fact, H_2S induces *S*-sulfhydration of Kir6.1 subunit of K_{ATP} channels, and dithiothreitol (DTT) reverses this *S*-sulfhydration (160). H_2S-induced *S*-sulfhydration of phosphatase PTP1B alters endoplasmic reticulum stress response (201). H_2S *S*-sulfhydrates p65 and mediates the anti-apoptotic effect of NF-κB (179). H_2S modulates cellular redox signaling via direct *S*-sulfhydration of Keap1/Nrf2 system, p66, and electrophiles, contributing to the beneficial effects of H_2S on cellular aging and heart failure (34,202,203). *S*-sulfhydration of MEK1 leads to PARP1 activation and DNA damage repair in endothelia cells (161).

Like protein phosphorylation and *S*-nitrosylation, *S*-sulfhydration is proposed to mediate or modulate transduction of myriad cellular signals (94,198,199). While decades of research have established the generality and broad physiological importance of protein phosphorylation and *S*-nitrosylation, our current understanding of the fundamental biology and chemistry of *S*-sulfhydration as a protein signaling modality is still in its infancy (170,204). Is H_2S *S*-sulfhydration of cysteine enzyme-regulated or a spontaneous process? How is the biochemical stability of *S*-sulfhydrated proteins? Which critical factors are involved and what conditions are required for the formation of *S*-sulfhydration? Is there any

possibility for desulfhydration and/or transsulfhydration and how are they regulated? Little is known about the nature of or even necessity for enzymatic mechanisms that may directly add or remove SH groups from cysteine thiols.

In the present study, we demonstrated that thioredoxin 1 (Trx1), an oxidoreductase found in both prokaryotes and eukaryotes, is essential for protein *S*-desulfhydration. Overexpression of Trx1 attenuated the basal level and H_2S-induced protein *S*-sulfhydration by direct interaction with *S*-sulfhydated proteins, i.e., glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and pyruvate carboxylase (PC). In contrast, blockage of Trx1 activity strengthened protein *S*-sulfhydration. Furthermore, mutation of cyseine-32 in Trx1 eliminated the binding of Trx1 with *S*-sulfhydated proteins and abolished the *S*-desulfhydrating effect of Trx1 on both GAPDH and PC.

3. 3 Materials and methods

Cell culture and transfection

HepG2 (a human hepatocellular liver carcinoma cell line) and HEK293 cells were purchased from American Type Culture Collection **(**Manassas, VA**)** and maintained in Dulbecco's Modified Eagle's medium (Sigma, Oakville, ON) supplemented with 10% fetal bovine serum (Clontech, Mountain View, CA) and 1% penicillin-streptomycin solution (Sigma). For cell transfection, wild-type Trx1 or mutant Trx1 was transfected into HEK293 or HepG2 cells using Lipofectamine^{TM} 2000 reagent as described by the manufacturer's protocol (Invitrogen, Burlington, ON).

Biotin switch assay of *S*-sulfhydration

BSA was carried-out as described previously with some modifications (94). Briefly, the cells were homogenized in HEN buffer (250 mM HEPES (pH 7.7), 1 mM EDTA, and 0.1 mM Neocuproine) supplemented with 100 μM deferoxamine and centrifuged at 13,000 × *g* for 30 minutes at 4°C. The lysates were added to blocking buffer (HEN buffer adjust to 2.5% SDS and 20 mM methyl methanethiosulfonate (MMTS)) at 50°C for 20 minutes with frequent vortexing. The MMTS was then removed by acetone and the proteins were precipitated at -20°C for 20 min. Proteins were resuspended in HENS buffer (HEN buffer containing 1 % SDS) and 4 mM biotin-N-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio) propinamide (HPDP) in DMSO without ascorbic acid. After incubation for 2 hours at 25°C, biotinylated proteins were purified by streptavidin-agarose beads, which were then washed with HENS buffer. The biotinylated proteins were eluted by SD*S*-PAGE sample buffer and subjected to Western blotting analysis with antibody against GAPDH or PC.

Construction of Trx1 mutants

The plasmid Myc-TRX was purchased from Addgene (MA, USA) (205). Single mutation of cyteine-32 (Trx1-C32) and cysteine-35 (Trx1-C35) to serine were conducted using the Quick Change Site-Directed Mutagenesis kit (Stratagene, CA, USA) (161). The oligonucleotides using for mutagenesis were 5'-CTTCTCAGCCACGTGGAGTGGGCCTTGCAAAATG-3' (forward) and 5'-CATTTTGCAAGG-CCCACTCCACGTGGCTGAGAAG-3' (reverse) for cysteine-32, and 5'-CGTGGTGTGGGCC-TAGCAAAATGATCAAGC-3' (forward) and 5'-GCTTGATCATTTTGCTAGGCCCACACCACG-3' for cysteine-35. The correct mutant was confirmed by DNA sequencing at the Paleo-DNA laboratory in Lakehead University, ON, Canada.

Co-Immunoprecipitation (Co-IP)

The cells were lysed in IP lysis/wash buffer (25 mM Tris, 0.15M NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, pH7.4) including protease inhibitor cocktail (Sigma) (34). The proteins (500 μg) were then incubated with 5 μg anti-PC antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-GAPDH (Santa Cruz Biotechnology) for overnight at 4 ^{o}C. Protein A/G agarose was incubated with lysates for 1 hour with gentle end-over-end mixing. Agarose resin was washed three times with IP lysis/wash buffer. The resin was then washed one time with saline solution (0.15 M NaCl). Immune complex was eluted with 2 × SDS PAGE sample buffer (62.5 mM Tri*s*-Cl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue) at 100 ^{o}C for 5 minutes. The samples were subjected to Western immunoblotting analysis.

Western immunoblotting

The cells were harvested and lysed in a cell lysis buffer including protease inhibitor

cocktail (Sigma). Extracts were separated by centrifugation at 14,000 g for 15 min at 4°C. SD*S*-PAGE and Western blot analysis were performed. Briefly, equal amount of proteins were boiled in 1 × SDS sample buffer and run in a 10% SD*S*-PAGE gel, and transferred onto pure nitrocellulose blotting membranes (Pall Corporation). Immunoblots were probed with mouse monoclonal anti-PC (1:500 dilution; Santa Cruz Biotechnology), anti-GAPDH (1:1,000 dilution; Santa Cruz Biotechnology) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse secondary antibody (1:10,000 dilution; Sigma). Anti-β-actin primary antibody (1:10,000 dilution; Sigma) and HRP-conjugated rabbit anti-mouse secondary antibody (1:5,000 dilution; Sigma) were employed to normalize protein expression. The blots were developed using chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences).

Statistical analysis

Data were presented as means ± SEM, representing at least three independent experiments. Statistical comparisons were made using Excel 2007 (Microsoft, Redmond, WA) with Student's *t-test* to evaluate the difference between two groups, and the difference between multiple groups were analyzed by using SigmaPlot 12.0 (Systat Software Inc. San Jose, CA) with ANOVA and post-hoc Tukey test. Significance level was set at $p<0.05$.

3.4 Results

Trx1 suppresses protein *S*-sulfhydration

Protein *S*-sulfhydration can be detected by the modified BSA (94). Following the procedures descried in the Methods, the *S*-sulfhydrated thiols were labeled with biotin-HPDP and then detected by western blotting with the antibody against either biotin or specific protein. We first detected the basal labeling of multiple proteins using an antibody against biotin, and the signaling became even stronger after treatment with 50 μM NaHS (a well known H_2S donor) for 2 hours in HEK293 cells. We further found that Trx1 overexpression in HEK293 cells markedly decreases H_2S-induced protein *S*-sulfhydration (Fig. 3. 1A). Probing with antibodies to specific proteins, such as PC (Fig. 3. 1B) and GAPDH (Fig. 3. 1C), it was clearly showed that both GAPDH and PC proteins were basically *S*-sulfhydrated and further strengthened by exogenously applied NaHS, while transfection of HEK293 cells with Trx1 cDNA significantly reversed the basal and H_2S-initiated *S*-sulfhydration of PC and GAPDH, suggesting Trx1 acts as a *S*-desulfhydrase. Identical results were further validated in another cell line, HepG2 cells (Fig. 3. 1D and 1E).

Inhibition of Trx1 activity stimulates protein *S*-sulfhydration

PX12, an irreversible and competitive inhibitor of Trx1, significantly enhanced the *S*-sulfhydration of PC and GAPDH when compared with the control HepG2 cells (Fig. 3. 2A and 2B). Similarly, 2,4-dinitrochlorobenzene (DNCB), an irreversible inhibitor of thioredoxin reductase (TrxR), also increased *S*-sulfhydrated PC and GAPDH (Fig. 3. 2C and 2D), suggesting that thioredoxin reductase cooperates with Trx1 contributing to protein *S*-desulfhydration. Either PX12 or DNCB did not further increase H_2S-stimulated PC and GAPDH *S*-sulfhydration (Fig. 3. 2A-D). Furthermore, we observed that PX12 or DNCB significantly reverses the inhibitory role of Trx1 overexpression on *S*-desulfhydration of PC

and GAPDH (Fig. 3. 3A-D).

Cysteine-32 is requisite for Trx1 desulfhydration of proteins

Trx1 has a highly conserved active site, Trp-Cys^{32}–Gly–Pro–Cys^{35}, which is required for its redox activity (206,207). Here we found that mutation of cysteine-32 but not cysteine-35 in the Trp-Cys^{32}–Gly–Pro–Cys^{35} motif eliminates the *S*-desulfhydration activity of Trx1 on both PC and GAPDH (Fig. 3. 4A and 4B), suggesting cysteine-32 is requisite for Trx1 *S*-desulfhydration of proteins. Trx1 directly interacted with PC and GAPDH, which was not altered in the presence of NaHS. Interestedly, mutation of cysteine-32 but not cysteine-35 abolished the binding of Trx1 with PC (Fig. 3. 4C) or GAPDH (Fig. 3. 4B).

3. 5 Discussion

H_2S regulates cellular processes largely through *S*-sulfhydration of active cysteine residues within proteins (198,199). *S*-sulfhydration of proteins has been demonstrated to be involved in a broad range of signaling and metabolic pathways, including cell death and differentiation, enzymatic activity, protein localization, protein–protein interactions, and protein stability, etc (34,94,160,161,178,179,201,203,208). Given the importance of protein *S*-sulfhdyration in both health and diseases, much less is known about the systems governing protein *S*-desulfhydration. In our present report, we demonstrated that Trx1 is essential for breakage of hydropersulfide group from the cysteine thiol in *S*-sulfhdyrated proteins by direct interaction with Trx1 at cysteine-32.

Trx1, a ubiquitous 12 kDa proteins with general anti-oxidative properties, is involved in a large number of cellular functions such as cell proliferation and differentiation, redox and metabolic pathways, gene transcription, embryogenesis, etc (162,169,173,209,210). In recent years, Trx1 has been suggested to mediate cysteine *S*-denitrosylation by reducing inter- and intramolecular disulfide bonds in proteins (153,162). By analogy to *S*-nitrosylation, the extent of *S*-sulfhydration of a given protein must be governed by the equilibrium between *S*-sulfhydration and *S*-desulfhydration reactions (162). Protein *S*-sulfhydration is mainly dependent on the activity of H_2S-producing enzymes (179,211). Here we showed that Trx1 is clearly involved in protein *S*-desulfhydration, because overexpression of Trx1 suppressed but blockage of Trx1 activity enhanced protein *S*-sulfhydration. Trx1 catalyzes thiol-disulfide oxidoreductions by using redox-active cysteine residues present in a Trp-Cys^{32}-Gly-Pro-Cys^{35} sequence motif (153,169,212). The active cysteine residues form a disulfide in oxidized Trx1, while the disulfide is broken in reduced Trx1 with the aid of TrxR using electrons from NADPH (169,175). In our study, inhibition of TrxR activity by DNCB must block the transition of reduced Trx1 to oxidized Trx1, which diminishes the inhibitory role of Trx1 on

protein *S*-desulfhydration. These findings indicate that protein *S*-desulfhydration is indeed mediated by the Trx1/TrxR system.

We further provided evidence that Trx1 contributes to protein *S*-desulfhydration via protein-to-protein association between its substrate and Trx1 at cysteine-32. Co-immunoprecipitation results demonstrated that Trx1 directly interacts with *S*-sulfhydrated proteins, and single mutation of cysteine-32 to serine abolished the inhibitory role of Trx1 on protein *S*-desulfhydration. Mutation of cysteine-32 also eliminated the binding of Trx1 with *S*-sulfhydrated proteins. In contrary, mutation of cysteine-35 in the same Trp-Cys^{32}-Gly-Pro-Cys^{35} sequence motif failed to change the *S*-desulfhydration activity of Trx1. There may be several reasons for this difference. Firstly, formation of the cysteine-32/cystine-35 disulfide bridge leads to a rotation of the side-chain of cysteine-32 away from cysteine-35 in the reduced form (207,210). Mutation of cysteine-32 will induce formation of new disulfide between cysteine-35 and other cysteine residues within Trx1, which would change the position of cysteine 32 and block the interaction of cysteine-32 with *S*-sulfhydrated proteins. Secondly, the thiol of cysteine-32 has a low pK_a value in comparison with cysteine-35, and cysteine-32 is more easily attacked by *S*-sulfhydrated proteins for disulfide reduction (169,175,212). Thirdly, Trx-interacing protein (Txnip), a constitutively expressed protein, forms a mixed disulfide with the active site cysteine-32 of Trx1 (162,174,206). Mutation of cysteine-32 would interrupt the interaction of Txnip with Trx1, thereby affecting the oxidoreductase activity of Trx1. More studies are required to elucidate the precise interaction of Trx1 cysteine-32 and *S*-sulfhydrated proteins. In addition, we cannot exclude the possibility that other cysteine residues within Trx1 are involved in this process.

Two major pathways for enzymatic denitrosylation have recently been recognized (162). Besides with Trx system, *S*-nitrosoglutathione (GSNO) reductase also can lower the levels of protein *S*-nitrosothiols (162,207,213). Unlike Trx1, which directly target specific *S*-

nitrosylated proteins for cysteine reduction, GSNO reductase indirectly decreases cell protein *S*-nitrosylation through GSNO metabolism. In addition, glutathione, the most abundant intracellular thiols, plays a major role in protein *S*-denitrosylation by GSNO reductase. We had data showed that the supplemental of glutathione has no effect on protein *S*-desulfhydration. Therefore, GSNO reductase would not act as an *S*-desulfhydrase.

Based on these results above, we conclude that the *S*-desulfhydration of proteins is associated with the Trx1/TrxR system, and cysteine-32 within Trx1 is responsible for the direct interaction of Trx1 and *S*-sulfhydrated proteins following the breakage of hydropersulfide group. Further investigation on the role of Trx system in relation to protein *S*-desulfhydation and their biological relevance needs to be explored.

Acknowledgment: This study was supported by a grant-in-aid from the Heart and Stroke Foundation of Canada. G.Y. was supported by a New Investigator award from the Heart and Stroke Foundation of Canada.

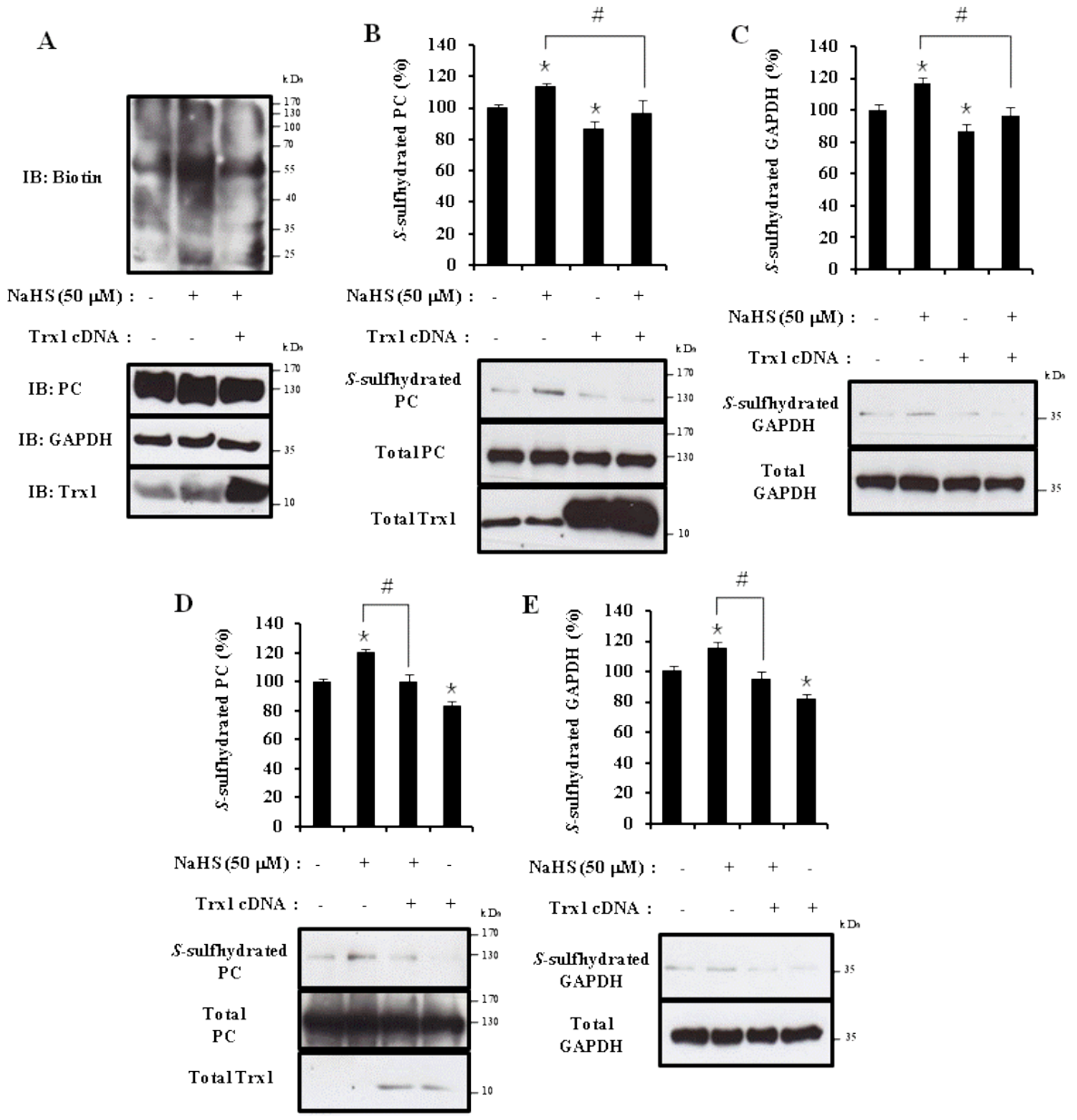


Figure 3. 1 Trx1 acts as an *S*-desulfhydrase. (A), (B), and (C) Trx1 overexpression inhibited the *S*-sulfhydration level of total proteins, PC and GAPDH in HEK293 cells. HEK293 cells were transfected with Trx1 cDNA for 24 hours, and then treated with 50 μM NaHS for 2 hours. After that, the cells were collected for BSA using antibody against biotin (A), PC (B), and GAPDH (C), respectively. * $p<0.05$ versus all other groups; # $p<0.05$ versus control. n=4. (D) and (E) Trx1 overexpression inhibited PC and GAPDH *S*-sulfhydration in HepG2 cells. HepG2 cells were transfected with Trx1 cDNA for 24 hours, and then treated

with 50 μM NaHS for 2 hours. After that, the cells were collected for BSA using antibody against PC (D) and GAPDH (E), respectively. * $p<0.05$ versus all other groups; # $p<0.05$ versus control. n=4.

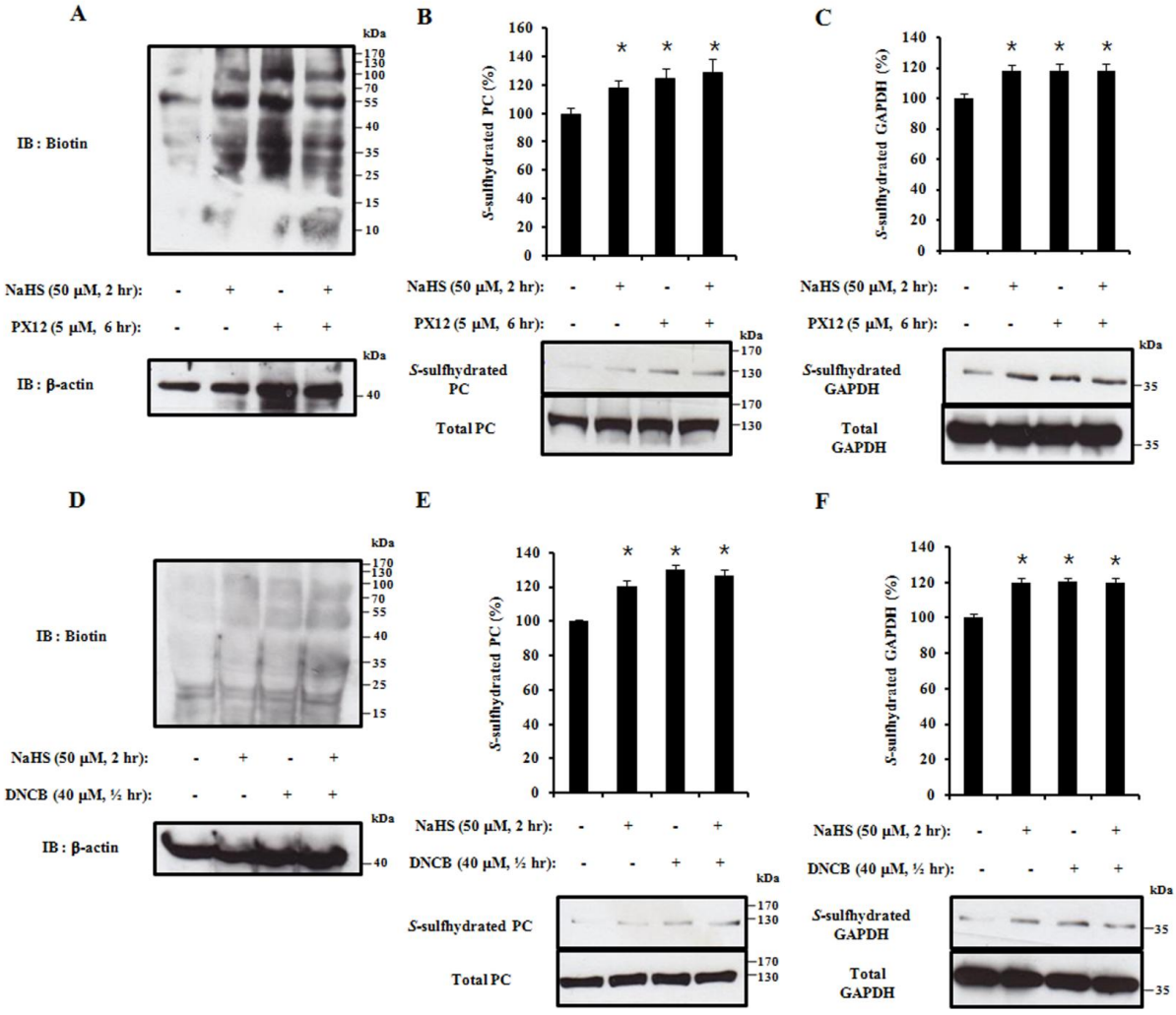


Figure 3. 2 Inhibition of Trx system enhances protein *S*-sulfhydration. (A) and (B) PX12 induces PC and GAPDH *S*-sulfhydration. HepG2 cells were firstly treated with 5 μM PX12 for 4 hours, and then 50 μM NaHS was added for additional 2 hours. After that, the cells were collected for BSA using antibody against biotin PC (A) and GAPDH (B), respectively. * $p<0.05$ versus control. n=4. (C) and (D) DNCB induces PC and GAPDH *S*-sulfhydration. HepG2 cells were treated with 40 μM DNCB or 30 minutes and/or 50 μM NaHS for 2 hours. After that, the cells were collected for BSA using antibody against biotin PC (C) and GAPDH (D), respectively. * $p<0.05$ versus control. n=4.

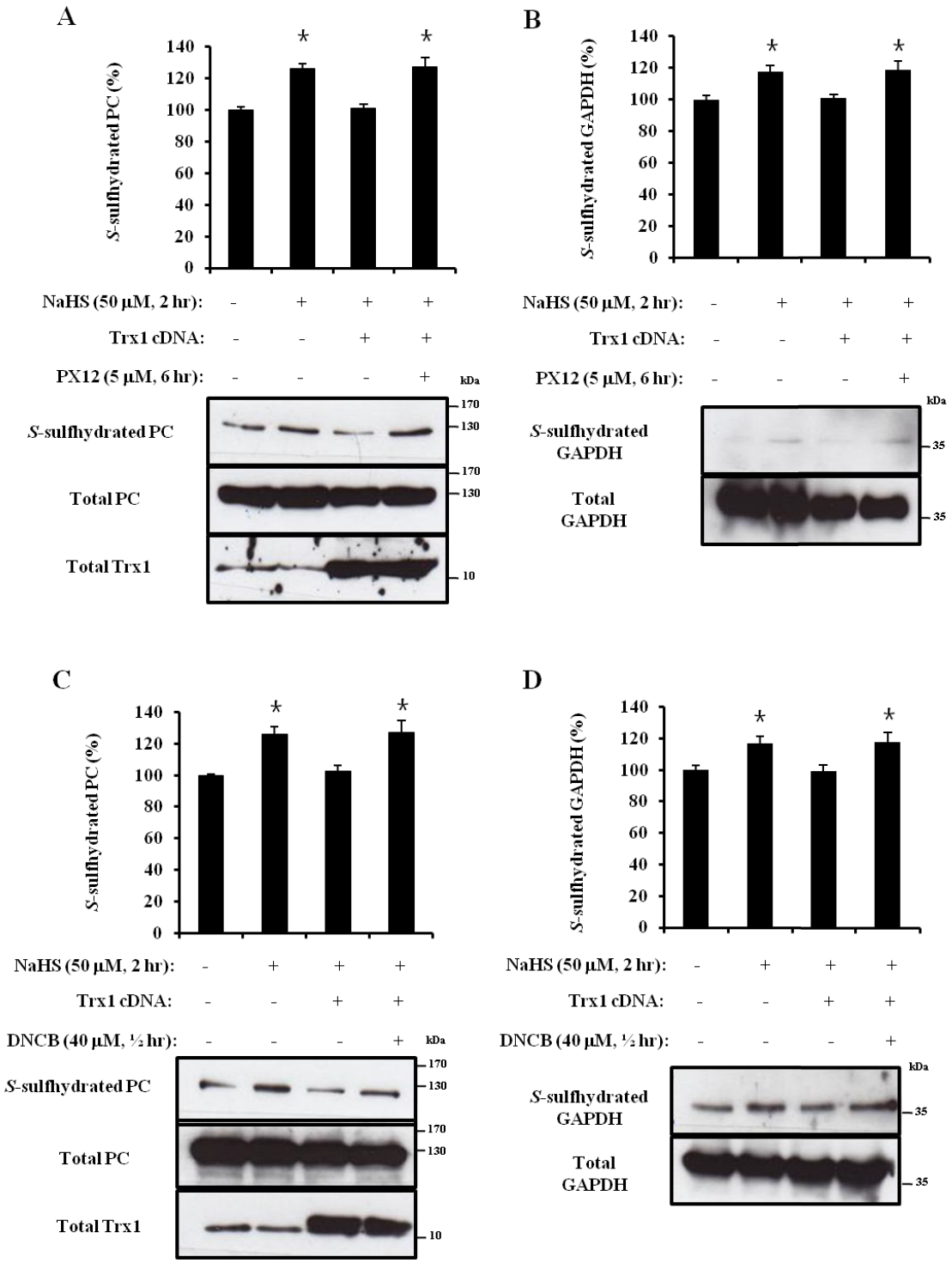


Figure 3. 3 PX12 and DNCB reverse the inhibitory effect of Trx1 on protein *S*-desulfhydration. (A) and (B) PX12 abolished the inhibitory effect of Trx1 on PC and GAPDH *S*-desulfhydration. HEK293 cells were firstly transfected with Trx1 cDNA for 24

hours following incubation with 5 μM PX12 for 4 hours and/or 50 μM NaHS for 2 hours. After that, the cells were collected for BSA using antibody against biotin PC (A) and GAPDH (B), respectively. * $p<0.05$ versus control. n=4. (C) and (D) DNCB reversed the inhibitory effect of Trx1 on PC and GAPDH *S*-desulfhydration. HEK293 cells were firstly transfected with Trx1 cDNA for 24 hours following incubation with 40 μM DNCB or 30 minutes and/or 50 μM NaHS for 2 hours. After that, the cells were collected for BSA using antibody against biotin PC (C) and GAPDH (D), respectively. * $p<0.05$ versus control. n=4.

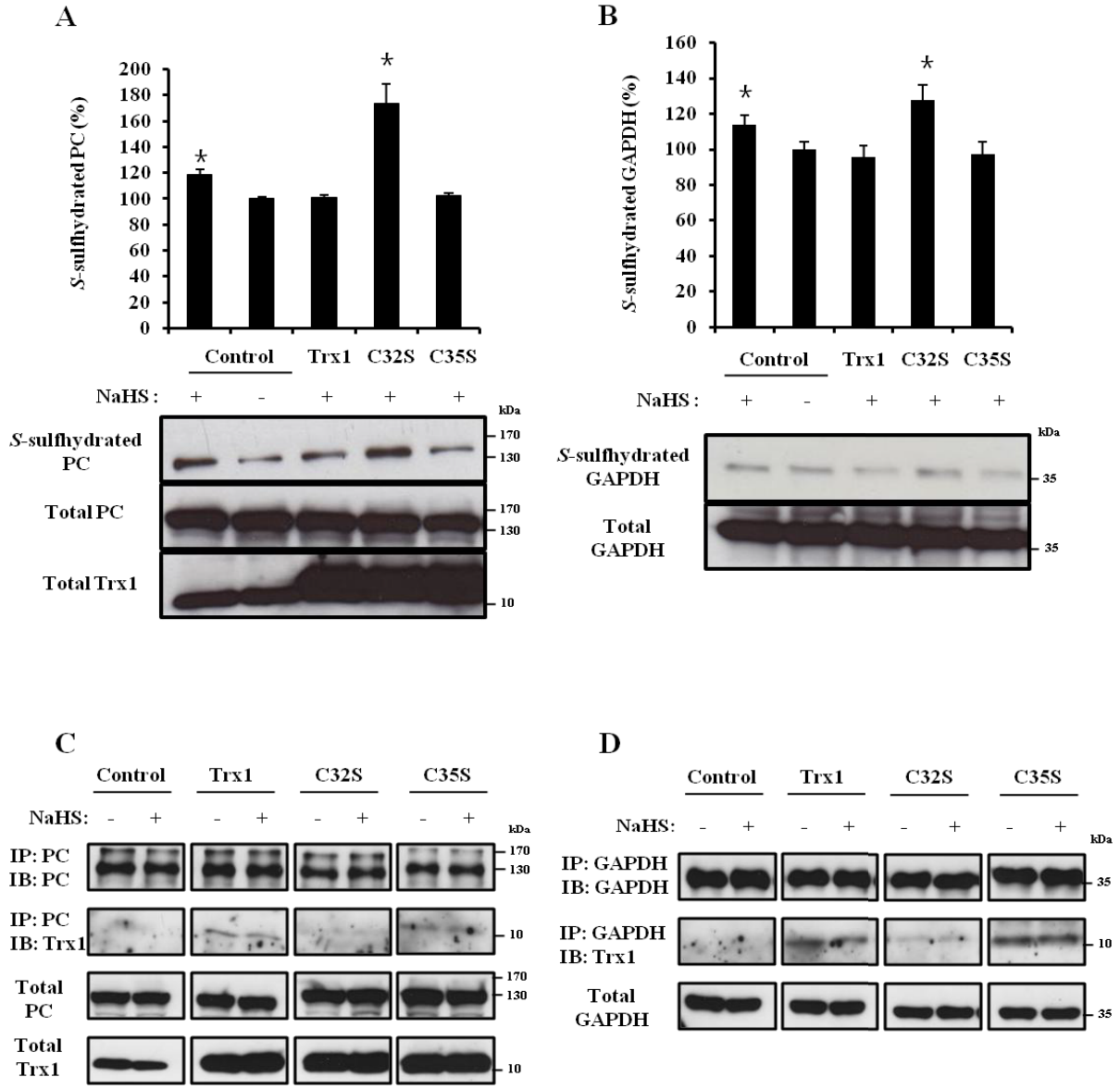


Figure 3. 4 Cysteine-32 in Trx1 is responsible for Trx1 *S*-desulfhydrating proteins.

(A) and (B) cysteine-32 mutation abolished the inhibitory role of Trx1 on protein *S*-desulfhydration. HEK293 cells were transfected with Trx1 cDNA and mutated Trx1 cDNA (C32S and C35S) for 24 hours following incubation with 50 μM NaHS for 2 hours. After that, the cells were collected for BSA using antibody against PC (A) and GAPDH (B), respectively. * $p<0.05$ versus the control without NaHS treatment. n=4. (C) and (D) cysteine-32 mutation disassociated Trx1 binding with *S*-sulfhydrated PC and GAPDH. HEK293 cells were transfected with Trx1 cDNA and mutated Trx1 cDNA (C32S and C35S) for 24 hours

following incubation with 50 μM NaHS for 2 hours. After that, the cells were collected for co-immunoprecipitation assay as indicated in the figure. n=3.

CHAPTER 4

DISCUSSION AND CONCLUSION

GENERAL DISCUSSION

H_2S is considered as the third gasotransmitter along with NO and CO. Similar to NO and CO, H_2S plays diverse roles in metabolic and signaling pathways. Recently, it is recognized that H_2S *S*-sulfhydration of protein mediates most of H_2S activities in various cellular functions. Protein *S*-sulfhydration is analogous to protein *S*-nitrosylation by NO. Protein *S*-sulfhydration is just newly discovered, many things remain unclear about protein *S*-sulfhydration, i.e., the regulatory and forming mechanism of *S*-sulfhydration, the interaction between *S*-nitrosylation and *S*-sulfhydration, and their alterations in health and diseases. Here we demonstrated that H_2S regulates liver gluconeogenesis through *S*-sulfhydration of PC and Trx1 is involved in the regulation of protein *S*-desulfhydration.

In chapter 2, we found that *S*-sulfhydration of PC induced by H_2S increases gluconeogenesis in HepG2 and mouse primary liver cells. To date, the post-translational modification of PC is poorly understood. The present study showed that PC *S*-sulfhydration occurs in cysteine 265 of human PC, and *S*-sulfhydration induces PC activity. H_2S had no effect on the expression of PC. *S*-sulfhydration of PC is regulated by CSE in liver cells and it was further found that high glucose inhibits CSE expression and *S*-sulfhydration of PC. CSE/H_2S pathway is involved in the regulation of gluconeogenesis through PC *S*-sulfhydration and it is modulated by glucose in liver cells. In the last decade, CSE/H_2S pathway has been gotten attentions for their pathological and physiological roles in liver. However, most studies of H_2S in liver are related to its anti-oxidant, bioenergetic, and detoxification effects (95). In our previous studies, it was shown that CSE/H_2S pathway plays critical roles for the metabolisms in liver, such as lipid metabolism (95,96) and glucose metabolism (98). Unfortunately, the detail regulation of metabolisms by H_2S in liver was not fully determined yet. Here, based on my results, we suggest that PC *S*-sulfhydration regulated by CSE/H_2S pathway in liver cells is one of the regulatory mechanisms for the glucose

metabolism, especially gluconeogenesis. Gluconeogenesis is associated with metabolic syndromes, such as diabetes, obesity, and hypertension. For metabolic syndromes, insulin resistance is considered as a main pathogenic factor. In our previous studies, it was shown that H_2S plays a pathological role in the induction of hepatic insulin resistance (98,214). Until now, the effect and regulation of CSE/H_2S pathway in metabolic syndromes are not well determined. In addition, malfunction of CSE/H_2S pathway in liver may be related to the development of metabolic syndromes because abnormality of CSE/H_2S system is often found in metabolic syndromes (215). Therefore, protein *S*-sulfhydration regulated by CSE/H_2S pathway in liver will be a critical process to regulate metabolic pathways and syndromes through CSE/H_2S pathway, and protein *S*-sulfhydration will be an useful therapeutic target for metabolic syndromes through the regulation of gluconeogenesis.

In chaper 3, we reported that *S*-sulfhydration of GAPDH and PC are regulated by Trx1. Trx1 is a well-known redox related small protein which plays a role as a denitrosylase and transnitrosylase. However, the role of Trx1 in regulation of protein *S*-sulfhydration is not clear. Here we found that Trx1 reverses *S*-sulfhydration of GAPDH and PC and acts as a *S*-desulfhydrase. Especially, cysteine 32 but not cysteine 35 in Trx1 is a critical residue for the desulfhdyration activity of Trx1. The regulatory mechanism of desulfhydration activity in Trx1 by cysteine 32, not cysteine 35, results from the different properties between these two cysteines (216). Firstly, in the folding structure of Trx1, cysteine 35 is buried and cysteine 32 is freely accessible for the interaction with substrate and target residue in biomolecules. Thus, cysteine 32 is able to access the *S*-sulfhydrated cysteine residue. Secondly, cysteine 32 is deprotonated at physiological pH and can be allowed for nucleophilic attack on target, such as disulfide bond. Persulfide in protein *S*-sulfhydration shares similar properties with disulfide bond. Consequently, cysteine 32 can play a nucleophilic attacker for the persulfide in protein *S*-sulfhydration. Finally, in physiological pH, cysteine 32 acts as an acid and

cysteine 35 as a base. Deprotonated and acidic cysteine 32 uptakes HS^- from persulfide in protein *S*-sulfhydration and cysteine 35 then plays a role for the release of H_2S from Trx1, as a base (Fig. 4). The study for the regulatory and forming mechanism of protein *S*-sulfhydration is important to understand the effects and roles of H_2S in pathological and physiological conditions. Unfortunately, up to now there is no report about the regulatory and forming mechanism of protein *S*-sulfhydration. The present study first explored the regulatory mechanism of protein *S*-sulfhydration and demonstrated that Trx1 is essential for removal of protein *S*-sulfhydration .

The forming mechanism is an important matter for the study of protein *S*-sulfhydration. As mentioned in Chapter 1, the study of forming mechanism of protein *S*-sulfhydration is rarely reported. It is unknown whether protein *S*-sulfhydration is enzyme-catalyzed reaction or automatic redox reaction but it is clear that many intermediates must be in involved the formation of protein *S*-sulfhydration. RSSH including GSSH and CSSH is a highly potential intermediate for the forming of protein *S*-sulfhydration (162). Furthermore, the correlation between H_2S and GSSH was determined in different tissues, such as brain, kidney, and liver, from mouse. It was suggested that H_2S producing enzymes, CSE and CBS, are involved in the regulation of GSSH levels and may influence on the forming protein *S*-sulfhydration (162). Therefore, further investigation on the forming mechanism of protein *S*-sulfhydration will be required to determine regulation of protein *S*-sulfhydration and understand its roles and effects on diverse pathophysiological conditions.

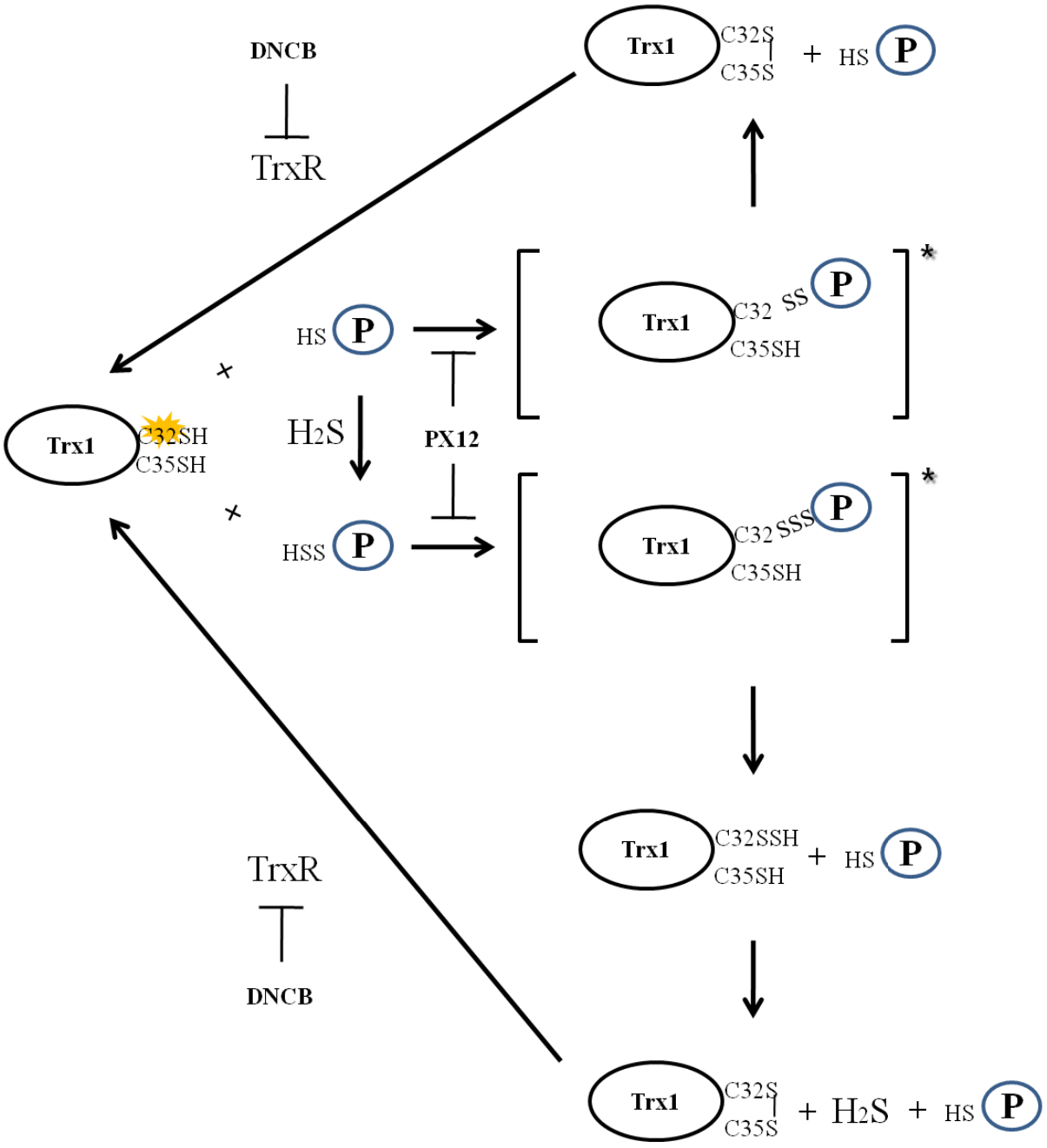


Figure 4. 1 The signal pathway underlying Trx1-regulated protein *S*-desulfhydration.

CONCLUSION

The present studies showed that H_2S plays an important role in up-regulation of gluconeogenesis, through PC *S*-sulfhydration, and Trx1 acts as a protein *S*-desulfhydrase.

In liver, CSE is mainly responsible for H_2S production and contributes to PC *S*-sulfhydration. I further found that glucose decreases CSE expression in liver and inhibits PC and gluconeogenesis. Malfunction of gluconeogenesis, elevated gluconeogenic activity, is a critical factor in metabolic syndromes. Gluconeogenesis is a therapeutic target of these syndromes through the inhibitory regulation of gluconeogenesis. Therefore, CSE/H_2S pathway in liver is a potential therapeutic target for metabolic syndromes and PC *S*-sulfhydration is a useful approach to understand the roles and effects of H_2S on gluconeogenesis.

The PC and GAPDH *S*-sulfhydration are regulated by Trx1. Cysteine 32 among active sites in Trx1, but not cysteine 35, plays a critical role for the regulation of protein *S*-sulfhydration (Fig. 4). I expect that the present finding about the regulatory mechanism of protein *S*-sulfhydration by Trx1 will provide the basis for the understanding of protein *S*-sulfhydration in both health and diseases.

SIGNIFICANCE OF THE STUDY

Diabetes is a metabolic disease and it is estimated that 387 million people who suffer diabetes worldwide as of 2014. Type 2 diabetes makes up about 90 % of the cases which is equal to 8.3 % of the adult population. Diabetes can cause many complications, such as diabetic ketoacidosis, nonketotic hyperosmolarcoma, cardiovascular disease, stroke, kidney failure, foot ulcers, and damage to the eyes. Diabetes resulted in an estimated 1.5 to 4.9 million deaths per year in the years 2012 to 2014. In Canada, diabetes is involved in over 41,500 deaths each year. Furthermore, the cost for the diabetes is huge and direct cost ranges from $ 1,000 to $ 15,000 per year. In the United States, diabetes cost $ 245 billion in 2012. In Canada, Canadian healthcare system estimated that the cost will be $ 16.9 billion per year by 2020.

Until now, the pathogenic mechanisms of diabetes have not been fully understood. Clinical therapies according to current mechanisms are not effective as much as expected and it is hard to prevent multiple complications caused by diabetes. Increased rates of endogenous glucose production by gluconeogenesis were commonly observed in patients with poorly controlled type 2 diabetes. Metaformin is a well known therapeutic drug for type 2 diabetes. Metaformin exerts its function in type 2 diabetes via inhibition of gluconeogenesis (89). It has been shown that H_2S is involved in the development and progression of diabetes (217,218). The detailed mechanism of H_2S in diabetes is still unclear. The regulation of CSE/H_2S system on glucose balance in liver cells as a cellular response will be beneficial to understand the mechanism of diabetes and acts as a potential therapeutic target for diabetes. In addition, we demonstrated that H_2S is involved in the regulation of gluconeogenesis through *S*-sulfhydration of PC, which provide a direct link for the role of CSE/H_2S in

diabetes. In diabetic rat model, CSE expression and H_2S production are increased (219). Pathogenic effects of CSE/H_2S pathway in Zucker diabetic rats are found as well (217). Therefore, PC *S*-sulfhydration may act as a pathogenic factor in diabetes. Once detail regulation of PC *S*-sulfhydation and gluconeogenic pathway regulated by H_2S is determined, it will provide the beneficial way for the therapy of diabetes.

S-sulfhydration is a novel posttranslational modification of proteins by H_2S. Here we firstly reported that protein *S*-sulfhydration is regulated by Trx1. Further studies on the regulatory mechanisms of protein *S*-sulfhydration will provide the strategies for designing novel drug against diverse diseases linked to abnormal protein *S*-sulfhydration.

FUTURE DIRECTION

Future directions suggested as a follow-up on this study are:

1. To further investigate the regulatory mechanism of *S*-sulfhydration. It has been well known that *S*-nitrosylation is extensively regulated via denitrosylation or transnitrosylation. I fully believe Trx1 is one of the factors involved for *S*-sulfhydration regulation. In order to further investigate the regulatory mechanism of protein *S*-sulfhydration, two concerns are required. Firstly, the regulatory mechanism of protein *S*-sulfhydration by Trx1 may be commonly occurred in various proteins based on redox status of cells since Trx1 is related to control redox environment in the cells. Secondly, the possibility of other enzymes, except for Trx1, will be considered for further investigation of regulatory mechanism of protein *S*-sulfhydration since GAPDH also mediates protein *S*-nitrosylation (220).

2. To further investigate the forming mechanism of *S*-sulfhydration. Unlike phosphorylation, the forming mechanism of *S*-sulfhydration is unclear. The relationship among GSSH, glutathione persulfide, and protein *S*-sulfhydration is critical for the regulation of protein *S*-sulfhydration (162). Acid-base motif is considered as a potential forming mechanism of protein *S*-nitrosylation. RSNO derived from NO including GSNO is known as a NO donor for protein *S*-nitrosylation. Therefore, GSSH or other persulfides derived from H_2S may be involved in forming of protein *S*-sulfhydration, similar to the forming mechanism of protein *S*-nitrosylation by GSNO through acid-base motif.

3. To elucidate the relationship between protein *S*-nitrosylation and *S*-sulfhydration. Methodological approach is important for this study. Up to date, the study for the relationship between protein *S*-nitrosylation and *S*-sulfhydration was determined with comparison of protein *S*-nitrosylation and *S*-sulfhydration in separate samples (94,178). Development of detection methods for protein *S*-nitrosylation and *S*-sulfhydration at the same time will be necessary for the further study. Protein *S*-nitrosylation and *S*-sulfhydration possess an opposite role in GAPDH and eNOS (94,178). In addition, protein *S*-nitrosylation and *S*-sulfhydration share the same cysteine residue in GAPDH and eNOS. Therefore, the balance of protein *S*-nitrosylation and *S*-sulfhydration in the cells may be involved in the various diseases which are related to protein *S*-nitrosylation and *S*-sulfhydration, such as Parkin's and Alzheimer's disease.

PUBLICATIONS

Manuscripts and abstracts have been published or submitted for publication in the peer reviewed journals or communicated in the national and international conferences during my PhD program. These publications are listed below.

Articles published in peer-reviewed journals

1. Ling Zhang, Guangdong Yang, Ashley Untereiner, Youngjun Ju, Lingyun Wu, and Rui Wang*. (2013) Hydrogen Sulfide Impairs Glucose Utilization and Increases Gluconeogenesis in Hepatocytes. *Endocrinology* **154**, 114-126. *(Impact factor: 4.64)*

2. Youngjun Ju, Weihua Zhang, Yanxi Pei, and Guangdong Yang*. (2013) H_2S signaling in redox regulation of cellular functions. *Can. J. Physiol. Pharmacol.* **91**, 8-14. *(Impact factor: 1.55)*

3. Guanghua Tang, Guangdong Yang, Bo Jiang, Youngjun Ju, Lingyun Wu, and Rui Wang*. (2013) H_2S Is an Endothelium-Derived Hyperpolarizing Factor. *Antioxid Redox Signal.* **19**,1634-1646. *(Impact factor: 7.67)*

4. Guangdong Yang, Kexin Zhao, Youngjun Ju, Sarathi Mani, Qiuhui Cao, Stephanie Puukila, Neelam Khaper, Lingyun Wu, and Rui Wang*. (2013) Hydrogen Sulfide Protects Against Cellular Senescence via *S*-Sulfhydration of Keap1 and Activation of Nrf2. *Antioxid Redox Signal.* **18**,1906-1919. *(Impact factor: 7.67)*

5. Kexin Zhao, YoungJun Ju, Shuangshuang Li, Zaid Al Tanny, Rui Wang, and Guangdong Yang*. (2014) *S*-sulfhydration of MEK1 leads to PARP-1 activation and DNA damage repair. *EMBO Rep.* **15**,792-800. *(Impact factor: 7.86)*

6. Zaid Altaany, YoungJun Ju, Guangdong Yang, Rui Wang*. (2014) The coordination of *S*-

sulfhydration, *S*-nitrosylation, and phosphorylation of endothelial nitric oxide synthase by hydrogen sulfide. *Sci Signal.* **7**, ra87. *(Impact factor: 7.65)*

Articles accepted and submitted in peer-reviewed journals

1. YoungJun Ju, Ashley Untereiner, Lingyun Wu, Guangdong Yang*. H_2S-induced *S*-sulfhydration of pyruvate carboxylase contributes to gluconeogenesis in liver cells. (Accepted in BBA-GEN.)

2. Youngjun Ju, Guangdong Yang*. Thioredoxin 1 is essential for protein *S*-desulfhydration. (Submitted to BBREP and under revision.)

3. YoungJun Ju, Ming Fu, Lingyun Wu, Guangdong Yang*. Strategies and tools for detection of protein *S*-nitrosylation and *S*-sulfhydration. (Submitted to OMCL)

4. Peipei Wang, Lingyun Wu, YoungJun Ju, Rui Wang*. Early onset of allergic asthma due to age-dependent expressional deficiency of cystathionine gamma-lyase in young mice. (Submitted to JACI and under revision.)

5. Ashley Untereiner, Rui Wang, YoungJun Ju, Lingyun Wu*. H_2S-induced increase in hepatic glucose production and the underlying mechanisms. (Accepted in ARS.)

Abstract communicated in conferences

1. Katalin Módis, YoungJun Ju, Zaid Altaany, Ashley A. Untereiner, Guangdong Yang, Lingyun Wu, Csaba Szabo, Rui Wang*. Hydrogen sulfide-induced *S*-sulfhydration of ATP synthase stimulates mitochondrial bioenergetics. **3rd European Conference on the Biology of Hydrogen Sulfide**, 2015, Athens, Greece. 47:S53-S54.

REFERENCES

1. Linden, D. R., Levitt, M. D., Farrugia, G., and Szurszewski, J. H. (2010) Endogenous production of H_2S in the gastrointestinal tract: still in search of a physiologic function. *Antioxid Redox Signal* **12**, 1135-1146
2. Wang, R. (2002) Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter? *FASEB J* **16**, 1792-1798
3. Wang, R. (2014) Gasotransmitters: growing pains and joys. *Trends Biochem Sci* **39**, 227-232
4. Szabó, C. (2007) Hydrogen sulphide and its therapeutic potential. *Nat Rev Drug Discov* **6**, 917-935
5. Wang, R. (2012) Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Physiol Rev* **92**, 791-896
6. Beauchamp, R. O., Bus, J. S., Popp, J. A., Boreiko, C. J., and Andjelkovich, D. A. (1984) A critical review of the literature on hydrogen sulfide toxicity. *Crit Rev Toxicol* **13**, 25-97
7. Zhao, W., Zhang, J., Lu, Y., and Wang, R. (2001) The vasorelaxant effect of H_2S as a novel endogenous gaseous K_{ATP} channel opener. *EMBO J* **20**, 6008-6016
8. Zhao, W., Ndisang, J. F., and Wang, R. (2003) Modulation of endogenous production of H_2S in rat tissues. *Can J Physiol Pharmacol* **81**, 848-853
9. Leffler, C. W., Parfenova, H., Basuroy, S., Jaggar, J. H., Umstot, E. S., and Fedinec, A. L. (2011) Hydrogen sulfide and cerebral microvascular tone in newborn pigs. *Am J Physiol Heart Circ Physiol* **300**, H440-447
10. Teague, B., Asiedu, S., and Moore, P. K. (2002) The smooth muscle relaxant effect of hydrogen sulphide in vitro: evidence for a physiological role to control intestinal contractility. *Br J Pharmacol* **137**, 139-145
11. Riedel, G., Platt, B., and Micheau, J. (2003) Glutamate receptor function in learning and memory. *Behav Brain Res* **140**, 1-47
12. Kimura, H. (2002) Hydrogen sulfide as a neuromodulator. *Mol Neurobiol* **26**, 13-19
13. Abe, K., and Kimura, H. (1996) The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* **16**, 1066-1071
14. Yang, W., Yang, G., Jia, X., Wu, L., and Wang, R. (2005) Activation of K_{ATP} channels by H_2S in rat insulin-secreting cells and the underlying mechanisms. *J Physiol* **569**, 519-531
15. Ali, M. Y., Whiteman, M., Low, C. M., and Moore, P. K. (2007) Hydrogen sulphide reduces insulin secretion from HIT-T15 cells by a K_{ATP} channel-dependent pathway. *J Endocrinol* **195**, 105-112
16. Wang, R. (2010) Hydrogen sulfide: the third gasotransmitter in biology and medicine. *Antioxid Redox Signal* **12**, 1061-1064
17. Tang, C., Li, X., and Du, J. (2006) Hydrogen sulfide as a new endogenous gaseous transmitter in the cardiovascular system. *Curr Vasc Pharmacol* **4**, 17-22
18. Nagahara, N., Ito, T., Kitamura, H., and Nishino, T. (1998) Tissue and subcellular distribution of mercaptopyruvate sulfurtransferase in the rat: confocal laser fluorescence and immunoelectron microscopic studies combined with biochemical analysis. *Histochem Cell Biol* **110**, 243-250
19. Ogasawara, Y., Isoda, S., and Tanabe, S. (1994) Tissue and subcellular distribution of bound and acid-labile sulfur, and the enzymic capacity for sulfide production in the rat. *Biol Pharm Bull* **17**, 1535-1542
20. Shibuya, N., Tanaka, M., Yoshida, M., Ogasawara, Y., Togawa, T., Ishii, K., and Kimura, H. (2009) 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. *Antioxid Redox Signal* **11**, 703-714
21. Shibuya, N., Mikami, Y., Kimura, Y., Nagahara, N., and Kimura, H. (2009) Vascular endothelium expresses 3-mercaptopyruvate sulfurtransferase and produces hydrogen sulfide. *J Biochem* **146**, 623-626
22. Searcy, D. G., and Lee, S. H. (1998) Sulfur reduction by human erythrocytes. *J Exp Zool* **282**, 310-322
23. Łowicka, E., and Bełtowski, J. (2007) Hydrogen sulfide (H_2S) - the third gas of interest for pharmacologists. *Pharmacol Rep* **59**, 4-24
24. Lakhan, S. E., and Kirchgessner, A. (2010) Gut inflammation in chronic fatigue syndrome. *Nutr Metab (Lond)* **7**, 79
25. Kruger, W. D., Evans, A. A., Wang, L., Malinow, M. R., Duell, P. B., Anderson, P. H., Block, P. C., Hess, D. L., Graf, E. E., and Upson, B. (2000) Polymorphisms in the CBS gene associated with decreased risk of coronary artery disease and increased responsiveness to total homocysteine lowering by folic acid. *Mol Genet Metab* **70**, 53-60
26. Kery, V., Bukovska, G., and Kraus, J. P. (1994) Transsulfuration depends on heme in addition to pyridoxal 5'-phosphate. Cystathionine beta-synthase is a heme protein. *J Biol Chem* **269**, 25283-25288

27. Kimura, H., Shibuya, N., and Kimura, Y. (2012) Hydrogen sulfide is a signaling molecule and a cytoprotectant. *Antioxid Redox Signal* **17**, 45-57
28. Jhee, K. H., and Kruger, W. D. (2005) The role of cystathionine beta-synthase in homocysteine metabolism. *Antioxid Redox Signal* **7**, 813-822
29. Austin, R. C., Lentz, S. R., and Werstuck, G. H. (2004) Role of hyperhomocysteinemia in endothelial dysfunction and atherothrombotic disease. *Cell Death Differ* **11 Suppl 1**, S56-64
30. Braunstein, A. E., and Goryachenkova, E. V. (1984) The beta-replacement-specific pyridoxal-P-dependent lyases. *Adv Enzymol Relat Areas Mol Biol* **56**, 1-89
31. Rao, A. M., Drake, M. R., and Stipanuk, M. H. (1990) Role of the transsulfuration pathway and of gamma-cystathionase activity in the formation of cysteine and sulfate from methionine in rat hepatocytes. *J Nutr* **120**, 837-845
32. Reed, D. J., and Orrenius, S. (1977) The role of methionine in glutathione biosynthesis by isolated hepatocytes. *Biochem Biophys Res Commun* **77**, 1257-1264
33. Lee, Z. W., Low, Y. L., Huang, S., Wang, T., and Deng, L. W. (2014) The cystathionine γ-lyase/hydrogen sulfide system maintains cellular glutathione status. *Biochem J* **460**, 425-435
34. Yang, G., Zhao, K., Ju, Y., Mani, S., Cao, Q., Puukila, S., Khaper, N., Wu, L., and Wang, R. (2013) Hydrogen sulfide protects against cellular senescence via *S*-sulfhydration of Keap1 and activation of Nrf2. *Antioxid Redox Signal* **18**, 1906-1919
35. Pallardo, F. V., Sastre, J., Asensi, M., Rodrigo, F., Estrela, J. M., and Viña, J. (1991) Physiological changes in glutathione metabolism in foetal and newborn rat liver. *Biochem J* **274 (Pt 3)**, 891-893
36. Hosoki, R., Matsuki, N., and Kimura, H. (1997) The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem Biophys Res Commun* **237**, 527-531
37. Yang, G., Wu, L., Jiang, B., Yang, W., Qi, J., Cao, K., Meng, Q., Mustafa, A. K., Mu, W., Zhang, S., Snyder, S. H., and Wang, R. (2008) H_2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. *Science* **322**, 587-590
38. Li, L., Rose, P., and Moore, P. K. (2011) Hydrogen sulfide and cell signaling. *Annu Rev Pharmacol Toxicol* **51**, 169-187
39. Renga, B. (2011) Hydrogen sulfide generation in mammals: the molecular biology of cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). *Inflamm Allergy Drug Targets* **10**, 85-91
40. Ichinohe, A., Kanaumi, T., Takashima, S., Enokido, Y., Nagai, Y., and Kimura, H. (2005) Cystathionine beta-synthase is enriched in the brains of Down's patients. *Biochem Biophys Res Commun* **338**, 1547-1550
41. Taub, J. W., Huang, X., Matherly, L. H., Stout, M. L., Buck, S. A., Massey, G. V., Becton, D. L., Chang, M. N., Weinstein, H. J., and Ravindranath, Y. (1999) Expression of chromosome 21-localized genes in acute myeloid leukemia: differences between Down syndrome and non-Down syndrome blast cells and relationship to in vitro sensitivity to cytosine arabinoside and daunorubicin. *Blood* **94**, 1393-1400
42. Li, M., Nie, L., Hu, Y., Yan, X., Xue, L., Chen, L., Zhou, H., and Zheng, Y. (2013) Chronic intermittent hypoxia promotes expression of 3-mercaptopyruvate sulfurtransferase in adult rat medulla oblongata. *Auton Neurosci* **179**, 84-89
43. Zhao, H., Chan, S. J., Ng, Y. K., and Wong, P. T. (2013) Brain 3-Mercaptopyruvate Sulfurtransferase (3MST): Cellular Localization and Downregulation after Acute Stroke. *PLoS One* **8**, e67322
44. Módis, K., Asimakopoulou, A., Coletta, C., Papapetropoulos, A., and Szabo, C. (2013) Oxidative stress suppresses the cellular bioenergetic effect of the 3-mercaptopyruvate sulfurtransferase/hydrogen sulfide pathway. *Biochem Biophys Res Commun* **433**, 401-407
45. Crawhall, J. C. (1985) A review of the clinical presentation and laboratory findings in two uncommon hereditary disorders of sulfur amino acid metabolism, beta-mercaptolactate cysteine disulfideuria and sulfite oxidase deficiency. *Clin Biochem* **18**, 139-142
46. Kolluru, G. K., Shen, X., Bir, S. C., and Kevil, C. G. (2013) Hydrogen sulfide chemical biology: pathophysiological roles and detection. *Nitric Oxide* **35**, 5-20
47. Smith, R. P., and Abbanat, R. A. (1966) Protective effect of oxidized glutathione in acute sulfide poisoning. *Toxicol Appl Pharmacol* **9**, 209-217
48. Ackermann, M., Kubitza, M., Hauska, G., and Piña, A. L. (2014) The vertebrate homologue of sulfide-quinone reductase in mammalian mitochondria. *Cell Tissue Res* **358**, 779-792
49. Vande Weghe, J. G., and Ow, D. W. (1999) A fission yeast gene for mitochondrial sulfide oxidation. *J Biol Chem* **274**, 13250-13257
50. Rej, R. (1977) Aminooxyacetate is not an adequate differential inhibitor of aspartate aminotransferase isoenzymes. *Clin Chem* **23**, 1508-1509
51. Asimakopoulou, A., Panopoulos, P., Chasapis, C. T., Coletta, C., Zhou, Z., Cirino, G., Giannis, A.,

Szabo, C., Spyroulias, G. A., and Papapetropoulos, A. (2013) Selectivity of commonly used pharmacological inhibitors for cystathionine β synthase (CBS) and cystathionine γ lyase (CSE). *Br J Pharmacol* **169**, 922-932
52. Mok, Y. Y., Atan, M. S., Yoke Ping, C., Zhong Jing, W., Bhatia, M., Moochhala, S., and Moore, P. K. (2004) Role of hydrogen sulphide in haemorrhagic shock in the rat: protective effect of inhibitors of hydrogen sulphide biosynthesis. *Br J Pharmacol* **143**, 881-889
53. Bhatia, M., Sidhapuriwala, J., Moochhala, S. M., and Moore, P. K. (2005) Hydrogen sulphide is a mediator of carrageenan-induced hindpaw oedema in the rat. *Br J Pharmacol* **145**, 141-144
54. Bhatia, M., Wong, F. L., Fu, D., Lau, H. Y., Moochhala, S. M., and Moore, P. K. (2005) Role of hydrogen sulfide in acute pancreatitis and associated lung injury. *FASEB J* **19**, 623-625
55. Hellmich, M. R., Coletta, C., Chao, C., and Szabo, C. (2015) The therapeutic potential of cystathionine β-synthetase/hydrogen sulfide inhibition in cancer. *Antioxid Redox Signal* **22**, 424-448
56. DeLeon, E. R., Stoy, G. F., and Olson, K. R. (2012) Passive loss of hydrogen sulfide in biological experiments. *Anal Biochem* **421**, 203-207
57. Lee, Z. W., Zhou, J., Chen, C. S., Zhao, Y., Tan, C. H., Li, L., Moore, P. K., and Deng, L. W. (2011) The slow-releasing hydrogen sulfide donor, GYY4137, exhibits novel anti-cancer effects in vitro and in vivo. *PLoS One* **6**, e21077
58. Grambow, E., Mueller-Graf, F., Delyagina, E., Frank, M., Kuhla, A., and Vollmar, B. (2013) Effect of the hydrogen sulfide donor GYY4137 on platelet activation and microvascular thrombus formation in mice. *Platelets* **25**, 166-174
59. Liu, Z., Han, Y., Li, L., Lu, H., Meng, G., Li, X., Shirhan, M., Peh, M. T., Xie, L., Zhou, S., Wang, X., Chen, Q., Dai, W., Tan, C. H., Pan, S., Moore, P. K., and Ji, Y. (2013) The hydrogen sulfide donor, GYY4137, exhibits anti-atherosclerotic activity in high fat fed apolipoprotein E(-/-) mice. *Br J Pharmacol* **169**, 1795-1809
60. Liu, L., Cui, J., Song, C. J., Bian, J. S., Sparatore, A., Soldato, P. D., Wang, X. Y., and Yan, C. D. (2012) H_2S-releasing aspirin protects against aspirin-induced gastric injury via reducing oxidative stress. *PLoS One* **7**, e46301
61. Rossoni, G., Manfredi, B., Tazzari, V., Sparatore, A., Trivulzio, S., Del Soldato, P., and Berti, F. (2010) Activity of a new hydrogen sulfide-releasing aspirin (ACS14) on pathological cardiovascular alterations induced by glutathione depletion in rats. *Eur J Pharmacol* **648**, 139-145
62. Zhang, H., Guo, C., Zhang, A., Fan, Y., Gu, T., Wu, D., Sparatore, A., and Wang, C. (2012) Effect of *S*-aspirin, a novel hydrogen-sulfide-releasing aspirin (ACS14), on atherosclerosis in apoE-deficient mice. *Eur J Pharmacol* **697**, 106-116
63. Frantzias, J., Logan, J. G., Mollat, P., Sparatore, A., Del Soldato, P., Ralston, S. H., and Idris, A. I. (2012) Hydrogen sulphide-releasing diclofenac derivatives inhibit breast cancer-induced osteoclastogenesis in vitro and prevent osteolysis ex vivo. *Br J Pharmacol* **165**, 1914-1925
64. Elsheikh, W., Blackler, R. W., Flannigan, K. L., and Wallace, J. L. (2014) Enhanced chemopreventive effects of a hydrogen sulfide-releasing anti-inflammatory drug (ATB-346) in experimental colorectal cancer. *Nitric Oxide* **41**, 131-137
65. Blackler, R., Syer, S., Bolla, M., Ongini, E., and Wallace, J. L. (2012) Gastrointestinal-sparing effects of novel NSAIDs in rats with compromised mucosal defence. *PLoS One* **7**, e35196
66. Wallace, J. L., Caliendo, G., Santagada, V., and Cirino, G. (2010) Markedly reduced toxicity of a hydrogen sulphide-releasing derivative of naproxen (ATB-346). *Br J Pharmacol* **159**, 1236-1246
67. Ekundi-Valentim, E., Mesquita, F. P., Santos, K. T., de Paula, M. A., Florenzano, J., Zanoni, C. I., Rodrigues, L., de Nucci, G., Teixeira, S. A., Ferreira, H. H., Wallace, J. L., Costa, S. K., and Muscará, M. N. (2013) A comparative study on the anti-inflammatory effects of single oral doses of naproxen and its hydrogen sulfide (H_2S)-releasing derivative ATB-346 in rats with carrageenan-induced synovitis. *Med Gas Res* **3**, 24
68. Lee, M., Tazzari, V., Giustarini, D., Rossi, R., Sparatore, A., Del Soldato, P., McGeer, E., and McGeer, P. L. (2010) Effects of hydrogen sulfide-releasing L-DOPA derivatives on glial activation: potential for treating Parkinson disease. *J Biol Chem* **285**, 17318-17328
69. Liu, Y. Y., Sparatore, A., Del Soldato, P., and Bian, J. S. (2011) ACS84, a novel hydrogen sulfide-releasing compound, protects against amyloid β-induced cell cytotoxicity. *Neurochem Int* **58**, 591-598
70. Iciek, M., Kwiecień, I., Chwatko, G., Sokołowska-Jeżewicz, M., Kowalczyk-Pachel, D., and Rokita, H. (2012) The effects of garlic-derived sulfur compounds on cell proliferation, caspase 3 activity, thiol levels and anaerobic sulfur metabolism in human hepatoblastoma HepG2 cells. *Cell Biochem Funct* **30**, 198-204
71. Li, L., Salto-Tellez, M., Tan, C. H., Whiteman, M., and Moore, P. K. (2009) GYY4137, a novel hydrogen sulfide-releasing molecule, protects against endotoxic shock in the rat. *Free Radic Biol Med*

47, 103-113
72. Li, L., Whiteman, M., Guan, Y. Y., Neo, K. L., Cheng, Y., Lee, S. W., Zhao, Y., Baskar, R., Tan, C. H., and Moore, P. K. (2008) Characterization of a novel, water-soluble hydrogen sulfide-releasing molecule (GYY4137): new insights into the biology of hydrogen sulfide. *Circulation* **117**, 2351-2360
73. Martelli, A., Testai, L., Citi, V., Marino, A., Pugliesi, I., Barresi, E., Nesi, G., Rapposelli, S., Taliani, S., Da Settimo, F., Breschi, M. C., and Calderone, V. (2013) Arylthioamides as H_2S Donors: l-Cysteine-Activated Releasing Properties and Vascular Effects in Vitro and in Vivo. *ACS Med Chem Lett* **4**, 904-908
74. Zhao, Y., Bhushan, S., Yang, C., Otsuka, H., Stein, J. D., Pacheco, A., Peng, B., Devarie-Baez, N. O., Aguilar, H. C., Lefer, D. J., and Xian, M. (2013) Controllable hydrogen sulfide donors and their activity against myocardial ischemia-reperfusion injury. *ACS Chem Biol* **8**, 1283-1290
75. Maga, G., Falchi, F., Radi, M., Botta, L., Casaluce, G., Bernardini, M., Irannejad, H., Manetti, F., Garbelli, A., Samuele, A., Zanoli, S., Esté, J. A., Gonzalez, E., Zucca, E., Paolucci, S., Baldanti, F., De Rijck, J., Debyser, Z., and Botta, M. (2011) Toward the discovery of novel anti-HIV drugs. Second-generation inhibitors of the cellular ATPase DDX3 with improved anti-HIV activity: synthesis, structure-activity relationship analysis, cytotoxicity studies, and target validation. *ChemMedChem* **6**, 1371-1389
76. Xu, L. L., Zheng, C. J., Sun, L. P., Miao, J., and Piao, H. R. (2012) Synthesis of novel 1,3-diaryl pyrazole derivatives bearing rhodanine-3-fatty acid moieties as potential antibacterial agents. *Eur J Med Chem* **48**, 174-178
77. Alegaon, S. G., Alagawadi, K. R., Sonkusare, P. V., Chaudhary, S. M., Dadwe, D. H., and Shah, A. S. (2012) Novel imidazo[2,1-b][1,3,4]thiadiazole carrying rhodanine-3-acetic acid as potential antitubercular agents. *Bioorg Med Chem Lett* **22**, 1917-1921
78. Lisjak, M., Srivastava, N., Teklic, T., Civale, L., Lewandowski, K., Wilson, I., Wood, M. E., Whiteman, M., and Hancock, J. T. (2010) A novel hydrogen sulfide donor causes stomatal opening and reduces nitric oxide accumulation. *Plant Physiol Biochem* **48**, 931-935
79. Robinson, H., and Wray, S. (2012) A new slow releasing, H_2S generating compound, GYY4137 relaxes spontaneous and oxytocin-stimulated contractions of human and rat pregnant myometrium. *PLoS One* **7**, e46278
80. Zhao, Y., Wang, H., and Xian, M. (2011) Cysteine-activated hydrogen sulfide (H_2S) donors. *J Am Chem Soc* **133**, 15-17
81. Hasegawa, U., and van der Vlies, A. J. (2014) Design and synthesis of polymeric hydrogen sulfide donors. *Bioconjug Chem* **25**, 1290-1300
82. Szczesny, B., Módis, K., Yanagi, K., Coletta, C., Le Trionnaire, S., Perry, A., Wood, M. E., Whiteman, M., and Szabo, C. (2014) AP39, a novel mitochondria-targeted hydrogen sulfide donor, stimulates cellular bioenergetics, exerts cytoprotective effects and protects against the loss of mitochondrial DNA integrity in oxidatively stressed endothelial cells in vitro. *Nitric Oxide* **41**, 120-130
83. Whiteman, M., and Moore, P. K. (2009) Hydrogen sulfide and the vasculature: a novel vasculoprotective entity and regulator of nitric oxide bioavailability? *J Cell Mol Med* **13**, 488-507
84. Pfeffer, M., and Ressler, C. (1967) Beta-cyanoalanine, an inhibitor of rat liver cystathionase. *Biochem Pharmacol* **16**, 2299-2308
85. Ubuka, T., Abe, T., Fujiwara, M., and Wróbel, M. (1995) Inhibition of sulfate excretion by (aminooxy)acetate induced stimulation of taurine excretion in rats. *Amino Acids* **8**, 345-352
86. Porter, D. W., and Baskin, S. I. (1995) Specificity studies of 3-Mercaptopyruvate sulfurtransferase. *J Biochem Toxicol* **10**, 287-292
87. Merighi, S., Gessi, S., Varani, K., Fazzi, D., and Borea, P. A. (2012) Hydrogen sulfide modulates the release of nitric oxide and VEGF in human keratinocytes. *Pharmacol Res* **66**, 428-436
88. Whiteman, M., Li, L., Rose, P., Tan, C. H., Parkinson, D. B., and Moore, P. K. (2010) The effect of hydrogen sulfide donors on lipopolysaccharide-induced formation of inflammatory mediators in macrophages. *Antioxid Redox Signal* **12**, 1147-1154
89. Hundal, R. S., Krssak, M., Dufour, S., Laurent, D., Lebon, V., Chandramouli, V., Inzucchi, S. E., Schumann, W. C., Petersen, K. F., Landau, B. R., and Shulman, G. I. (2000) Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes* **49**, 2063-2069
90. Lehninger, A. L., Nelson, D. L., and Cox, M. M. (2000) *Principles of Biochemistry*, Worth Publishers Inc., N.Y.
91. Söling, H. D., Willms, B., and Kleineke, J. (1970) Regulation of gluconeogenesis in rat and guinea pig liver. *Hoppe Seylers Z Physiol Chem* **351**, 291
92. Seitz, H. J., Kaiser, M., Krone, W., and Tarnowski, W. (1976) Physiologic significance of

glucocorticoids and insulin in the regulation of hepatic gluconeogenesis during starvation in rats. *Metabolism* **25**, 1545-1555
93. Bagby, S. P. (2004) Obesity-initiated metabolic syndrome and the kidney: a recipe for chronic kidney disease? *J Am Soc Nephrol* **15**, 2775-2791
94. Mustafa, A. K., Gadalla, M. M., Sen, N., Kim, S., Mu, W., Gazi, S. K., Barrow, R. K., Yang, G., Wang, R., and Snyder, S. H. (2009) H_2S signals through protein *S*-sulfhydration. *Sci Signal* **2**, ra72
95. Mani, S., Cao, W., Wu, L., and Wang, R. (2014) Hydrogen sulfide and the liver. *Nitric Oxide* **41**, 62-71
96. Mani, S., Li, H., Untereiner, A., Wu, L., Yang, G., Austin, R. C., Dickhout, J. G., Lhoták, Ń., Meng, Q. H., and Wang, R. (2013) Decreased endogenous production of hydrogen sulfide accelerates atherosclerosis. *Circulation* **127**, 2523-2534
97. Wu, D., Zheng, N., Qi, K., Cheng, H., Sun, Z., Gao, B., Zhang, Y., Pang, W., Huangfu, C., Ji, S., Xue, M., Ji, A., and Li, Y. (2015) Exogenous hydrogen sulfide mitigates the fatty liver in obese mice through improving lipid metabolism and antioxidant potential. *Med Gas Res* **5**, 1
98. Zhang, L., Yang, G., Untereiner, A., Ju, Y., Wu, L., and Wang, R. (2013) Hydrogen sulfide impairs glucose utilization and increases gluconeogenesis in hepatocytes. *Endocrinology* **154**, 114-126
99. Módis, K., Coletta, C., Erdélyi, K., Papapetropoulos, A., and Szabo, C. (2013) Intramitochondrial hydrogen sulfide production by 3-mercaptopyruvate sulfurtransferase maintains mitochondrial electron flow and supports cellular bioenergetics. *FASEB J* **27**, 601-611
100. Fiorucci, S., Distrutti, E., Cirino, G., and Wallace, J. L. (2006) The emerging roles of hydrogen sulfide in the gastrointestinal tract and liver. *Gastroenterology* **131**, 259-271
101. Gawrieh, S., Opara, E. C., and Koch, T. R. (2004) Oxidative stress in nonalcoholic fatty liver disease: pathogenesis and antioxidant therapies. *J Investig Med* **52**, 506-514
102. Poli, G. (2000) Pathogenesis of liver fibrosis: role of oxidative stress. *Mol Aspects Med* **21**, 49-98
103. Tan, G., Pan, S., Li, J., Dong, X., Kang, K., Zhao, M., Jiang, X., Kanwar, J. R., Qiao, H., Jiang, H., and Sun, X. (2011) Hydrogen sulfide attenuates carbon tetrachloride-induced hepatotoxicity, liver cirrhosis and portal hypertension in rats. *PLoS One* **6**, e25943
104. Jha, S., Calvert, J. W., Duranski, M. R., Ramachandran, A., and Lefer, D. J. (2008) Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: role of antioxidant and antiapoptotic signaling. *Am J Physiol Heart Circ Physiol* **295**, H801-806
105. Wexler, I. D., Kerr, D. S., Du, Y., Kaung, M. M., Stephenson, W., Lusk, M. M., Wappner, R. S., and Higgins, J. J. (1998) Molecular characterization of pyruvate carboxylase deficiency in two consanguineous families. *Pediatr Res* **43**, 579-584
106. UTTER, M. F., and KEECH, D. B. (1960) Formation of oxaloacetate from pyruvate and carbon dioxide. *J Biol Chem* **235**, PC17-18
107. Jitrapakdee, S., St Maurice, M., Rayment, I., Cleland, W. W., Wallace, J. C., and Attwood, P. V. (2008) Structure, mechanism and regulation of pyruvate carboxylase. *Biochem J* **413**, 369-387
108. Jitrapakdee, S., Walker, M. E., and Wallace, J. C. (1996) Identification of novel alternatively spliced pyruvate carboxylase mRNAs with divergent 5'-untranslated regions which are expressed in a tissue-specific manner. *Biochem Biophys Res Commun* **223**, 695-700
109. Adina-Zada, A., Zeczycki, T. N., and Attwood, P. V. (2012) Regulation of the structure and activity of pyruvate carboxylase by acetyl CoA. *Arch Biochem Biophys* **519**, 118-130
110. Jitrapakdee, S., and Wallace, J. C. (1999) Structure, function and regulation of pyruvate carboxylase. *Biochem J* **340 (Pt 1)**, 1-16
111. Wimhurst, J. M., and Manchester, K. L. (1970) A comparison of the effects of diabetes induced with either alloxan or streptozotocin and of starvation on the activities in rat liver of the key enzymes of gluconeogenesis. *Biochem J* **120**, 95-103
112. Ballard, F. J., Hanson, R. W., and Kronfeld, D. S. (1968) Factors controlling the concentration of mitochondrial oxaloacetate in liver during spontaneous bovine ketosis. *Biochem Biophys Res Commun* **30**, 100-104
113. Lemons, J. A., Moorehead, H. C., and Hage, G. P. (1986) Effects of fasting on gluconeogenic enzymes in the ovine fetus. *Pediatr Res* **20**, 676-679
114. Weinberg, M. B., and Utter, M. F. (1979) Effect of thyroid hormone on the turnover of rat liver pyruvate carboxylase and pyruvate dehydrogenase. *J Biol Chem* **254**, 9492-9499
115. Lietzan, A. D., Menefee, A. L., Zeczycki, T. N., Kumar, S., Attwood, P. V., Wallace, J. C., Cleland, W. W., and St Maurice, M. (2011) Interaction between the biotin carboxyl carrier domain and the biotin carboxylase domain in pyruvate carboxylase from Rhizobium etli. *Biochemistry* **50**, 9708-9723
116. Kumashiro, N., Beddow, S. A., Vatner, D. F., Majumdar, S. K., Cantley, J. L., Guebre-Egziabher, F., Fat, I., Guigni, B., Jurczak, M. J., Birkenfeld, A. L., Kahn, M., Perler, B. K., Puchowicz, M. A., Manchem, V. P., Bhanot, S., Still, C. D., Gerhard, G. S., Petersen, K. F., Cline, G. W., Shulman, G. I., and Samuel,

V. T. (2013) Targeting pyruvate carboxylase reduces gluconeogenesis and adiposity and improves insulin resistance. *Diabetes* **62**, 2183-2194
117. Lee, P., Leong, W., Tan, T., Lim, M., Han, W., and Radda, G. K. (2013) *In vivo* hyperpolarized carbon-13 magnetic resonance spectroscopy reveals increased pyruvate carboxylase flux in an insulin-resistant mouse model. *Hepatology* **57**, 515-524
118. Lynch, C. J., McCall, K. M., Billingsley, M. L., Bohlen, L. M., Hreniuk, S. P., Martin, L. F., Witters, L. A., and Vannucci, S. J. (1992) Pyruvate carboxylase in genetic obesity. *Am J Physiol* **262**, E608-618
119. Atkin, B. M., Utter, M. F., and Weinberg, M. B. (1979) Pyruvate carboxylase and phosphoenolpyruvate carboxykinase activity in leukocytes and fibroblasts from a patient with pyruvate carboxylase deficiency. *Pediatr Res* **13**, 38-43
120. Robinson, B. H., Oei, J., Saunders, M., and Gravel, R. (1983) [3H]biotin-labeled proteins in cultured human skin fibroblasts from patients with pyruvate carboxylase deficiency. *J Biol Chem* **258**, 6660-6664
121. Xu, J., Han, J., Long, Y. S., Epstein, P. N., and Liu, Y. Q. (2008) The role of pyruvate carboxylase in insulin secretion and proliferation in rat pancreatic beta cells. *Diabetologia* **51**, 2022-2030
122. Han, J., and Liu, Y. Q. (2010) Reduction of islet pyruvate carboxylase activity might be related to the development of type 2 diabetes mellitus in Agouti-K mice. *J Endocrinol* **204**, 143-152
123. Salto, R., Sola, M., Oliver, F. J., and Vargas, A. M. (1996) Effects of starvation, diabetes and carbon tetrachloride intoxication on rat kidney cortex and liver pyruvate carboxylase levels. *Arch Physiol Biochem* **104**, 845-850
124. Mishima, N., Sahara, N., Shirakawa, M., and Ozawa, H. (2002) Effect of streptozotocin-induced diabetes mellitus on alveolar bone deposition in the rat. *Arch Oral Biol* **47**, 843-849
125. Large, V., and Beylot, M. (1999) Modifications of citric acid cycle activity and gluconeogenesis in streptozotocin-induced diabetes and effects of metformin. *Diabetes* **48**, 1251-1257
126. Jitrapakdee, S., Gong, Q., MacDonald, M. J., and Wallace, J. C. (1998) Regulation of rat pyruvate carboxylase gene expression by alternate promoters during development, in genetically obese rats and in insulin-secreting cells. Multiple transcripts with 5'-end heterogeneity modulate translation. *J Biol Chem* **273**, 34422-34428
127. MacDonald, M. J., Longacre, M. J., Langberg, E. C., Tibell, A., Kendrick, M. A., Fukao, T., and Ostenson, C. G. (2009) Decreased levels of metabolic enzymes in pancreatic islets of patients with type 2 diabetes. *Diabetologia* **52**, 1087-1091
128. Branson, J. P., Nezic, M., Jitrapakdee, S., Wallace, J. C., and Attwood, P. V. (2004) Kinetic characterization of yeast pyruvate carboxylase isozyme Pyc1 and the Pyc1 mutant, C249A. *Biochemistry* **43**, 1075-1081
129. Attwood, P. V. (1995) The structure and the mechanism of action of pyruvate carboxylase. *Int J Biochem Cell Biol* **27**, 231-249
130. Ashman, L. K., and Keech, D. B. (1975) Sheep kidney pyruvate carboxylase. Studies on the coupling of adenosine triphosphate hydrolysis and CO_2 fixation. *J Biol Chem* **250**, 14-21
131. Foster, M. W., Forrester, M. T., and Stamler, J. S. (2009) A protein microarray-based analysis of *S*-nitrosylation. *Proc Natl Acad Sci U S A* **106**, 18948-18953
132. Wu, C., Liu, T., Chen, W., Oka, S., Fu, C., Jain, M. R., Parrott, A. M., Baykal, A. T., Sadoshima, J., and Li, H. (2010) Redox regulatory mechanism of transnitrosylation by thioredoxin. *Mol Cell Proteomics* **9**, 2262-2275
133. Pearlman, S. M., Serber, Z., and Ferrell, J. E. (2011) A mechanism for the evolution of phosphorylation sites. *Cell* **147**, 934-946
134. Cieśla, J., Frączyk, T., and Rode, W. (2011) Phosphorylation of basic amino acid residues in proteins: important but easily missed. *Acta Biochim Pol* **58**, 137-148
135. BURNETT, G., and KENNEDY, E. P. (1954) The enzymatic phosphorylation of proteins. *J Biol Chem* **211**, 969-980
136. Minagawa, H., Kakamu, Y., Yoshida, H., Tomita, F., Oshima, H., and Mizuno, D. (1988) Endogenous tumor necrosis factor induction with Bordetella pertussis vaccine as a triggering agent and its therapeutic effect on MM46 carcinoma-bearing mice. *Jpn J Cancer Res* **79**, 384-389
137. Beck-Sickinger, A. G., and Morl, K. (2006) Posttranslational modification of proteins. Expanding nautre's inventory. By Christpher T. Walsh. Angew. Chem. Int. Ed.
138. Grewal, S. I., and Rice, J. C. (2004) Regulation of heterochromatin by histone methylation and small RNAs. *Curr Opin Cell Biol* **16**, 230-238
139. Brosnan, J. T., and Brosnan, M. E. (2006) The sulfur-containing amino acids: an overview. *J Nutr* **136**, 1636S-1640S
140. Tajc, S. G., Tolbert, B. S., Basavappa, R., and Miller, B. L. (2004) Direct determination of thiol pKa by

isothermal titration microcalorimetry. *J Am Chem Soc* **126**, 10508-10509
141. Alegre-Cebollada, J., Kosuri, P., Rivas-Pardo, J. A., and Fernández, J. M. (2011) Direct observation of disulfide isomerization in a single protein. *Nat Chem* **3**, 882-887
142. Lo Conte, M., and Carroll, K. S. (2013) The redox biochemistry of protein sulfenylation and sulfinylation. *J Biol Chem* **288**, 26480-26488
143. Chung, H. S., Wang, S. B., Venkatraman, V., Murray, C. I., and Van Eyk, J. E. (2013) Cysteine oxidative posttranslational modifications: emerging regulation in the cardiovascular system. *Circ Res* **112**, 382-392
144. Hess, D. T., Matsumoto, A., Kim, S. O., Marshall, H. E., and Stamler, J. S. (2005) Protein *S*-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol* **6**, 150-166
145. Marozkina, N. V., and Gaston, B. (2012) *S*-Nitrosylation signaling regulates cellular protein interactions. *Biochim Biophys Acta* **1820**, 722-729
146. Nott, A., Watson, P. M., Robinson, J. D., Crepaldi, L., and Riccio, A. (2008) *S*-Nitrosylation of histone deacetylase 2 induces chromatin remodelling in neurons. *Nature* **455**, 411-415
147. Ding, S. Y., Tribble, N. D., Kraft, C. A., Markwardt, M., Gloyn, A. L., and Rizzo, M. A. (2010) Naturally occurring glucokinase mutations are associated with defects in posttranslational *S*-nitrosylation. *Mol Endocrinol* **24**, 171-177
148. Kaneki, M., Shimizu, N., Yamada, D., and Chang, K. (2007) Nitrosative stress and pathogenesis of insulin resistance. *Antioxid Redox Signal* **9**, 319-329
149. Poteser, M., Romanin, C., Schreibmayer, W., Mayer, B., and Groschner, K. (2001) *S*-nitrosation controls gating and conductance of the alpha 1 subunit of class C L-type Ca^{2+} channels. *J Biol Chem* **276**, 14797-14803
150. Choi, Y. B., Tenneti, L., Le, D. A., Ortiz, J., Bai, G., Chen, H. S., and Lipton, S. A. (2000) Molecular basis of NMDA receptor-coupled ion channel modulation by *S*-nitrosylation. *Nat Neurosci* **3**, 15-21
151. Stamler, J. S., Toone, E. J., Lipton, S. A., and Sucher, N. J. (1997) (S)NO signals: translocation, regulation, and a consensus motif. *Neuron* **18**, 691-696
152. Ascenzi, P., Colasanti, M., Persichini, T., Muolo, M., Polticelli, F., Venturini, G., Bordo, D., and Bolognesi, M. (2000) Re-evaluation of amino acid sequence and structural consensus rules for cysteine-nitric oxide reactivity. *Biol Chem* **381**, 623-627
153. Benhar, M., Forrester, M. T., and Stamler, J. S. (2009) Protein denitrosylation: enzymatic mechanisms and cellular functions. *Nat Rev Mol Cell Biol* **10**, 721-732
154. Foster, M. W., Hess, D. T., and Stamler, J. S. (2009) Protein *S*-nitrosylation in health and disease: a current perspective. *Trends Mol Med* **15**, 391-404
155. Sayed, N., Kim, D. D., Fioramonti, X., Iwahashi, T., Durán, W. N., and Beuve, A. (2008) Nitroglycerin-induced *S*-nitrosylation and desensitization of soluble guanylyl cyclase contribute to nitrate tolerance. *Circ Res* **103**, 606-614
156. Fioramonti, X., Deak, A., Deshpande, S., Carneiro, L., Zhou, C., Sayed, N., Orban, B., Berlin, J. R., Pénicaud, L., Leloup, C., Beuve, A., and Routh, V. H. (2013) Hypothalamic S-nitrosylation contributes to the counter-regulatory response impairment following recurrent hypoglycemia. *PLoS One* **8**, e68709
157. Yu, C. X., Li, S., and Whorton, A. R. (2005) Redox regulation of PTEN by *S*-nitrosothiols. *Mol Pharmacol* **68**, 847-854
158. Marino, S. M., and Gladyshev, V. N. (2010) Structural analysis of cysteine *S*-nitrosylation: a modified acid-based motif and the emerging role of trans-nitrosylation. *J Mol Biol* **395**, 844-859
159. Jaffrey, S. R., and Snyder, S. H. (2001) The biotin switch method for the detection of *S*-nitrosylated proteins. *Sci STKE* **2001**, pl1
160. Mustafa, A. K., Sikka, G., Gazi, S. K., Steppan, J., Jung, S. M., Bhunia, A. K., Barodka, V. M., Gazi, F. K., Barrow, R. K., Wang, R., Amzel, L. M., Berkowitz, D. E., and Snyder, S. H. (2011) Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulfhydrates potassium channels. *Circ Res* **109**, 1259-1268
161. Zhao, K., Ju, Y., Li, S., Al Tanny, Z., Wang, R., and Yang, G. (2014) *S*-sulfhydration of MEK1 leads to PARP-1 activation and DNA damage repair. *EMBO Rep* **15**, 792-800
162. Lu, C., Kavalier, A., Lukyanov, E., and Gross, S. S. (2013) *S*-sulfhydration/desulfhydration and *S*-nitrosylation/denitrosylation: a common paradigm for gasotransmitter signaling by H_2S and NO. *Methods* **62**, 177-181
163. Kabil, O., and Banerjee, R. (2012) Characterization of patient mutations in human persulfide dioxygenase (ETHE1) involved in H_2S catabolism. *J Biol Chem* **287**, 44561-44567
164. Mieyal, J. J., Gallogly, M. M., Qanungo, S., Sabens, E. A., and Shelton, M. D. (2008) Molecular mechanisms and clinical implications of reversible protein *S*-glutathionylation. *Antioxid Redox Signal* **10**, 1941-1988

165. Srivastava, S. K., Ramana, K. V., and Bhatnagar, A. (2005) Role of aldose reductase and oxidative damage in diabetes and the consequent potential for therapeutic options. *Endocr Rev* **26**, 380-392
166. Aracena-Parks, P., Goonasekera, S. A., Gilman, C. P., Dirksen, R. T., Hidalgo, C., and Hamilton, S. L. (2006) Identification of cysteines involved in *S*-nitrosylation, *S*-glutathionylation, and oxidation to disulfides in ryanodine receptor type 1. *J Biol Chem* **281**, 40354-40368
167. Salsbury, F. R., Knutson, S. T., Poole, L. B., and Fetrow, J. S. (2008) Functional site profiling and electrostatic analysis of cysteines modifiable to cysteine sulfenic acid. *Protein Sci* **17**, 299-312
168. Haendeler, J., Hoffmann, J., Tischler, V., Berk, B. C., Zeiher, A. M., and Dimmeler, S. (2002) Redox regulatory and anti-apoptotic functions of thioredoxin depend on *S*-nitrosylation at cysteine 69. *Nat Cell Biol* **4**, 743-749
169. Kelleher, Z. T., Sha, Y., Foster, M. W., Foster, W. M., Forrester, M. T., and Marshall, H. E. (2014) Thioredoxin-mediated denitrosylation regulates cytokine-induced nuclear factor κB (NF-κB) activation. *J Biol Chem* **289**, 3066-3072
170. Benhar, M., Forrester, M. T., Hess, D. T., and Stamler, J. S. (2008) Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins. *Science* **320**, 1050-1054
171. Wu, C., Parrott, A. M., Liu, T., Beuve, A., and Li, H. (2013) Functional proteomics approaches for the identification of transnitrosylase and denitrosylase targets. *Methods* **62**, 151-160
172. Sumbayev, V. V. (2003) S-nitrosylation of thioredoxin mediates activation of apoptosis signal-regulating kinase 1. *Arch Biochem Biophys* **415**, 133-136
173. Mitchell, D. A., Morton, S. U., Fernhoff, N. B., and Marletta, M. A. (2007) Thioredoxin is required for *S*-nitrosation of procaspase-3 and the inhibition of apoptosis in Jurkat cells. *Proc Natl Acad Sci U S A* **104**, 11609-11614
174. Wu, C., Parrott, A. M., Liu, T., Jain, M. R., Yang, Y., Sadoshima, J., and Li, H. (2011) Distinction of thioredoxin transnitrosylation and denitrosylation target proteins by the ICAT quantitative approach. *J. Proteomics* **74**, 2498-2509
175. Zaffagnini, M., Morisse, S., Bedhomme, M., Marchand, C. H., Festa, M., Rouhier, N., Lemaire, S. D., and Trost, P. (2013) Mechanisms of nitrosylation and denitrosylation of cytoplasmic glyceraldehyde-3-phosphate dehydrogenase from Arabidopsis thaliana. *J Biol Chem* **288**, 22777-22789
176. Zaman, K., Carraro, S., Doherty, J., Henderson, E. M., Lendermon, E., Liu, L., Verghese, G., Zigler, M., Ross, M., Park, E., Palmer, L. A., Doctor, A., Stamler, J. S., and Gaston, B. (2006) *S*-nitrosylating agents: a novel class of compounds that increase cystic fibrosis transmembrane conductance regulator expression and maturation in epithelial cells. *Mol Pharmacol* **70**, 1435-1442
177. Robert, K., Nehmé, J., Bourdon, E., Pivert, G., Friguet, B., Delcayre, C., Delabar, J. M., and Janel, N. (2005) Cystathionine beta synthase deficiency promotes oxidative stress, fibrosis, and steatosis in mice liver. *Gastroenterology* **128**, 1405-1415
178. Altaany, Z., Ju, Y., Yang, G., and Wang, R. (2014) The coordination of *S*-sulfhydration, *S*-nitrosylation, and phosphorylation of endothelial nitric oxide synthase by hydrogen sulfide. *Sci Signal* **7**, ra87
179. Sen, N., Paul, B. D., Gadalla, M. M., Mustafa, A. K., Sen, T., Xu, R., Kim, S., and Snyder, S. H. (2012) Hydrogen sulfide-linked sulfhydration of NF-κB mediates its antiapoptotic actions. *Mol Cell* **45**, 13-24
180. Norris, E. J., Culberson, C. R., Narasimhan, S., and Clemens, M. G. (2011) The liver as a central regulator of hydrogen sulfide. *Shock* **36**, 242-250
181. Young, J. W. (1977) Gluconeogenesis in cattle: significance and methodology. *J Dairy Sci* **60**, 1-15
182. Duran-Sandoval, D., Cariou, B., Percevault, F., Hennuyer, N., Grefhorst, A., van Dijk, T. H., Gonzalez, F. J., Fruchart, J. C., Kuipers, F., and Staels, B. (2005) The farnesoid X receptor modulates hepatic carbohydrate metabolism during the fasting-refeeding transition. *J Biol Chem* **280**, 29971-29979
183. Jitrapakdee, S., Vidal-Puig, A., and Wallace, J. C. (2006) Anaplerotic roles of pyruvate carboxylase in mammalian tissues. *Cell Mol Life Sci* **63**, 843-854
184. Yang, G., Cao, K., Wu, L., and Wang, R. (2004) Cystathionine gamma-lyase overexpression inhibits cell proliferation via a H_2S-dependent modulation of ERK1/2 phosphorylation and p21Cip/WAK-1. *J Biol Chem* **279**, 49199-49205
185. Klaunig, J. E., Goldblatt, P. J., Hinton, D. E., Lipsky, M. M., Chacko, J., and Trump, B. F. (1981) Mouse liver cell culture. I. Hepatocyte isolation. *In Vitro* **17**, 913-925
186. Liu, Y. Q., Jetton, T. L., and Leahy, J. L. (2002) beta-Cell adaptation to insulin resistance. Increased pyruvate carboxylase and malate-pyruvate shuttle activity in islets of nondiabetic Zucker fatty rats. *J Biol Chem* **277**, 39163-39168
187. Peters-Wendisch, P. G., Kreutzer, C., Kalinowski, J., Pátek, M., Sahm, H., and Eikmanns, B. J. (1998) Pyruvate carboxylase from *Corynebacterium glutamicum*: characterization, expression and inactivation of the pyc gene. *Microbiology* **144 (Pt 4)**, 915-927
188. WEBER, G., and CANTERO, A. (1954) Glucose-6-phosphatase studies in fasting. *Science* **120**, 851-

852
189. MCGILVERY, R. W., and MOKRASCH, L. C. (1956) Purification and properties of fructose-1, 6-diphosphatase. *J Biol Chem* **221**, 909-917
190. Thonpho, A., Sereeruk, C., Rojvirat, P., and Jitrapakdee, S. (2010) Identification of the cyclic AMP responsive element (CRE) that mediates transcriptional regulation of the pyruvate carboxylase gene in HepG2 cells. *Biochem Biophys Res Commun* **393**, 714-719
191. Untereiner, A. A., Dhar, A., Liu, J., and Wu, L. (2011) Increased renal methylglyoxal formation with down-regulation of PGC-1α-FBPase pathway in cystathionine γ-lyase knockout mice. *PLoS One* **6**, e29592
192. Sitbon, E., and Pietrokovski, S. (2007) Occurrence of protein structure elements in conserved sequence regions. *BMC Struct Biol* **7**, 3
193. Pang, C. N., Hayen, A., and Wilkins, M. R. (2007) Surface accessibility of protein post-translational modifications. *J Proteome Res* **6**, 1833-1845
194. Foltz, I. N., Gerl, R. E., Wieler, J. S., Luckach, M., Salmon, R. A., and Schrader, J. W. (1998) Human mitogen-activated protein kinase kinase 7 (MKK7) is a highly conserved c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) activated by environmental stresses and physiological stimuli. *J Biol Chem* **273**, 9344-9351
195. Nixon, B. T., Ronson, C. W., and Ausubel, F. M. (1986) Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes ntrB and ntrC. *Proc Natl Acad Sci U S A* **83**, 7850-7854
196. Peh, M. T., Anwar, A. B., Ng, D. S., Atan, M. S., Kumar, S. D., and Moore, P. K. (2014) Effect of feeding a high fat diet on hydrogen sulfide (H_2S) metabolism in the mouse. *Nitric Oxide* **15**, 138-145
197. Manna, P., Gungor, N., McVie, R., and Jain, S. K. (2014) Decreased Cystathionine-γ-lyase (CSE) Activity in Livers of Type 1 Diabetic Rats and Peripheral Blood Mononuclear Cells (PBMC) of Type 1 Diabetic Patients. *J Biol Chem* **289**, 11767-11778
198. Mustafa, A. K., Gadalla, M. M., and Snyder, S. H. (2009) Signaling by gasotransmitters. *Sci Signal* **2**, re2
199. Yang, G. (2014) *S*-sulfhydration as a major source of H_2S bioactivity. Receptors & Clinical Investigation
200. Zhang, D., Macinkovic, I., Devarie-Baez, N. O., Pan, J., Park, C. M., Carroll, K. S., Filipovic, M. R., and Xian, M. (2014) Detection of protein *S*-sulfhydration by a tag-switch technique. *Angew Chem Int Ed Engl* **53**, 575-581
201. Krishnan, N., Fu, C., Pappin, D. J., and Tonks, N. K. (2011) H_2S-Induced sulfhydration of the phosphatase PTP1B and its role in the endoplasmic reticulum stress response. *Sci Signal* **4**, ra86
202. Xie, Z. Z., Shi, M. M., Xie, L., Wu, Z. Y., Li, G., Hua, F., and Bian, J. S. (2014) Sulfhydration of p66Shc at cysteine59 mediates the anti-oxidant effect of Hydrogen Sulfide. *Antioxid Redox Signal*
203. Nishida, M., Sawa, T., Kitajima, N., Ono, K., Inoue, H., Ihara, H., Motohashi, H., Yamamoto, M., Suematsu, M., Kurose, H., van der Vliet, A., Freeman, B. A., Shibata, T., Uchida, K., Kumagai, Y., and Akaike, T. (2012) Hydrogen sulfide anion regulates redox signaling via electrophile sulfhydration. *Nat Chem Biol* **8**, 714-724
204. Roskoski, R. (2005) Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun* **331**, 1-14
205. Liu, H., Nishitoh, H., Ichijo, H., and Kyriakis, J. M. (2000) Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. *Mol Cell Biol* **20**, 2198-2208
206. Forrester, M. T., Seth, D., Hausladen, A., Eyler, C. E., Foster, M. W., Matsumoto, A., Benhar, M., Marshall, H. E., and Stamler, J. S. (2009) Thioredoxin-interacting protein (Txnip) is a feedback regulator of *S*-nitrosylation. *J Biol Chem* **284**, 36160-36166
207. Wahl, M. C., Irmler, A., Hecker, B., Schirmer, R. H., and Becker, K. (2005) Comparative structural analysis of oxidized and reduced thioredoxin from Drosophila melanogaster. *J Mol Biol* **345**, 1119-1130
208. Liu, Y., Yang, R., Liu, X., Zhou, Y., Qu, C., Kikuiri, T., Wang, S., Zandi, E., Du, J., Ambudkar, I. S., and Shi, S. (2014) Hydrogen sulfide maintains mesenchymal stem cell function and bone homeostasis via regulation of Ca^{2+} channel sulfhydration. *Cell Stem Cell* **15**, 66-78
209. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* **17**, 2596-2606
210. Sengupta, R., and Holmgren, A. (2012) The role of thioredoxin in the regulation of cellular processes by *S*-nitrosylation. *Biochim Biophys Acta* **1820**, 689-700

211. Vandiver, M. S., Paul, B. D., Xu, R., Karuppagounder, S., Rao, F., Snowman, A. M., Ko, H. S., Lee, Y. I., Dawson, V. L., Dawson, T. M., Sen, N., and Snyder, S. H. (2013) Sulfhydration mediates neuroprotective actions of parkin. *Nat Commun* **4**, 1626
212. Qu, Z. W., Miao, W. Y., Hu, S. Q., Li, C., Zhuo, X. L., Zong, Y. Y., Wu, Y. P., and Zhang, G. Y. (2012) N-methyl-D-aspartate receptor-dependent denitrosylation of neuronal nitric oxide synthase increase the enzyme activity. *PLoS One* **7**, e52788
213. Anand, P., and Stamler, J. S. (2012) Enzymatic mechanisms regulating protein *S*-nitrosylation: implications in health and disease. *J Mol Med (Berl)* **90**, 233-244
214. Tang, G., Zhang, L., Yang, G., Wu, L., and Wang, R. (2013) Hydrogen sulfide-induced inhibition of L-type Ca^{2+} channels and insulin secretion in mouse pancreatic beta cells. *Diabetologia* **56**, 533-541
215. Desai, K. M., Chang, T., Untereiner, A., and Wu, L. (2011) Hydrogen sulfide and the metabolic syndrome. *Expert Rev Clin Pharmacol* **4**, 63-73
216. Chivers, P. T., and Raines, R. T. (1997) General acid/base catalysis in the active site of *Escherichia coli* thioredoxin. *Biochemistry* **36**, 15810-15816
217. Wu, L., Yang, W., Jia, X., Yang, G., Duridanova, D., Cao, K., and Wang, R. (2009) Pancreatic islet overproduction of H_2S and suppressed insulin release in Zucker diabetic rats. *Lab Invest* **89**, 59-67
218. Yang, G., Tang, G., Zhang, L., Wu, L., and Wang, R. (2011) The pathogenic role of cystathionine γ-lyase/hydrogen sulfide in streptozotocin-induced diabetes in mice. *Am J Pathol* **179**, 869-879
219. Yusuf, M., Kwong Huat, B. T., Hsu, A., Whiteman, M., Bhatia, M., and Moore, P. K. (2005) Streptozotocin-induced diabetes in the rat is associated with enhanced tissue hydrogen sulfide biosynthesis. *Biochem Biophys Res Commun* **333**, 1146-1152
220. Kornberg, M. D., Sen, N., Hara, M. R., Juluri, K. R., Nguyen, J. V., Snowman, A. M., Law, L., Hester, L. D., and Snyder, S. H. (2010) GAPDH mediates nitrosylation of nuclear proteins. *Nat Cell Biol* **12**, 1094-1100