CYSTATHIONINE GAMMA-LYASE/HYDROGEN SULFIDE SYSTEM AND GLUCOSE HOMEOSTASIS

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by

LING ZHANG

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ABSTRACT

Research in the last twenty years has transformed the role of hydrogen sulfide (H_2S) being perceived from a toxic gas to a gaso-transmitter with diverse physiological and pathological significance. Cystathionine gamma-lyase (CSE) is the major enzyme responsible for the endogenous production of H_2S in pancreatic β cells and liver cells, which are two key types of cells to regulate glucose level. The hallmarks of type 2 diabetes mellitus consist of insulin resistance, pancreatic β-cell dysfunction, and increased endogenous glucose production by liver. Pathophysiological implications of the CSE/H_2S system in both type 1 and type 2 diabetes as well as diabetic complications have been reported before.

Previous studies in our laboratory have shown that endogenous H_2S mainly produced from CSE inhibited insulin release via activating K_{ATP} channels and decreased H_2S production was observed after exposure to elevated glucose level in pancreatic β cells. However, the effect of glucose on CSE gene expression in pancreatic β cells and the underlying mechanism have not been thoroughly explored. Liver, an important organ to control glucose level, has relatively high amount of H_2S production compared to other organs and CSE has been reported to be the major enzyme responsible for it. The aim of the current study was to investigate the effect of glucose on CSE expression in INS-1E cells (insulin-secreting β cells) and the role of H_2S on basal and insulin-stimulated glucose uptake, glycogen synthesis and gluconeogenesis in human hepatoma $HepG_2$ cells as well as the underlying mechanisms.

In the present study, a significant decrease of CSE mRNA and protein expression by high glucose was observed in INS-1E cells and freshly isolated rat pancreatic islets. This regulation required Sp1 (specific protein-1) and p38 MAPK phosphorylation. Glucose stimulated the phosphorylation of Sp1 via p38 MAPK activation, which led to decreased CSE promoter activity and subsequent downregulation of CSE gene expression. Diminished H_2S production through altered CSE activity and expression by high glucose may be involved in the fine control of glucose stimulated insulin secretion.

Besides insulin secretion, insulin actions through adipose tissues, skeletal muscle and liver are also critical for blood glucose control. In liver cells, incubation with NaHS (a H_2S donor) significantly impaired basal and insulin-stimulated glucose uptake and glycogen storage in $HepG_2$ cells. DL-propargylglycine (PPG), as an irreversible inhibitor of the H_2S synthesizing enzyme CSE, significantly improved glycogen storage in $HepG_2$ cells. These impairing effects of H_2S did not appear to be due to loss of cell viability, but were mediated by AMPK inhibition. We also found that H_2S increased gluconeogenesis in $HepG_2$ cells with pyruvate and lactate as substrate in association with increased phosphoenolpyruvate carboxykinase (PEPCK) activity. Decreased glucokinase activity and enhanced glycogenolysis were also observed when the cells were treated with NaHS. Moreover, adenovirus-mediated CSE overexpression had similar effects as that of low dose of exogenous H_2S (below 100 μM) on glycogen storage. These data suggest that CSE/H_2S system in the liver is an important potential target for regulating insulin sensitivity and glucose metabolism. A detailed understanding of the mechanisms correlating CSE/H_2S system and insulin secretion, insulin action as well as glucose homeostasis under physiological or diabetes like conditions may help develop improved

treatment strategies for diabetes, considering the importance of these events within the context of pancreatic β cell function and liver function.

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DEDICATION

The thesis is dedicated to my parents and my husband who support me all the way.

TABLE OF CONTENTS

PERMISSION TO USE STATEMENT....i

ABSTRACT....ii

ACKNOWLEDGEMENTS....v

DEDICATION....viii

TABLE OF CONTENTS....ix

LIST OF TABLES....xvi

LIST OF FIGURES....xvii

LIST OF ABBREVIATIONS....xix

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW....1

1. H_2S as a gasotransmitter....2

1.1 Biochemistry and physiological production of H_2S....2

1.2 Endogenous generation and metabolism of H_2S....6

1.2.1 Cystathionine gamma lyase (CSE)....9

1.2.2 Cystathionine beta synthase (CBS)....11

1.3 Mechanism of H_2S effect....12

1.4 Regulation of CSE gene expression and activity....16

2. General overview of insulin secretion, glucose homeostasis and diabetes....18

2.1 Insulin secretion....18

2.1.1 Biosynthesis of insulin....18

2.1.2 Regulation and mechanism of insulin secretion..19

2.1.2.1 The importance of K_{ATP} channel and voltage-dependent calcium channels (VDCC) in insulin secretion..21

2.1.2.2 Regulators of insulin secretion..23

2.2 Insulin action...25

2.2.1 PI3-K signalling pathway activated by insulin 26

2.2.2 Insulin and carbohydrate metabolism...27

2.3 Insulin resistance...28

2.4 Glucose homeostasis...29

2.4.1 Hepatic glucose metabolism.. 31

2.4.2 Gluconeogenesis...33

2.4.3 Glycogenolysis...34

2.4.4 Glucokinase (GK) 35

2.4.5 Phosphoenolpyruvate carboxykinase (PEPCK) and its relationship to diabetes.. 36

2.4.6 5'-AMP-activated protein kinase (AMPK) and its relationship to insulin resistance. 39

2.4.7 AMPK activation in glucose uptake...40

2.5 Definition and classification of diabetes……..41

3. Involvement of H_2S in diabetes and the underlying mechanism..........................43

3.1 Inhibition of glucose metabolism and insulin release by H_2S................................44

3.2 Proapoptotic and proinflammatory effect of H_2S..45

3.2.1 Apoptosis induced by H_2S in pancreatic acinar cells and β cells...................45

3.2.2 Proinflammatory effect of H_2S...46

3.3 H_2S as an opener of ATP-sensitive K^+ channels (K_{ATP} channels)..........................48

3.3.1 Molecular basis of K_{ATP} channels in pancreatic β cells.................................50

3.3.2 Mechanism of H_2S induced direct K_{ATP} activation.......................................50

3.4 H_2S, a novel regulator of insulin resistance..51

4. Glucose regulation of gene expression..54

4.1 Kinase cascade and role of MAPK….. 56

4.2 Glucose regulates gene transcription in pancreatic β cells….................................57

4.3 Transcriptional factors of the Sp1 family are involved in glucose responsiveness.59

5. Sp1 phosphorylation in gene regulation...60

5.1 Gene regulation by Sp1...61

5.2 Pathways of Sp1 phosphorylation...61

5.3 Phosphorylation sites of Sp1 correlating to functional changes............................62

6. Rationale and hypotheses...63

7. Objectives and experimental approaches...64

CHAPTER 2. GENERAL METHODOLOGY... 66

1. Cell culture 67

2. Animal preparation...67

3. Measurement of H_2S production in the medium and H_2S production rate...................68

4. Western blots..69

5. Cell viability assay... 70

6. Preparation of intact rodent pancreatic islets...71

7. Hepatocyte preparation……………………………………………........................73

8. Determination of mRNA level by real-time PCR……………………........................73

9. Short interfering RNA (siRNA) transfection... 74

10. Measurement of insulin secretion from isolated pancreatic islets............................. 74

11.Adenovirus-mediated CSE overexpression in $HepG_2$ cells....................................... 75

12.Measurement of enzyme activity……………. 76

12.1 PEPCK activity assay...76

12.2 GK activity assay…..……………………………...77

13. Materials and data analysis..……………………..77

CHAPTER 3. RAT PANCREATIC LEVEL OF CYSTATHIONINE GAMMA-LYASE IS REGULATED BY GLUCOSE LEVEL VIA SP1 PHOSPHORYLATION..78

1. Abstract.. 79

2. Introduction...80

3. Materials and methods...81

3.1 Cell culture...81

3.2 Preparation of intact islets. 82

3.3 Measurement of endogenous H_2S production. 82

3.4 Western blots...83

3.5 Short interfering RNA (siRNA) transfection.. 83

3.6 Determination of mRNA level by real-time PCR...83

3.7 Cloning of mouse CSE promoter region and construction of reporter plasmids.....84

3.8 Transient transfection and luciferase assay...85

3.9 Measurement of insulin secretion from INS-1E cells... 85

3.10 Materials and data analysis 86

4. Results.. 87

4.1 High glucose suppressed H_2S release from INS-1E cells and pancreatic islets. 87

4.2 High glucose decreased CSE mRNA and protein expression in INS-1E cells. 89

4.3 Effects of pharmacological treatments on the expression of CSE. 91

4.4 High glucose modulated CSE expression requires the phosphorylation of p38 MAPK and SP1. 93

4.5 Role of p38 MAPK in glucose-induced phosphorylation of SP1. 95

4.6 p38 MAPK-SP1 signalling pathway mediated high glucose-induced decrease of ` CSE promoter activity...97

4.7 PPG, but not pyruvate or 2-DG, enhances high glucose-induced insulin secretion...99

5. Discussion.. 101

CHAPTER 4. HYDROGEN SULFIDE IMPAIRS GLUCOSE UTILIZATION AND INCREASES GLUCONEOGENESIS IN HEPATOCYTES..........109

1. Abstract...110

2. Introduction...112

3. Materials and methods ………..113

3.1 Animal preparation...113

3.2 Cell culture and treatments...114

3.3 Hepatocyte preparation...114

3.4 Adenovirus-mediated CSE overexpression in $HepG_2$ cells....115

3.5 Measurement of glucose consumption and glucose uptake....115

3.6 Measurement of glycogen content... 117

3.7 Hepatic glucose production....117

3.7 Establishment of insulin resistant $HepG_2$ cell model 119

3.8 Measurement of endogenous H_2S production....119

3.9 Western blotting 120

3.10 Enzyme assays... 120

3.11 Materials and data analysis. 121

4. Results....121

4.1 Effects of H_2S on glucose consumption....121

4.2 Effects of H_2S on glycogen storage 125

4.3 Involvement of AMPK activation in H_2S-impaired glucose consumption 130

4.4 H_2S-mediated changes in PEPCK and GK activity on glucose generation. 132

4.5 H_2S induces insulin resistance in $HepG_2$ cells....135

4.6 Hormonal regulation of CSE expression and activity in $HepG_2$ cells....135

5. Discussion....139

.

CHAPTER 5. DISCUSSION AND CONCLUSION....148

General discussion.... 149

Conclusions....160

Significance of the study....162

Future directions..164

References...166

Appendix...197

LIST OF TABLES

Table 1-1. Pharmacology and biology of H_2S..............................………...........................4

Table 1-2. Regulators of insulin release..25

LIST OF FIGURES

Figure 1-1. Schematic representation of the metabolic pathways involved in production of H_2S from cysteine in mammalian cells..7

Figure 1-2. K_{ATP} dependent insulin secretion.. 22

Figure 1-3. Mechanisms of hyperglycemia...30

Figure 1-4. Routes of postprandial glucose disposal 31

Figure 1-5. Schematic metabolic pathways of PEPCK and GK involved in hepatic glucose metabolism.. 38

Figure 2-1. Flow chart of the islet isolation experiments..72

Figure 2-2. Coupled enzyme assay of PEPCK activity......…......................................76

Figure 3-1. High glucose inhibited H_2S production from INS-1E cells and freshly isolated rat islets... 88

Figure 3-2. High glucose downregulated *Cse* mRNA and CSE protein expression in INS-1E cells. 90

Figure 3-3. Effect of pharmacological treatments on the expression of CSE....................92

Figure 3-4. Glucose-stimulated phosphorylation of p38 MAPK and SP1.......................94

Figure 3-5. Role of SP1 in glucose-induced regulation of CSE.....................................96

Figure 3-6. Effect of glucose on mouse CSE promoter activity.....................................98

Figure 3-7. Inhibition of CSE activation enhanced glucose-induced insulin secretion...100

Figure 3-8. Schematic signal transduction pathways underlying glucose-repressed CSE expression...108
Figure 4-1. Effects of H_2S on glucose consumption and uptake in $HepG_2$ cells and primary hepatocytes...124
Figure 4-2. Effects of exogenous and endogenous H_2S on glycogen storage................128
Figure 4-3. Phosphorylation of AMPK mediated H_2S-impaired glucose consumption..131
Figure 4-4. H_2S-mediated enhanced glucose production and altered PEPCK and GK activities in hepatocytes……..……......... 134
Figure 4-5.H_2S induced insulin-resistant state was reversed by PPG application…….138
Figure 4-6. Schematic mechanism of H_2S induced insulin resistance. 147
Supplemental Fig. 4-1. Effects of increasing concentration of NaHS or PPG on $HepG_2$ glycogen content………………………………………..........129
Supplemental Fig. 4-2. Absence of CSE protein expression in primary hepatocytes isolated from CSE KO mice……….………………….........130
Figure 5-1. Regulation of H_2S on glucose stimulated insulin secretion in pancreatic β-cells 152
Figure 5-2. Schematic diagram of the pathogenic role of CSE/H_2S system in type 1 diabetes 154
Figure 5-3. Summarized mechanism of the role of CSE/H_2S system in glucose homeostasis 161

LIST OF ABBREVIATIONS

ADP – Adenosine diphosphate

AICAR– 5-aminoimidazole-4-carboxamide-riboside

AKT (PKB) – protein kinase B

AMPK – 5'-AMP-activated protein kinase

ATP – Adenosine triphosphate

BSA – Bovine serum albumin

cAMP – Cyclic adenosine monophosphate

CBS – Cystathionine beta-synthase

cDNA – Complementary deoxyribonucleic acid

CO – Carbon monoxide

CSE (CTH, CGL) – Cystathionine gamma-lyase

DM – Diabetes mellitus

DMEM – Dulbecco's modified Eagle's medium

DPD – *N*, *N*-dimethyl-*p*-phenylenediamine sulphate

ECL – Enhanced chemiluminescence

EDRF - Endothelial-derived relaxing factor

ELISA – Enzyme-linked immunosorbent assay

ERK – Extracellular signal-regulated kinase

FBS – Fetal bovine serum

G-1-P – Glucose-1-phosphate

G-6-P – Glucose-6-phosphate

G-6-Pase – Glucose-6-phosphatase

GK – Glucokinase

GLUT – Glucose transporter

GSH – Reduced glutathione

GSIS – Glucose stimulated insulin secretion

H_2S – Hydrogen sulfide

HRP – Horseradish peroxidase

INS-1E – Rat insulinoma β cell line

IR – Insulin resistance

IRS – Insulin receptor substrate

IU – International unit

JNK – Jun n-terminal kinase

K_{ATP} – Adenosine triphosphate-sensitive potassium channel

kDa – Kilodalton

Kir – inwardly-rectifying potassium channels

KO – Knockout

KRB – Krebs-Ringer Bicarbonate

MAPK – Mitogen-activated protein kinase

MOI - multiplicity of infection

mRNA – Messenger ribonucleic acid

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD^+/NADH – Nicotinamide adenine dinucleotide

NADPH – Nicotinamide adenine dinucleotide phosphate

2-NBDG – 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose

NO – Nitric oxide

NOS – Nitric oxide synthase

OGTT – Oral glucose tolerance test

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PEPCK - Phosphoenolpyruvate carboxykinase

PI3-K – Phosphatidylinositol (PI) 3-kinase

PLP- Pyridoxal-phosphate

PPG – D,L-propargylglycine

qPCR – Quantitative real-time polymerase chain reaction

RPMI medium – Roswell Park Memorial Institute medium

SD rats – Sprague-Dawley rats

SDS-PAGE – Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM – Standard error of the mean

siRNA – Short interfering mRNA

SMC – Smooth muscle cells

Sp1 – Specificity protein 1

STZ – Streptozotocin

SUR1 – Sulfonylurea receptor-1

T1DM – Type 1 diabetes mellitus

T2DM – Type 2 diabetes mellitus

TRIS – Tris (hydroxymethyl) aminomethane

VDCC – Voltage-dependent Ca^{2+} channels

VSMC – Vascular smooth muscle cells

WT – Wild-type

ZDF – Zuker diabetic fatty rats

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

1 Hydrogen sulphide (H_2S) as a gasotransmitter

1.1 Biochemistry and physiological production of H_2S

Gasotransmitters are important endogenous signalling molecules with multifaceted physiological significance (Wang, 2002). Gasotransmitters play a major role in physiological and pathological processes such as blood pressure regulation, neurotransmitter release, inflammatory processes, longevity and so on (Szabó, 2007). It was a great surprise for the scientific world when they found nitric oxide (NO), a simple inorganic molecule, functions as an important vertebrate biological messenger (Culotta, et al., 1992). Following the identification of NO and carbon monoxide (CO) as gasotransmitters, mounting evidence has demonstrated H_2S being the third one. The advances in H_2S researches may revolutionize many conventional doctrines in the biomedical field.

H_2S is a colorless, flammable, water-soluble and foul-smelling gas with an odour resembling that of rotten eggs. Its molecular weight is 34.08 and vapor density is 1.19, heavier than air (d=1). H_2S is the sulfur analog of water molecule, and can be oxidized into different forms such as SO_2, sulfates and elemental sulfur. It has a boiling point of -60.3 °C, melting point -82.3 °C, and freezing point of -86 °C (Wang, 2012). This “swamp gas” is generally associated with decaying vegetation, sewers and noxious industrial emissions. Its toxic effect has been known for at least 300 years (Beauchamp, et al., 1984). H_2S can cause a series of deleterious effects in humans and animals when inhaled

above safe levels, such as anxiety, irritation to eyes and respiratory tract, fatigue, headache, even death if above 1000 ppm.

Around 1990, a turning point came for H_2S. Endogenous "sulphide" was reported to exist in rat brain tissues and in normal human post-mortem brainstem (Warenycia, et al., 1989; Goodwin, et al., 1989). It is now established that H_2S is produced endogenously in mammals in the brain, blood vessels, liver and kidneys. H_2S is thought to be implicated in various physiological and pathological processes in our body like the other two gasotransmitters: NO and CO. As the most recently discovered gasotransmitter, H_2S has attracted a great deal of attention as well as controversy over the past few years. Ample evidence points to an important cell signalling role of H_2S that may be of fundamental importance for cellular functions.

In vitro, H_2S can be hydrolyzed to hydrosulfide and sulfide ions in the following sequential reactions:

$$H_2S \rightleftharpoons H^+ + HS^- \rightleftharpoons 2H^+ + S^{2-}$$

Even in aqueous solutions, about one third of H_2S remains undissociated at pH 7.4, which is close to the physiological environment. The other two thirds dissociate into H^+ and HS^-, which subsequently decompose to H^+ and S^{2-}. However, the latter reaction occurs only at high pH, thus S^{2-} does not occur *in vivo* substantially. Sodium hydrosulfide (NaHS) is commonly used as an H_2S donor since it dissociates into Na^+ and HS^-; the latter can partially binds H^+ to form undissociated H_2S (Wang, 2002).

Table 1-1. summarize the pharmacology and biology of H_2S in mammalian tissues.

Table1-1.

Pharmacology and biology of H_2S	
Environmental sources	Organic: toxic gas originating from sewers, septic tanks, swamps, water treatment plants and putrefaction (Lederer, et al., 1913). Inorganic: natural gas, petroleum refinery, paper and pulp mill plant (Haahatela, et al., 1992), sulfur deposits, sulfur springs.
Endogenous sources	Synthesized in various tissues from L-cysteine by cystathionine-beta-synthase (CBS) (Jhee, et al., 2005), cystathionine-gamma-lyase (CSE) (Zhao, et al., 2001), and 3-mercaptopyruvate sulfurtransferase (MST) (Kuo, et al., 1983) with cysteine aminotransferase (CAT) (EC 2.6.1.75) (Shibuya, et al., 2009a; Shibuya, et al., 2009b).
Pharmacological inhibitors	CSE inhibitors: (1) D,L-propargylglycine (PPG)(selective and specific effects are concentration dependent) (Whiteman, et al., 2011) (2) beta-cyanoalanine (BCA) (limited selectivity, unspecific side-effects)

	(Pfeffer, et al., 1967; Zhao, et al., 2001; Mok, et al., 2004) CBS inhibitors: (1) aminooxyacetate (AOA) (unspecific, inhibiting aminotranferase generally) (Rej, 1977); (2) hydroxylamine (HA) (Kimura, 2002)
Elimination kinetics	Half-life within minutes; metabolites comprise thiosulfate, sulfite, and sulfate (Wang, 2002)
Receptors and targets	ATP-dependent potassium channels (K_{ATP} channels); cytochrome c oxidase (Zhao, et al., 2001; Yang, et al., 2005)
Cardiovascular effects	(1) Chronotropic and inotropic effects on heart (Xu, et al., 2008) (2) Vasodilation or vasoconstriction (depending on local oxygen concentration) (Zhao, et al., 2001; Koenitzer, et al., 2007)
Biological effects	Radical scavenging, upregulation of heme oxygenase-1(HO-1), involving in long term synaptic potentiation in the hippocampus (Abe, et al., 1996) Toxicology: pulmonary irritant,

	damaging to mitochondrial by binding to cytochrome *c* oxidase (Wang, 2002; Khan, et al., 1990; Dorman, et al., 2002)
Inflammatory effects	Dose-dependent proinflammatory or anti-inflammatory effects (Yang, et al., 2007)

1.2 Endogenous generation and metabolism of H_2S

Physiological importance of H_2S emerged in the mid-1990s. It can be produced in mammals including humans, and it can be detected in significant amounts (Figure 1-1) (Yang G, et al., 2007a). The production of H_2S in mammalian tissues has been mainly attributed to two key enzymes responsible for metabolizing L-cysteine: CBS (EC 4.2.1.22) and CSE (EC 4.4.1.1). CBS and CSE are heme-containing enzymes whose activity depends on the cofactor pyridoxal 5′-phosphate (P5P). The substrate of CBS and CSE, L-cysteine, can be derived from alimentary sources or can be liberated from endogenous proteins.

It can also be synthesized endogenously from L-methionine through the trans-sulphuration pathway, with homocysteine being an intermediate in the process. In tissue homogenates, rates of sulphide production are in the range of 1–10 pmoles per second per mg protein (Doeller, et al., 2005). This results in low micromolar extracellular concentrations of sulfide. Sulfide can be rapidly consumed and degraded by various

mammalian tissues. H_2S cannot accumulate in the body and is rapidly metabolized. Sulphate is the major end product and is excreted through urine. Thiosulphate, another by-product of H_2S metabolism, is also excreted in urine and present in low concentrations. Thiosulphate serves as the marker of H_2S metabolism (Belardinelli, et al., 2001).

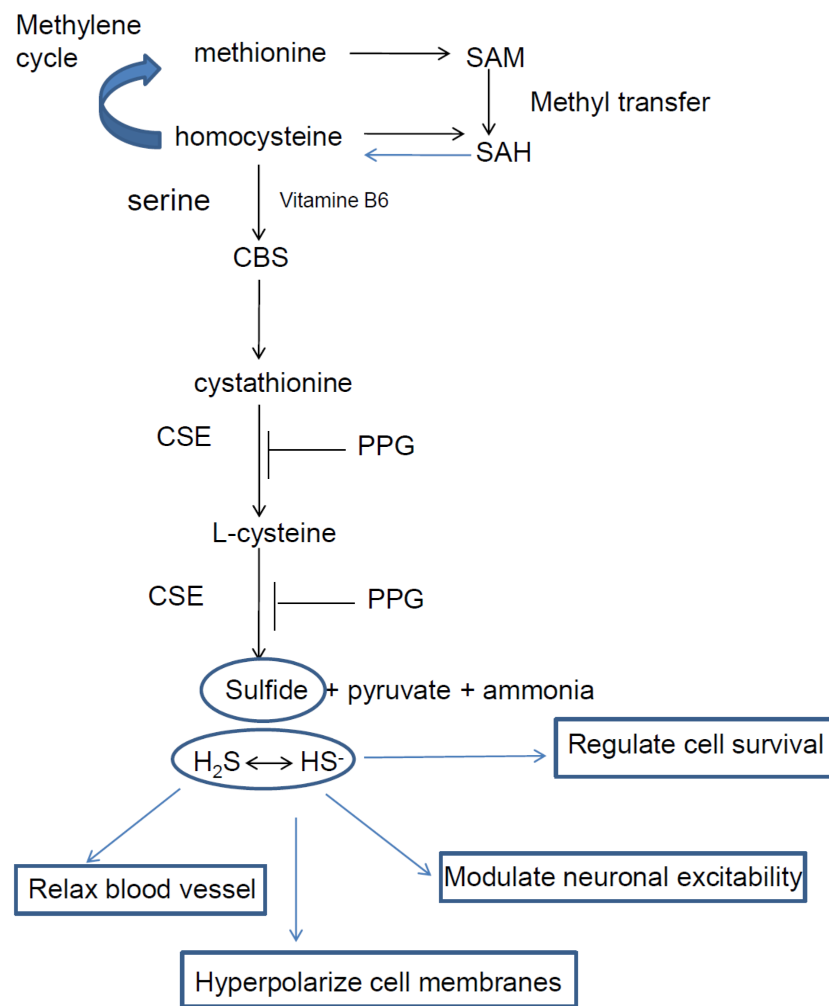


Figure 1-1. Schematic representation of the metabolic pathways involved in production of H_2S from cysteine in mammalian cells

A recent report proposed the existence of pyridoxal-phosphate (PLP)-independent pathway in neuronal tissue and endothelial cells from rodent large vessel (thoracic aorta) via utilizing the desulfuration of 3-mercaptopyruvate by MST (3-mercaptopyruvate sulfurtransferase; EC 2.8.1.2) with CAT (cysteine aminotransferase; EC 2.6.1.75) (Shibuya, et al., 2009a; Shibuya, et al., 2009b). However, the importance of this third pathway is not as clarified as CSE and CBS, and its role in generating H_2S in human tissues remains unknown.

The tissue-specific production of H_2S was determined both *in vivo* and *in vitro* using different pharmacological manipulations (Zhao, et al., 2003). The organ-specific expression and molecular regulation of CBS and CSE have been characterized by multiple groups in rodents before. The distributions of CBS and CSE were reported to vary from tissue to tissue. However, with more investigations researching on the role of H_2S in their specific system, the marked tissue specificity is not clear any more. The current literature agree that CBS is the predominant H_2S-producing enzyme in the central nervous system, highly concentrated in cerebellar purkinje and hippocampal neurons and is also densely expressed in liver and kidney. In comparison, CSE is mainly expressed in the liver and in vasculature (e.g.smooth muscle and endothelium) and non-vascular smooth muscle. CSE is also detected in the small intestine and stomach of rodents (Fiorucci, et al., 2006). It is important to note that substantial differences exist between the human and the mouse CBS and CSE enzymes (Fiorucci, et al., 2006; Miles, et al., 2004). The distributions of CSE and CBS are assumed to be much more widespread in humans, and the expressions of CSE, CBS, CAT and MST would be more similar in humans, highlighting the significance of H_2S synthesis in a variety of tissues. For

example, CSE has also been known to express in human brain including astrocytes (Lee, et al., 2009), pre-central cortex (Lee, et al., 2009), pyramidal neurons of CA3 and granule cell layer of dentate gyrus, and reticular neurons in midbrain (Diwakar, et al., 2007). CSE mRNA expression and catalytic activity was also detected in cerebellar and granule and Perkinje cells in human brain (Diwakar, et al., 2007).

Three pathways of H_2S degradation have been demonstrated: (1) mitochondrial oxidation to thiosulfate, which is further converted to sulfite and sulfate; (2) cytosolic methylation by thiol S-methyltransferase (TSMT) (EC 2.1.1.9) to methanethiol (CH_3SH) and dimethylsulfide (CH_3SCH_3); and (3) sulfhemoglobin formation after binding to hemoglobin (Wang, 2002). Similar to NO and CO, H_2S can also bind to hemoglobin. Hemoglobin was therefore termed the common sink for the three gaseous transmitters (Penney, et al., 1991). If this sink is filled with one gas, this binding might lead to enhanced plasma concentrations and, subsequently, the biological effects of the other gases (Wang, 2002). Nevertheless these biochemical means are available for H_2S catabolism. H_2S, as a powerful reducing agent, is likely to be consumed by endogenous oxidant species in the vasculature, such as peroxynitrite (Whiteman, et al., 2004), superoxide (Chang, et al., 2008), and hydrogen peroxide (Geng, et al., 2004).

1.2.1 Cystathionine gamma-lyase (CSE)

Cystahionine gamma-lyase (EC 4.4.1.1) has been conventionally abbreviated as CSE, CGL, or CTH. It is also known as gamma-cystathionase, cysteine lyase, cysteine desulfhydrase, cystathionase, cystathioninase, homoserine deaminase, or homoserine dehydratase, which catalyses the second step of transsulfuration: cleavage of the cystathionine C-gamma-S bond yielding L-cysteine, alpha-ketobutyrate (also called 2-

oxobutanoate, 2-ketobutyrate, and alpha-oxobutyrate), and ammonia. The rat CSE enzyme was reported to catalyze the formation of two gases, H_2S and NH_3 as well as pyruvate from L-cysteine (Braunstein, et al., 1984; Matsuo, et al., 1958). The trans-sulfuration pathway is critical for a sufficient supply of cysteine in *in vitro* culture of rat hepatocytes (Reed, et al., 1977; Rao, et al., 1990). Besides as an amino acid component of polypeptide chains, cysteine is necessary for synthesis of glutathione (GSH), a major intracellular antioxidant. Determination of CSE crystal structure has revealed that both yeast and human CSE are almost identical in their active sites to cystathionine gamma synthase (CGS) from *Escherichia coli* (Messerschmidt, et al., 2003).

Similar to CBS, CSE has been found to be exclusively localized in the cytosol (Ogasawara, et al., 1994). In the study of Ogasawara et al., rat liver and kidney tissues have been used to test the subcellular distribution of CSE. CSE activities and sulfide production capacity from cysteine were mainly detected in the cytosolic fractions of these tisseus.

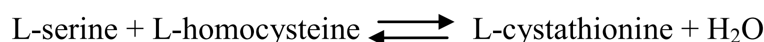
In human beings, there are two forms of CSE mRNA, of which the shorter form has an internal deletion of 132 bp, probably due to being products of different genes or splice variants. It was hypothesized that the subunit composition of the tetrameric CSE modulates the activity, and that the developmental changes in CSE activity are attributed to an alternation in relative expression of these two isoforms (Levonen, et al., 2000). It has been reported that CSE activity (Sturman, et al., 1970) as well as immunoreactive protein (Gaull, et al., 1972) is absent from human fetal liver. In rat liver, CSE activity is low during fetal development but increases rapidly during the last 3 days of gestation (Heinonen, et al., 1973). The rate of GSH synthesis from methionine is six times lower in

fetal than in adult rat hepatocytes, and the difference was concluded as a result of low CSE activity (Pallardo, et al., 1991). Genetic CSE deficiency in human beings has been associated with cystathioninuria, hypercystathioninemia and increased risk of developing atherosclerosis and bladder cancer (Renga, 2011). Regulation of CSE expression and activity has been elaborated in Section 1.4.

The fact that CSE expression and activity gradually increased in liver tissues during development has been observed before (Ishii, et al., 2004). CSE expression emerged after birth at very low levels at embryonic stage and gradually increased until 21 days postnatal (weaning age) and maintained this level at least until 84 days postnatal in mouse liver tissues, suggesting that CSE gene may involve in liver function after maturation. Indeed, CSE activity is vitamine B_6 dependent, considering all three forms of vitamin B_6 are precursors of pyridoxal 5'-phosphate (PLP), which is the co-factor of CSE and CBS. When rats are fed a vitamin B6-deficient diet, they have a lower level of blood glucose compared to fed controls (Beaton, et al., 1954; Huber, et al., 1964).

1.2.2. Cystathionine beta synthase (CBS)

CBS (EC 4.2.1.22) is a member of the beta family of PLP-dependent enzymes. It is the enzyme to catalyze the first irreversible step of the transsulfuration pathway, from homocysteine to cystathionine:



L-serine + L-homocysteine $\rightleftarrows$ L-cystathionine + H_2O

Cystathionine is further converted to cysteine by CSE. Alternatively, CBS is involved in the condensation of cysteine with homocysteine forming cystathionine and H_2S (Jhee, et al., 2005). CBS enzyme activity can be allosterically regulated by S-adenosyl-L-methionine (SAM).

In mammals, CBS contains a heme cofactor to modulate its enzyme activity in response to changes in the redox potential (Banerjee, et al., 2005). CBS is a multidomain enzyme composed of an N-terminal heme domain followed by two CBS domain in the regulatory C-terminal region. The activity of CBS is regulated presumably at the transcriptional level by glucocorticoids and cyclic AMP. The activity of CBS can also be directly inhibited by NO and CO (Puranik, et al., 2006). It can be activated by the NO donor sodium nitroprusside in a manner that paradoxically does not involve NO, but involves a chemical modification of the enzyme (Eto, et al., 2002). The regulation of CBS has been reviewed elsewhere (Miles, et al., 2004).

1.3. Mechanism of H_2S effect

H_2S exerts its various effects in biological systems through a variety of interconnected mechanisms (Szabó, et al., 2007). H_2S promotes numbers of cellular signals which regulate metabolism, cardiac function and cell survival. Previous studies have demonstrated that both CSE and CBS are critical for the maintenance of cardiovascular function and that treatment of animals with exogenous forms of H_2S has demonstrated very robust protection to various organs in a variety of cardiovascular diseases including: ischemia-reperfusion injury, various forms of shock, stroke, inflammatory disorders and models of ischemia-induced angiogenesis (Mok, et al., 2004; Elrod, et al., 2007; Szabó, et al., 2007; Kimura, et al., 2006; Jha, et al., 2008). H_2S is also a regulator of the N-methyl-D-aspartate (NMDA) receptor and may be central in long-term potentiation of neuronal circuitry for learning and memory (Kimura, et al., 2000; Boehning, et al., 2003).

Until now, the knowledge we gain about biology of H_2S largely derives from *in vitro* studies from different cell lines and isolated organ systems, either using CSE inhibitors such as PPG and BCA, or application of H_2S gas or H_2S donors such as sodium disulfide (Na_2S) or sodium hydrosulfide (NaHS). Applying H_2S in a high (micromolar to millimolar) concentration even for a short time can invariably cause cytotoxic consequences (Cheung, et al., 2007), which is the result of blockage of mitochondrial oxidative phosphorylation (Reiffenstein, et al., 1992). H_2S at low concentration has been shown to exert either cytoprotective (antinecrotic or antiapoptotic) effects (Elrod, et al., 2007; Sodha, et al., 2008; Jha, et al., 2008) or proapoptotic effects (Cao, et al., 2006; Baskar, et al., 2007; Adhikari, et al., 2008), depending on the cell type and experimental conditions.

Cytochrome c oxidase, or called Complex IV as a large transmembrane protein complex which can be found in bacteria and the mitochondrion at the end of the respiratory electron transport chain and it is also an intracellular target of H_2S (Khan, et al., 1990; Dorman, et al., 2002). Inhibition of this enzyme would lead to termination of oxidative phosphorylation, a major source of adenosine triphosphate (ATP) synthesis. The toxic effects of H_2S and the induction of a so-called “suspended animation” (Blackstone, et al., 2005; Roth, et al., 2005) are both referred to derive from this inhibition of mitochondrial respiration (Hill, et al., 1984; Cooper, et al., 2008), and therefore may underlie a potential mechanism for the regulation of cellular oxygen consumption (Leschelle, et al., 2005). The hypothesis that H_2S may be an oxygen-sensing molecule (Olson, et al., 2010), if confirmed, would fill up a major gap in our understanding on how the oxygen-sensing cells sense oxygen level change and then

initiates corresponding reactions to regain a desirable oxygen level. Chemoreceptors and chromaffin cells are well-known oxygen-sensing cells in vertebrate cardio-respiratory systems. It is speculated that oxidation of endogenously produced H_2S triggers the cardiorespiratory reflexes and relaxes smooth muscles to increase local blood perfusion and/or ventilation. Though this theory is not mature, lines of evidence have been amounted to support.

Activation of ATP-sensitive potassium channels (K_{ATP} channels) is another major mechanism underlying H_2S effect, which has been introduced as the molecular basis for vasodilation, preconditioning against ischemia/reperfusion injury, myocardial protection (Zhang Z, et al., 2007) and insulin release inhibition (Wu L, et al., 2009) (it will be discussed in detail later). Various findings support this notion (Szabo, et al., 2007; Lowicka, et al., 2007; Pryor, et al., 2006). K_{ATP} channel blockers (sulfonylurea derivates –such as glibenclamide) attenuated the H_2S-induced vasodilation both *in vivo* and *in vitro* (Tang, et al., 2005; Zhao, et al., 2001), and stimulation of K_{ATP} by H_2S was proved in the myocardium, pancreatic β cells, neurons and the carotid sinus (Lowicka, et al., 2007). Moreover, glibenclamide reversed the otherwise significant H_2S-induced hepatic arterial buffer response counteracting the reduction of portal venous flow, whereas inhibition of CSE by PPG decreased this compensatory mechanism (Siebert, et al., 2008).

Apart from the role of H_2S to open K_{ATP} channels, H_2S has a wide interaction with various ion channels to exert specific functions. In summary, H_2S regulated intracellular Ca^{2+} levels by inhibiting L-type Ca^{2+} channels in cardiomyocytes but activated the same channel in neurons. H_2S stimulated small and medium conductance K_{Ca} channels in peripheral resistance vessel - mesentery artery beds (Cheng, et al., 2004) to exert its

vasorelaxant effect. In addition, the activation of transient receptor potential vanilloid 1 (TRPV1, also called capsaicin receptor) and transient receptor potential ankyrin 1(TRPA1) by H_2S is believed to be responsible for the contraction of nonvascular smooth muscles and increased colonic luminal Cl^- secretion. H_2S-induced hyperalgesia and pro-nociception is known to be associated with the activation of T-type Ca^{2+} channels and TRPV1 channels respectively. Application of H_2S donor activates TRPV1 ion channels in the gastrointestinal tract, airway, pancreas and the urinary bladder, leading to colonic mucosal Cl^- secretion, gut motility, airway constriction, acute pancreatitis, detrusor muscle contraction and bladder contractility response respectively (Patacchini, et al., 2005; Bhatia, et al., 2005; Bhatia, et al., 2005; Streng, et al., 2008; Trevisani, et al., 2005). Activation of Cl^- channel by H_2S has been suggested to protect neurons from oxidative stress (Kimura, et al., 2006).

The evidence that H_2S acts on small to medium conductance K_{Ca} channels in endothelial cells points to the role of H_2S as an endothelium-derived hyperpolarizing factor (EDHF) (Wang, 2009). H_2S fits the principal criteria as an EDRF in many ways (Yang, et al., 2008). As the major enzyme to produce H_2S in the vascular tissues, CSE is selectively located in the endothelium of blood vessels. It is further demonstrated that endothelial CSE is activated in a calcium/calmodulin dependent manner (Yang, et al., 2008). Methacholine induced relaxation of the mesenteric artery is largely reduced in CSE knockout vessels and about half in heterozygotes, establishing H_2S as a major physiological EDRF. Eventually, CSE knockout mice developed hypertension with age, which largely reflects the gradually impaired endothelium-dependent vasorelaxation in these mice (Yang, et al., 2008; Wang, 2009).

H_2S is also a reducing agent and can interact with oxidative species. It was reported that H_2S significantly inhibited HOCl-mediated toxicity at physiological relevant concentrations in the brain (Whiteman, et al., 2005). Direct scavenge of peroxynitrate and inhibition of peroxynitrate-mediated processes by H_2S has been reported (Whiteman, et al., 2004). Its antioxidant capacity for peroxynitrate scavenging is comparable to that of GSH (Beckman, et al., 1994; Whiteman, et al., 1996).

A novel posttranslational modification mechanism, called S-sulfhydration, has been proposed by Mustafa et al., which has potential to alter the activity or function of numerous signalling proteins (Mustafa, et al., 2009). It is well known that S-nitrosylation is a process to transfer a nitric oxide (NO) group to a cysteine residue. In contrast, S-sulfhydration is a covalent modification by a sulfhydryl group, which is transferred to the cysteine residue of a protein. The outcome will be the formation of a hydropersulfide moiety (–SSH). It is demonstrated that H_2S appears to signal predominantly through S-sulfhydration of cysteines in its target proteins, which is comparable to S-nitrosylation by NO (Mustafa, et al., 2009). Even though S-nitrosylation usually inhibits enzyme activities, S-sulfhydration in most cases stimulates it. Intriguingly, the existence of endogenous desulfhydration molecules has not been confirmed. It is hypothesized that H_2S itself may function such a role due to its reducing property, whereas other strong endogenous reducing molecules cannot be excluded (Wang, 2012).

1.4 Regulation of CSE gene expression and activity

In recent years, there has been more and more understanding about how CSE can be regulated. Specifically, rodent CSE gene regulation has been well characterized. Zhao, et al. discovered that NO enhanced CSE activity in rat vascular tissues (Zhao, et al., 2001)

via two possible mechanisms. It is hypothesized that NO increases the activity of cGMP-dependent protein kinase, further stimulates CSE; or alternatively NO may directly act upon CSE protein. Additionally, NO-induced H_2S production also depended on upregulated CSE expression. In Zhao et al., study, incubation of cultured vascular SMCs with NO donor (S-nitroso-N-Acetylpenicillamine, abbreviated as SNAP) for 6 hour markedly increased the transcription level of CSE (Zhao, et al., 2001). In summary, their study for the first time demonstrated that NO might be a physiological modulator of the endogenous production of H_2S by increasing the CSE expression and activity. Conceivably, the interaction of NO and H_2S may act as a molecular switch for regulating vascular tone. In another study, NO increased the production of hydrogen sulphide in rat fetal membranes. The augmentation of hydrogen sulphide production in human intrauterine tissues in a low oxygen environment could have a role in pathophysiology of pregnancy (Patel, et al., 2009).

There is evidence that myeloid zinc finger 1 (MZF1) and specificity protein 1 (Sp1; also known as Sp1 transcription factor) play roles in the basal transcriptional activity of mouse CSE gene (Ishii, et al., 2004). Exposure to nonsteroidal anti-inflammatory drug (NSAIDs) inhibited CSE expression (mRNA and protein) and activity in the rat gastric mucosa by reduced Sp1 binding to CSE promoter (Fiorucci, et al., 2005a). As to the human counterpart, there are less reports regarding the regulation of CSE expression and enzyme activity compared to rodent CSE. CSE protein expression and activity can be up-regulated by butyrate in WiDr cells, a human colon carcinoma cell line (Cao, et al., 2004). Recently it has been demonstrated that liver CSE transcription is upregulated by bile acid mediated by the nuclear receptor Farnesoid X Receptor (FXR) in

human hepatoma $HepG_2$ cells (Renga, 2009), which is a potential protective mechanism against portal hypertension and endothelial dysfunction in livers. A very recent finding has indicated that PI3K/Akt elevated the expression of CSE at transcription level via Sp1 in human hepatoma cell lines, which is of potential importance to understand the effect of PI3K/Akt and CSE/H_2S system on tumorigenesis (Yin, et al., 2012).

2. General overview of insulin secretion, glucose homeostasis and diabetes

2.1 Insulin secretion

2.1.1 Biosynthesis of insulin

In mammals, insulin is synthesized in large quantities solely in pancreatic β cells of the islets of Langerhans. Around one to three million islets of Langerhans (pancreatic islets) distributed throughout the organ form the endocrine part of the pancreas in a healthy adult human, which occupies only 2% of the total mass of the pancreas (Saito, et al., 1978). Within the pancreatic islets, β cells constitute 60–80% of all the cells. The other cells include glucagon-producing α (alpha) cells, somatostatin-generating δ (delta) cells, pancreatic polypeptide-producing PP cells, and ghrelin-producing ε (epsilon) cells (Elayat, et al., 1995).

The insulin mRNA is translated into a single chain peptide named preproinsulin, and excision of its signal peptide generates proinsulin in the endoplasmic reticulum (Bell, et al., 1980). After folding and disulfide bond oxidizing, proinsulin is transported to the Golgi apparatus. The proteolysis of the prohormone starts there and the mature hormone is concentrated, sorted and packed into secretory granules, ready for extracellular release. Inside the endoplasmic reticulum, proinsulin is exposed to several specific proteases

which excise the C peptide, and generate mature insulin. Insulin and free C peptide are separately packaged in the Golgi as secretory granules and accumulate in the cytoplasm. When the β cell is stimulated, insulin is secreted from the cell through exocytosis and diffuses into islet capillary blood. Meanwhile, C peptide is also released into the blood, but has no known biological activity (Guest, et al., 1991; Davidson, 2004).

To be specific, unless "human" β cells were mentioned, most of the studies cited in this thesis have utlilized rat-, mouse- or hamster-derived insulinoma β-cell lines to study function *in vitro*, a small proportion of studies are using rodent islets. It is due to the inherent difficulty in maintaining primary rodent islet β-cell mass and function for more than a few days *in vitro* and also due to the scarcity of human islets for research purposes. As yet, no suitable human β-cell lines are available for unrestricted *in vitro* studies. Prevalent use of the major rodent β-cell lines have provided substantial data and insights into β cell function in physiological or pathogenic conditions.

2.1.2 Regulation and mechanism of insulin secretion

Pancreatic β cells in the islets release insulin in two distinct phases. The first phase release is triggered by elevated blood glucose levels within 10 minutes. In this phase, β cells discharge an immediately releasable pool of insulin granules which comprises the first phase of glucose stimulated insulin secretion (GSIS). The rate-limiting step for this process lies between Ca^{2+} sensing and exocytosis. The second phase is a stable, slow release of newly formed vesicles which lasts between one and three hours. Glucose-stimulated biphasic insulin secretion has drawn researchers and clinicians' attention since the phenomenon was first observed (Porte, et al., 1969). The major differences between two phases of insulin secretion are that the first phase represents

insulin release from a population of secretory vesicles which are “docked” and “primed” at the β-cell membrane and awaiting a glucose-dependent calcium signal for immediate release. The second phase represents replenishment of exocytosis-competent secretory vesicles. In fact, it is rare under physiological conditions because an abrupt increase of blood glucose level is mandatory to elicit the response—but such an increase does not normally occur following a meal (Ashcroft, et al., 1984).

Insulin is secreted largely in response to elevated blood glucose. Glucose is transported into the β cells by facilitated diffusion through GLUT2 transporter; elevated glucose concentrations in extracellular fluid thus permeate into the β cell. The increase of glucose metabolism via glycolysis and the Krebs cycle (also known as TCA cycle) where multiple high-energy ATP molecules are produced can lead to higher intracellular [ATP]/[ADP] ratio, closure of K_{ATP} channels, and membrane depolarization. Depolarization results in increased Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCC), the increase in $[Ca^{2+}]i$ eventually leads to insulin release (Hohmeier, et al., 2000; Antunes, et al., 2000). Thus, the K_{ATP} channel becomes the bridge between glucose metabolism and the stimulation of insulin secretion. This pathway is presently known as the K_{ATP} channel-dependent pathway (Rorsman, et al., 1985; Santos, et al., 1991; Koster, et al., 2005). It is now recognized as the major mechanism to stimulate insulin secretion. Another glucose-signalling pathway that augments the secretory response to increased $[Ca^{2+}]i$ was discovered more than 10 years ago (Aizawa, et al., 1998; Komatsu, et al., 2001). This is currently called the K_{ATP} channel–independent augmentation pathway which is mainly responsible for the second phase of GSIS. Nevertheless, when the clonal β cell lines-HIT-TI5, INS-1, and βHC9 were examined for presence of the K_{ATP} channel–

independent pathway, it was concluded that this pathway did not exist in these cells (Straub, et al., 2002).

Many diabetic drugs target on K_{ATP} channels to exert its therapeutic benefit. Opening of this channel is predicted to dampen the electrical trigger of insulin secretion. Sulfonylurea drugs promote, but diazoxide represses insulin secretion by binding to the regulatory sulfonylurea receptor-1 (SUR1) subunit to inhibit or activate K_{ATP} channel current, respectively (Bryan, et al., 2005).

2.1.2.1 The importance of K_{ATP} channel and voltage-dependent calcium channels (VDCC) in insulin secretion

Ion channels in pancreatic β cells modulate secretory activity triggered by metabolic, pharmacologic, or neural signals via controlling the electrical activity of ion channels, specifically permeability to K^+ and Ca^{2+}. K_{ATP} channels are a small family of potassium selective ion channels assembled of four inward rectifier pore-forming subunits, Kir6.x, paired with four sulfonylurea receptors (SURs) which are members of the ATP-binding cassette superfamily. This name is being called because the activity of these channels can be gated by the ratio of ADP to ATP, which is driven by cell metabolism. In this way, it provides a connection to link membrane electrical activity with metabolism. Apart from vascular smooth muscle cells and cardiomyocytes, K_{ATP} channels are abundantly expressed in insulin-secreting pancreatic β cells. In contrast to vascular K_{ATP} channels which consist of Kir 6.2 and sulfonylurea receptor SUR2B, pancreatic K_{ATP} channels contain Kir 6.2 and SUR1 (Ashcroft, et al., 1989). Pancreatic K_{ATP} channel plays a major role in the regulation of insulin secretion (see Figure 1-2).

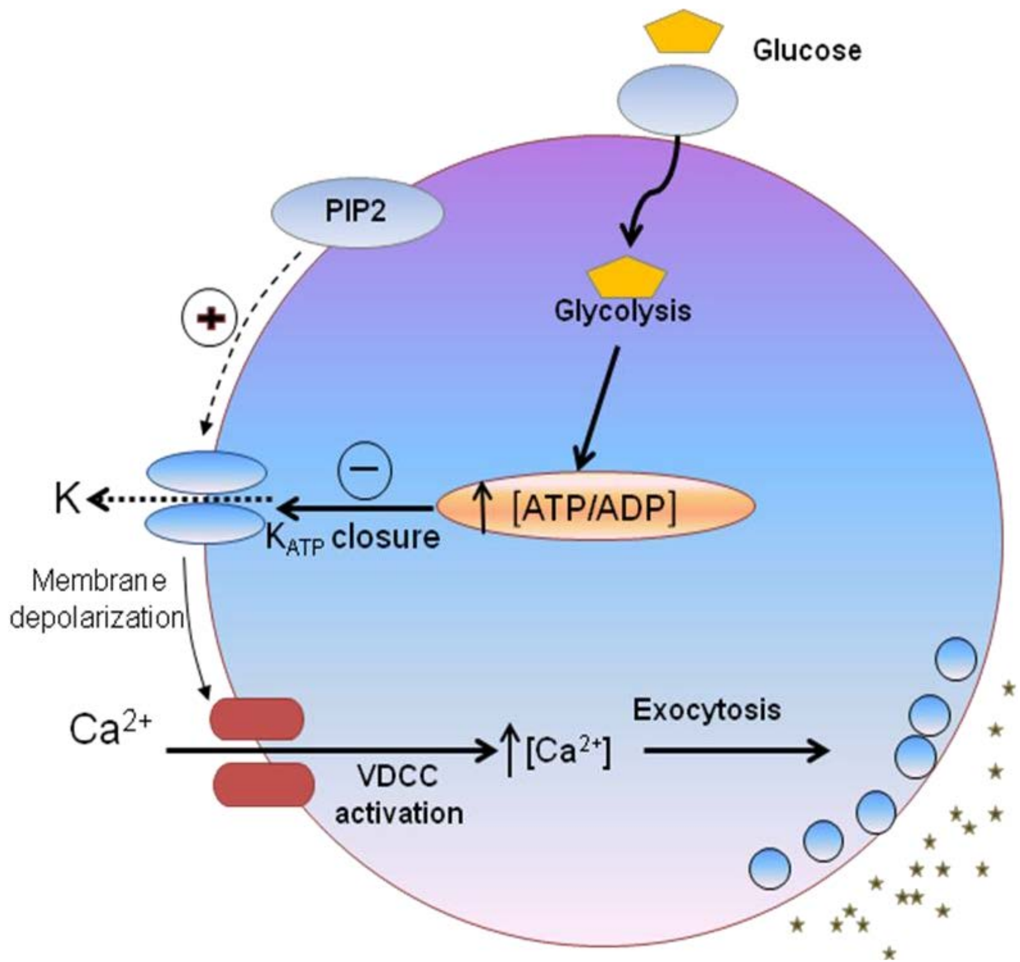


Figure 1-2. K_{ATP} dependent insulin secretion.

It is generally accepted that two signalling pathways – K_{ATP} dependent and K_{ATP} independent pathways – may cooperate in glucose induced stimulation of insulin secretion from pancreatic β cells.

Voltage-dependent calcium channels (VDCC) belong to a family of voltage-gated ion channels and exist in electrically excitable cells (e.g., skeletal muscle, glial cells, neurons and pancreatic β cells, etc.) with a permeability to Ca^{2+} ion (Catterall, et al., 2005). VDCCs are composed of a complex of different subunits: α1, α2δ, β1-4, and γ.

The α1 subunit is the pore forming subunit while the associated subunits modulate the activity of gating. VDCCs are normally closed at resting membrane potential. They can only be activated at depolarized membrane potentials, and this is why it's termed "voltage-dependent channels" (Yang SN, et al., 2006). Activation of particular VDCCs allows Ca^{2+} flux into the cell. Depending on the cell type, it leads to muscular contraction, excitation of neurons, up-regulation of gene expression, etc. In pancreatic β cells particularly, activation of VDCC results in release of insulin.

In summary, the K_{ATP} channels act as a switch in response to fuel secretagogues or sulfonylureas to initiate depolarization. VDCC is opened consequently by depolarization to increase the free cytosolic Ca^{2+} levels ($[Ca^{2+}]i$), then trigger exocytosis of insulin. In contrast, hyperpolarization of the β cell or any receptor-mediated, G-protein-coupled effects to decrease VDCC activity will inhibit insulin secretion. Membrane potential is recovered by repolarization which is initiated by voltage- and Ca^{2+}-activated K^+ channels. Both K_{ATP} channel and VDCC play critical roles in the regulation of insulin secretion in normal and diabetic β cells (Drews, et al., 2010). Understanding the regulatory mechanism of them should provide insight into the β cell metabolism and insulin secretion, which is fundamental for treating diabetes.

2.1.2.2 Regulators of insulin secretion

Insulin release from β cells is elegantly regulated by an interconnected network of factors, including hormones, autacoids, neurotransmitters, and nutrients. Most of them function by modulating the levels of their relevant intracellular signalling molecules to enhance insulin release. In our study, H_2S is demonstrated to be a physiological inhibitor of insulin secretion.

Nutrients regulate insulin secretion via various mechanisms. For instances, glucose induces insulin secretion through its oxidation by glycolysis and TCA cycle, further leading to increased ATP/ADP ratio and closure of the K_{ATP} channels. The presence of some fatty acids in pancreatic β-cells is necessary for glucose-stimulated insulin secretion; fatty acids also have enormous capacity to amplify glucose-stimulated insulin secretion, which becomes more important in situations of β-cell compensation for insulin resistance. Fatty acids stimulate exocytosis of insulin vesicles via generating acyl-CoA, which leads to acylation of proteins (Nolan, et al., 2006). Amino acids provide anaplerotic substrates to TCA cycle that increase ATP/ADP ratio, further resulting in an increase of insulin secretion. Nutrients may stimulate release of the incretins GIP and GLP-1 from the gut to induce insulin secretion (Drucker, et al., 2006). As to the mechanisms, GIP activates voltage-gated Ca^{2+} channel in β-cells via cAMP. GLP-1 mediates insulin transcription via PI3-K (Baggio, et al., 2007). However, glucagon and ghrelin prevent insulin gene transcription and insulin exocytosis, respectively (Torres, et al, 2009).

Some amino acids such as leucine and glutamine provide anaplerotic substrates to the TCA cycle which can increase ATP/ADP ratio and induce insulin secretion (Sener A, et al., 1980). Other amino acids such as alanine (Dunne MJ, et al., 1990; McClenaghan NH, et al., 1998) and arginine (Sener A, et al., 2000) are cotransported with Na^{+} into the β-cell provoking a membrane depolarization to activate voltage-gated Ca^{2+} channels, further leading to insulin vesicles exocytosis.

Regulating factors of insulin release including carbohydrate nutrients, noncarbohydrate nutrients, hormonal factors and neural factors are listed in the following table (See Table 1-2).

Table 1-2. Regulators of insulin release

Stimulants of insulin release	**Inhibitors of insulin release**
Sugar: glucose, mannose; Fatty acids (Nolan, et al., 2006) Amino acids: leucine, glutamine (Torres, et al, 2009) Vagus nerve stimulation Sulfonylureas, KCl, leptin	Neural: alpha-adrenergic effect of catecholamines (epinephrine, norepinephrine) Humoral: somatostatin (paracrine action), ghrelin (Egido, et al., 2002), prostaglandins (Robertson, et al., 1983), cytokines (IL-1β) (Tran, et al., 1999) Gasotransmitter: H_2S (Yang, et al., 2005)
Amplifiers of glucose induced insulin release	
Enteric hormones: Eg., gastrin,secretin, cholecystokinin (Kahil, et al., 1970) Neural amplifiers: β-adrenergic effect of catecholamine; Amino acids: alanine and arginine Torres, et al, 2009) Incretins: glucose-dependent insulinotropic peptide (GIP) glucagon-like peptide-1 (GLP-1) (Drucker, et al., 2006)	

2.2 Insulin action.

Insulin is a highly pleiotropic hormone which plays a pivotal role in the regulation of energy expenditure and glucose metabolism in the body by direct and indirect actions. Its anabolic action is mediated through a tightly regulated signalling cascade in a variety of tissues including kidney, muscle, and adipose tissue (O'Brien, et al., 1991). The signalling pathways downstream of binding of insulin to its receptor regulate a wide spectrum of biological events which are essential for cell survival, normal growth and development, and for glucose homeostasis, fat and protein metabolism. The signalling pathway involves a complex cascade of protein kinases and regulatory proteins, of which insulin receptor substrate (IRS)-1 and IRS-2 are the most important. Insulin promotes glucose transport and glycogen synthesis in the liver and skeletal muscle; thereby glucose is stored as glycogen in these tissues. Selectivity of final responses to insulin derives both from cell-specific expression of effector proteins and by activation of different signalling pathways.

The final outcomes can be summarized as follows:

Insulin causes (1) suppression of glucose release from liver and kidney (Woods, et al., 1979), (2) translocation of glucose transporters in muscle and adipose tissue to increase their glucose uptake (Sipols, et al., 1995) and (3) inhibition of release of FFA into the circulation due to suppression of the activity of hormone-sensitive lipase and a simultaneous increase in their clearance from the circulation (Schwartz, et al., 1991).

2.2.1 PI3-K signalling pathway activated by insulin

When insulin binds the insulin receptor, insulin action is initiated. The insulin receptor belongs to a family of receptor tyrosine kinases (RTKs) and is a heterotetrameric glycoprotein with two α-subunits (135 kDa) as the binding site and two β-subunits (95

kDa) as the tyrosine kinase. The receptor increases its kinase activity via autophosphorylation. IRS proteins are then phosphorylated by activated insulin receptor, leading to activation of PI3-K (Dupont, et al., 2001). Activation of PI3-K can recruit PI3-K dependent kinase and Akt from the cytoplasm to the plasma membrane. In this way, conformational changes occur in Akt, which allows the phosphorylation of Thr308 and Ser473 of AKT by PI3-K-dependent kinase. The final outcome of activation of Akt is the activation of glycogen synthesis and the translocation of GLUT 4 to the cell membrane for glucose transport (Tremblay, et al., 2001). The status of phosphorylation or dephosphorylation of these signal proteins, including the insulin receptor and many downstream signal proteins such as Akt, may alter insulin function. As a matter of fact, insulin resistance has been associated with impaired Akt activation (Tomás, et al., 2002).

2.2.2 Insulin and carbohydrate metabolism

Glucose is usually obtained from disaccharides like starch or sucrose in the diet. By hydrolysis in the small intestine, these disaccharides are then absorbed into the bloodstream as glucose. Elevated levels of blood glucose stimulate insulin release, and insulin then acts on target cells to promote glucose uptake, utilization and storage into glycogen. Insulin exerts tissue specific effects on glucose metabolism, which can be briefly divided into two important effects:

1. Insulin facilitates glucose uptake into muscle, adipose and several other tissues. The only mechanism of glucose uptake is by facilitated diffusion through a family of GLUT transporters. Muscle - being a case in point - the major transporter used for

glucose uptake (called GLUT4) is translocated into the plasma membrane through the action of insulin (Olson, et al., 1996).

GLUT4 transporters are mainly present in intracellular vesicles in muscle and adipose cells when insulin concentrations are low. Insulin action on these cells leads to rapid fusion of those vesicles with the plasma membrane and insertion of the glucose transporters, thereby making the cell available for taking up glucose. Under the condition when insulin level decreases and insulin receptors are free from binding, the glucose transporters are recycled back into the cytoplasm.

2. In liver tissues, insulin prompts the storage of glucose in the form of glycogen. A large portion of glucose absorbed from the small intestine is utilized by hepatocytes to convert into the storage of polymer glycogen.

2.3 Insulin resistance

Insulin resistance (IR) generally refers to a condition in which normal amount of insulin cannot produce adequate metabolic effects of insulin, including the suppressive effects of insulin on endogenous glucose production, the stimulatory effects of insulin on peripheral (predominantly skeletal muscle) glucose uptake and glycogen synthesis, and the inhibitory effects of insulin on adipose tissue lipolysis. Insulin resistance has been frequently linked to a number of diseases, including chronic infection, human obesity, and type 2 diabetes (Virkamäki, et al., 1999).

Insulin resistance in liver cells is featured by impaired glycogen synthesis and a failure to suppress glucose production. Hyperglycemia and hyperinsulinemia due to IR is considered to be the cause of metabolic syndrome and type 2 diabetes. To alleviate

insulin resistance by increasing insulin sensitivity is still one of the key avenues to cure type 2 diabetes (Le Roith, et al., 2001; Gillies CL, et al., 2007).

2.4 Glucose homeostasis

Glucose metabolism is adjusted for the maintenance of blood glucose levels within a normal range. The stable status of glucose level is called glucose homeostasis. Arterial plasma glucose values during a 24-h period average around 5 mM, with a maximum not exceeding 9.17 mM such as after meal (Rizza, et al., 1980) and staying above 3.05 mM after exercise (Wahren, et al., 1978) or fasting for 60 h (Consoli, et al., 1987). This relative stability is in contrasts to the case of other physiological substrates such as glycerol, lactate, free fatty acids, and ketone bodies which have much wider fluctuations (Gerich, et al., 1993).

Glucose homeostasis is so critical because glucose is the obligate metabolic fuel for the brain under normal conditions, whereas free fatty acids are the main fuel for most other organs. This is because of low circulating levels of other possible alternative substrates (e.g., ketone bodies) or because of limitations of transport across the blood-brain barriers (e.g., free fatty acids). After prolonged fasting, a rise in ketone bodies in the circulation contributes to brain energy to a large extent.

Glucose homeostasis is accomplished via highly complicated mechanisms involving the anabolic hormone insulin, several insulin-like growth factors and multiple organs and cell types (i.e., cells in the liver, pancreas, muscle, adipose tissues, brain and gut). Delicate signalling network including nutrient, hormone, neurotransmitter and specialized cell types have evolved to orchestrate a whole-body response to glucose level. Many catabolic hormones (glucagon, catecholamine, cortisol, and growth hormone) exert

the opposite action of insulin; they are known as anti-insulin or counter-regulatory hormones (Kahn, et al., 1994).

Blood glucose levels beyond the normal range (hyperglycemia) is probably an indicator for diabetes and its mechanism has been summarized (Figure 1-3).

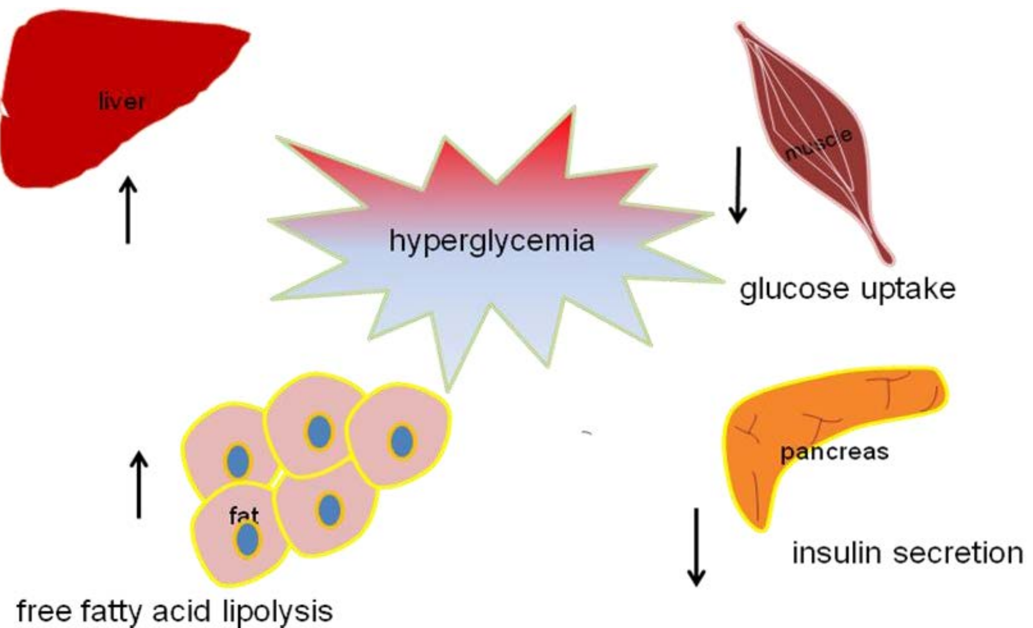


Figure1-3. Mechanisms of hyperglycemia.

With respect to routes and sites of glucose disposal after diet, glucose uptake in various tissues has been studied. In human beings, glucose taken up by tissues is largely stored as glycogen or goes through glycolysis pathway. The glucose undergoing glycolysis pathway is then either oxidized or converted to gluconeogenic intermediates. Among the glucose undergoing glycolysis, some will be oxidized; the remaining portion will undergo nonoxidative glycolysis which results in the formation of lactate, pyruvate,

and alanine. These substrates will then be available to undergo gluconeogenesis and either be stored in glycogen via the indirect pathway or be released into plasma as free glucose (Figure 1-4).

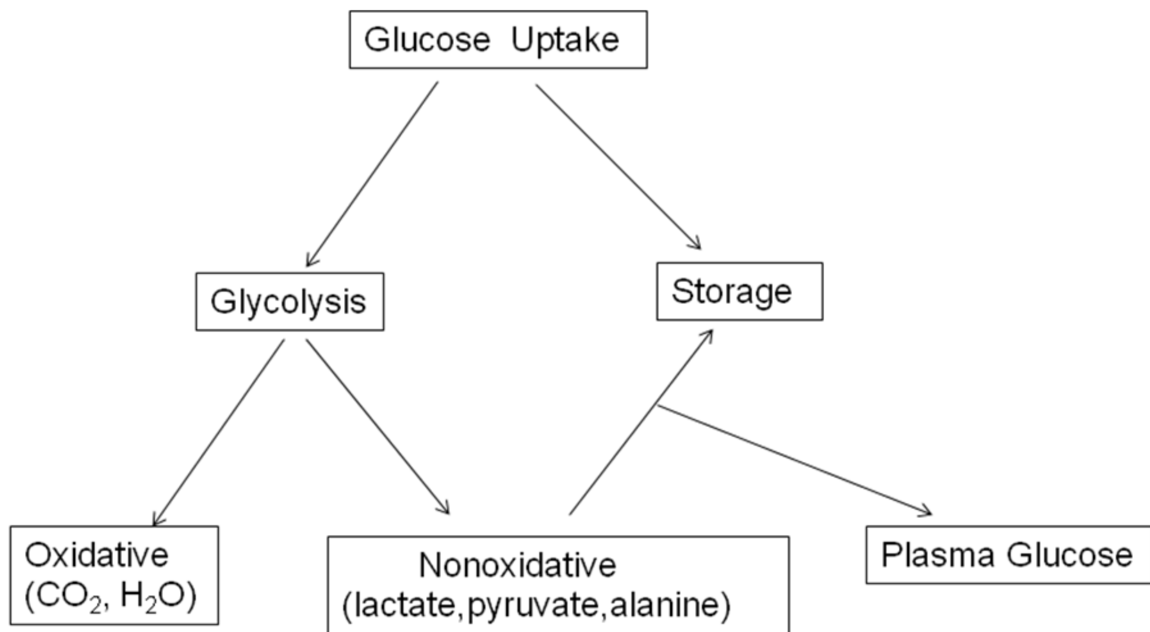


Figure 1-4. Routes of postprandial glucose disposal.

(**Adapted from** Woerle HJ, Meyer C, Dostou JM, et al. Pathways for glucose disposal after meal ingestion in humans. *Am J Physiol Endocrinol Metab.*2003; 284:E716–E725)

2.4.1 Hepatic glucose metabolism

The liver plays a dominant role in the regulation of glucose metabolism: glucose level in the circulation is the net result of peripheral glucose disposal and liver glucose output. Several hormones, including insulin, glucagon, growth hormone, cortisol, and

catecholamines are involved in the regulation of hepatic glucose metabolism. The key role of liver in glucose homeostasis is partially due to its central location, ideally fit to control systemic supply of nutrients. Another reason, it's one of only two organs that can both consume and produce glucose. Numerous evidence now established the fact that the liver takes up 1/3 of an oral glucose load, skeletal muscle and adipose tissue extract around 1/3, and the non-insulin-dependent tissues take up one-third (Meyer, et al., 2002).

Glucose enters hepatocytes by facilitated diffusion in an insulin independent way via GLUT2 transporters, which is in contrast to adipocyte and muscle cells through GLUT4 transporters (Pessin, et al., 1992). In the liver, the production of G-6-P is mediated by glucokinase (GK), a low affinity enzyme specifically expressed in liver and pancreas (HK type IV). Because GK activity rises rapidly as the glucose concentration elevates, it functions as a metabolic switch to shift hepatic glucose metabolism between fed and fasting states. G-6-P can then be stored as glycogen or metabolized in glycolytic or pentose phosphate pathway (Antoine, et al., 1997).

Glucose output predominantly occurs in the liver from glycogen breakdown. The biochemical and central anatomical location of the liver is adapted to release glucose in response to decrease in plasma glucose level and is modulated by various hormones. Hepatic glucose production (HGP) is an essential mechanism for the maintenance of circulating blood glucose levels and supply of glucose to the brain in fasting conditions and is delicately controlled to manage whole body homeostasis. HGP is suppressed primarily by insulin through modulation of glycogen metabolism, leading to increased glycogen synthesis and decreased glycogen breakdown (Ramnanan, et al., 2010).

However, glucose homeostasis is disrupted in diabetic condition, and the blood glucose is elevated due to unregulated HGP in the fasting state and impaired suppression of HGP in the feeding state (Agius, et al., 2007; Boden, et al., 2001). Specifically speaking, alterations in glycogenolysis and gluconeogenesis were observed in type-2 diabetic patients (Baynes, et al., 2004; Atkinson, et al., 1994). The consequence of glucose and fatty acids overproduction in liver is further stimulation of insulin secretion from the pancreatic β cells, and further peripheral insulin resistance, thereby becoming a vicious circle.

2.4.2 Gluconeogenesis

Gluconeogenesis is the process of glucose generation especially in the liver from non-carbohydrate carbon substrates. When the glucose level drops (hypoglycemia), glycogen is mobilized and degraded (glycogenolysis) immediately to supplement endogenous glucose. This supply is complemented by gluconeogenesis, the other source of endogenous glucose. In animals, gluconeogenesis takes place mainly in the liver with the cortex of kidneys contributing to a lesser extent (only after prolonged fasting) (Gerich, et al., 2001).

The substrates for gluconeogenesis include lactate, glucogenic amino acids and glycerol, which originate from anaerobic glycolysis in the skeletal muscle (lactate), the breakdown of either muscle protein (alanine) or adipose tissue triglycerides (glycerol). In big contrast to liver, muscle does not have glucose-6-phosphatase (G-6-Pase) thus cannot release free glucose into the circulation. Instead, glycogen is used as an important fuel for contracting skeletal muscle during prolonged strenuous exercise. However, muscle

contributes to endogenous glucose production by releasing lactate, which can be transported to the liver as substrate for gluconeogenesis (Nurjhan, et al., 1992).

Most of the enzymes required for gluconeogenesis can be found in the cytoplasm; pyruvate carboxylase (PC) and PEPCK being an exception. The former solely exists in mitochondrial and the latter acts as an isozyme distributed in both the mitochondrion and the cytosol (Chakravarty, et al., 2005). The rate of gluconeogenesis is ultimately controlled by the action of two enzyme, fructose-1,6-bisphosphatase and G-6-Pase.

Increased rates of endogenous glucose production due to increased rates of gluconeogenesis have been observed in patients with poorly controlled type 2 diabetes. Hence, gluconeogenesis is a therapeutic target for type II diabetes. Indeed, metformin, a widely used drug for type 2 diabetes, is known to exert its function primarily by inhibiting hepatic gluconeogenesis (Hundal, et al., 2000).

2.4.3 Glycogenolysis

Glycogenolysis (also known as “Glycogenlysis”) is the catabolism of glycogen polymers into glucose monomers by removal of a glucose monomer through cleavage with inorganic phosphate to produce glucose-1-phosphate (G-1-P), which is then converted to glucose-6-phosphate (G-6-P), an intermediate in glycolysis. Glycogen phosphorylase catalyzes the rate-limiting step in glycogenolysis by catalyzing the phosphorylytic cleavage of the 1-4 linkages of glycogen to yield G-1-P. Glycogenolysis occurs in the liver and muscle tissue, where glycogen is stored as energy and broken down into glucose in response to a hormonal stimulant like epinephrine (e.g., adrenergic stimulation) and/or glucagon. The combined action of phosphorylase and other enzymes leads to the complete breakdown of glycogen. The reaction catalyzed by

phosphoglucomutase is reversible, so that G-6-P can be formed from G-1-P. In liver and kidney, but not in muscle, G-6-Pase hydrolyzes G-6-P, exports free glucose into the bloodstream. Muscle cells cannot release glucose, instead use G-6-P in glycolysis (Bollen, et al., 1998; Ramnanan, et al., 2011).

Glycogenolysis occupies a large proportion of glucose output at the beginning of an overnight fast, but it is gradually replaced by gluconeogenesis with fasting goes on: The proportion of glycogenolysis contributing to the overall glucose release into the circulation was estimated to be about 45% in overnight-fasted humans, whereas that of gluconeogenesis around 55% (Gay, et al., 1994).

2.4.4 Glucokinase

Glucokinase (GK) is a type IV isoenzyme which is a member of family of hexokinases (ATP: D-hexose 6-phosphotransferase; EC 2.7.1.1), but is a distinct form of hexokinase mainly expressed in liver and pancreatic β cells. It facilitates the formation of G-6-P in eukaryotic cells. This isoenzyme (Dipietro, et al., 1962) has a molecular mass of 52 kDa and low affinity for glucose than the other hexokinases. It is not regulated by negative feedback by G-6-P as a product, which allows continued signal output (e.g., to trigger insulin release) and product formation. GK activity is localized in multiple tissues, including liver (Printz, et al., 1993), pancreatic islets of Langerhans (Matschinsky, et al., 1968), jejunal enterocytes, neuroendocrine cells, and brain of humans and most other vertebrates (Jetton, et al., 1994; Navarro, et al., 1996; Roncero, et al., 2004).

Mutations of the gene of this enzyme can cause specific forms of diabetes or hypoglycemia. For example, maturity onset diabetes of the young 2 (MODY2) is

associated with GK mutations (Fajans, et al., 2001). GK mutations that decreased its activity have deleterious effects on insulin secretion and hepatic glucose metabolism. Increased GK in the hepatocyte was shown to be glycogenic, because only G-6-P produced by GK promoted the activation of glycogen synthase.

GK is encoded by a single gene in the rat, but the alternative promoters allow this protein to be expressed in various types of cells with differential regulation. Thus, due to tissue specific promoter expression, liver GK is regulated by insulin (Printz, et al., 1993), whereas the pancreatic enzyme seems to be controlled posttranslationally by glucose levels (Magnuson, et al., 1989). In the liver, GK is found exclusively in hepatocytes, which are the predominant cells in liver tissues. GK is demonstrated to be key and rate-limiting enzyme in the regulation of glucose utilization by hepatocytes.

2.4.5 Phosphoenolpyruvate carboxykinase (PEPCK) and its relationship to diabetes

PEPCK (EC 4.1.1.32) converts oxaloacetate into phosphoenolpyruvate (PEP) and carbon dioxide and has been found in two forms: cytosolic and mitochondrial.

In animals, PEPCK is the rate-limiting enzyme catalyzing the first step of hepatic gluconeogenesis, the process whereby glucose is synthesized from metabolic precursors and responsible for hepatic glucose output. The enzyme has therefore been considered to be critical in glucose homeostasis, as evidenced by laboratory mice which manifest symptoms of Type 2 diabetes mellitus as a result of the overexpression of PEPCK (Valera, et al., 1994).

PEPCK in the liver is controlled by two different hormonal mechanisms that modulate the rate of gluconeogenesis. PEPCK activity is enhanced upon the secretion of cortisol and glucagon. As a hormone produced from the alpha cells of the islets of

Langerhans in pancreas, glucagon elevates the expression of PEPCK mediated by increased cAMP level in the liver which consequently phosphorylates cAMP response element binding (CREB) protein. CREB can bind to CRE (CREB regulatory element) upstream of the PEPCK gene and induce PEPCK transcription. On the other hand, cortisol (a hormone released from the adrenal cortex) can permeate into liver cells (due to its hydrophobic nature) and then binds to a glucocorticoid receptor (GR). GR dimerizes and the cortisol/GR complex then translocates into the nucleus and binds to the Glucocorticoid Response Element (GRE) region in a similar fashion to CREB and has similar results (synthesis of more PEPCK). Cortisol and glucagon can have huge synergistic results if applying together. On the other hand, insulin strongly represses PEPCK transcription via the activation of the PI3-K pathway (Hall, et al., 1999).

In normal people, the elevation in postprandial blood glucose stimulates insulin secretion from the pancreas. This increase in blood insulin concentration leads to the downregulation of PEPCK gene expression and then, the decrease in gluconeogenesis by the liver (Hall, et al., 1999). However, insulin-resistant hepatocytes are unable to convey the insulin signal effectively to decrease PEPCK mRNA transcription. Thus, the *de novo* glucose synthesis maintains in spite of high blood glucose (Quinn, et al., 2005). Development of compounds that are able to repress PEPCK expression and reverse insulin resistance is crucial for devising strategies to cure or treat diseases associated with defects in insulin-mediated glucose disposal.

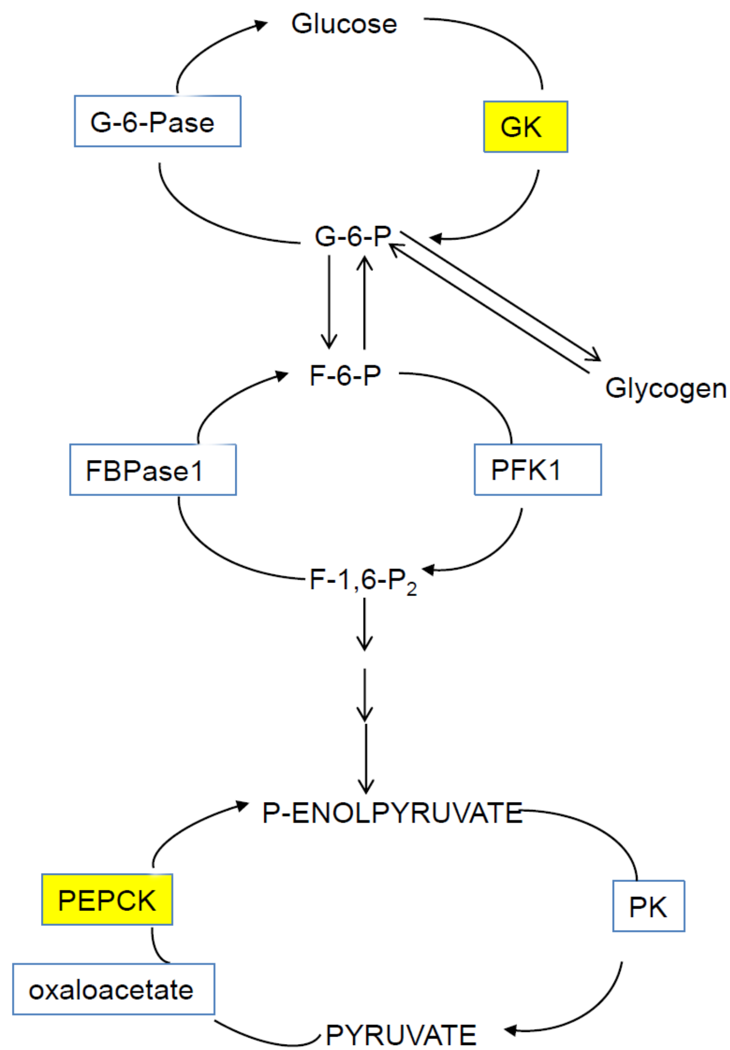


Figure 1-5. Schematic metabolic pathways of PEPCK and GK involved in hepatic glucose metabolism.

G-6-P, glucose-6-phosphate;

F-6-P, fructose-6-phosphate;

F-1-P, fructose-1-phosphate;

F-1, 6-P_2, fructose-1,6-bisphosphate;

GK, glucokinase;

G-6-Pase, glucose-6- phosphatase;

FBPase, fructose-1,6-bisphosphatase;

PFK 1, phosphofructokinase-1;

PEPCK, phosphoenolpyruvate carboxykinase;

PK, pyruvate kinase.

2.4.6. 5'-AMP-activated protein kinase (AMPK) and its relationship to insulin resistance

The AMPK system is emerging as a metabolic master switch and sensor of cellular energy status in both insulin-sensitive and other tissues that are conserved in all eukaryotic cells including mammals and lower-class organisms. It serves as a key metabolic sensor (Hardie, et al., 1998) that is capable of responding to metabolic stresses (like depletion of intracellular ATP) by shutting down the synthesis. Alterations in AMPK activity have been demonstrated to regulate glucose transport in skeletal muscle and glucose production by the liver. The AMPK system is known to be partially responsible for the beneficial effect of exercise and is the target for the well-known antidiabetic drug metformin (Hundal, et al., 2000). It is also a potential therapeutic target for obesity, type 2 diabetes, and metabolic syndrome (Hardie, et al., 1998).

To be specific, AMPK acts as a key regulator of one or more transcription factors associated with a monogenic form of diabetes mellitus. AMPK regulates PEPCK gene expression via direct phosphorylation of a novel transcription factor referred to as AICAR responsive element binding protein (AREBP) (Inoue, et al., 2006). The AMPK alpha2 catalytic subunit controls whole-body insulin sensitivity (Viollet, et al., 2003). The G-6-Pase and PEPCK genes are both downregulated by activation of AMPK (Lochhead, et al., 2000).

The heterotrimeric protein AMPK is formed by α, β and γ subunits with specific roles. The α subunit has catalytic activity and the β subunit contains a glycogen-sensing domain, and most importantly, the γ subunit includes cystathionine-beta-synthase (CBS) tandem domains (also called Bateman domains) that bind to the regulatory nucleotides AMP and ATP in a mutually exclusive manner. Hormones (e.g., insulin, leptin and adiponectin) and cytokines can interact with AMPK system.

Decreased AMPK activity has been reported to mediate the development of insulin resistance in high fat diet (HFD)-fed animal model (Lindholm CR, et al., 2012). Both isoforms of AMPK acitivities were substantially decreased by HFD in white adipose tissue, heart, and liver of SD rats. To summarize, the HFD-induced decrease in AMPK activity was correlated with systemic insulin resistance and hyperleptinemia (Lindholm CR, et al., 2012). AMPK phosphorylation, a measure of its activity, was demonstrated to be significantly decreased in both red muscle and liver after glucose infusion in parallel with the appearance of insulin resistance (Kraegen, et al., 2006). AMPK activation has been reported to inhibit the malonyl-CoA formation (Woods, et al., 2000), diacylglycerol (DAG) synthesis (Carling, et al., 1987), NF-κB activation, and oxidative stress (Zimmet, et al., 2001), all of which have been linked to insulin resistance. Additionally, decreased AMPK activity has been observed in muscle and liver of insulin-resistant rodents, including the ZDF rats, fa/fa rats, ob/ob mouse and IL-6 knockout mice (De Vit, et al., 1997; Woods, et al., 2000). Taken together, it is highly suggested that the attenuated AMPK activity contributed to the development of insulin resistance in multiple tissues including liver, skeletal muscle, white adipose tissue and heart.

2.4.7. AMPK activation in glucose uptake

Pharmacologically, AMPK can be activated by AICAR and the antidiabetic drug metformin. It is reported that the α2 isoform of AMPK could be activated in muscle by leptin injection in vivo in rats, accounting for its ability to stimulate these pathways. Lines of evidence support that AMPK is essential in the activation of glucose uptake in an insulin-independent fashion in isolated rat muscle but not in adipocytes (Musi N, et al., 2003; Sakoda H, et al., 2002). AMPK pathway has also been reported to mediate adiponectin induced glucose uptake in skeletal muscle (Yamauchi, et al., 2002). Application of AICAR in rodents activated AMPK, which results in lower blood glucose, partially due to enhanced muscle glucose uptake. Moreover, activation of AMPK in skeletal muscle also led to enhanced insulin sensitivity and oxidation of fatty acids (Musi N, et al., 2002). Many factors affect this process including glycogen content, exercise training and fiber type (Musi N, et al., 2003). The role of AMPK activation in the liver is to facilitate fatty acid oxidation and inhibit glucose production. All of these properties make AMPK a promising and potential pharmacological target for the treatment of type 2 diabetes.

2.5 Definition and classification of diabetes

Diabetes mellitus (DM) is a spectrum of disorders that is featured by hyperglycemia and glucose intolerance, owing to insulin deficiency and/or impaired insulin action.

Two major forms of diabetes, named type 1 and type 2 diabetes, have been defined. Type 1 diabetes (T1DM) is characteristic of absolute insulin deficiency mainly due to the autoimmune-mediated or unknown factor induced destruction of pancreatic β cells. The immune-mediated T1DM, previously named insulin-dependent diabetes, type

1 diabetes, or juvenile-onset diabetes, comprises approximately 5% to 10% of cases in the diabetes syndrome. Autoimmune destruction of β-cells is associated with multiple genetic susceptabilities and environmental factors that are still poorly understood. Patients in this category are rarely obese. These patients are often prone to other autoimmune disorders such as Graves' disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, celiac sprue, autoimmune hepatitis, myasthenia gravis, and pernicious anemia. Generally speaking, patients with T1DM depend on external insulin for their survival because the hormone is not produced internally any more. (American Diabetes Association, 2012).

The hallmark of type 2 diabetes (T2DM) is insulin resistance of the target tissues, and more than 90% of the diabetic patients suffered from it. It is caused by both genetic and non-genetic factors that result in insulin resistance and relative insulin deficiency. The specific genes are not known but intense investigation is underway. Aging, excessive caloric intake, overweight, central obesity, and sedentary lifestyle form the non-genetic factors. It occupies around 90% to 95% of cases in the diabetes syndrome. Type 2 diabetes has reached epidemic proportions not only in North America, but worldwide. Patients with T2DM are insulin resistant, due to such resistance, they may suffer from a relative insulin deficiency. However, for some patients with T2DM, insulin might be the last resort if all other medications failed to control symptoms, though this is relatively unusual. The hyperglycemia is resulted from insulin deficiency and/or resistance of cells to the action of insulin. Very often it's accompanied by disturbances of carbohydrate, fat, and protein metabolism. The major factors contributing to the hyperglycemia of T2DM are secondary to the failure of pancreatic β-cells to compensate for the insulin resistance

adequately, characterized by decreased whole body insulin-mediated glucose utilization and elevated hepatic glucose output (American Diabetes Association, 2012).

The classification system also included a heterogeneous etiologic category named as other types of diabetes, whose causes are established or partially known such as pancreatic diseases, endocrinopathies, genetic syndrome, insulin receptor abnormalities, infection, immune-mediated reaction and drugs or chemicals induced pancreatic alterations.

Gestational DM (GDM) comprised a fourth category resulted from insulin resistance and relative insulin deficiency associated with pregnancy and led to higher possibilities of complications for the fetus and mother. Around 7% of pregnant women are complicated by GDM, which is totally more than 200,000 cases annually worldwide (American Diabetes Association, 2012).

3. Involvement of H_2S in diabetes and the underlying mechanism.

In the recent decade, attention has been focused on the pathological role of CSE/H_2S system in diabetes. H_2S formation in rat pancrease and liver was significantly increased after diabetes induction by streptozotocin (STZ) injection. CSE and CBS mRNAs were overexpressed in liver of diabetic animals. Meanwhile, the level of pancreatic CBS mRNA was also elevated in diabetic rats and insulin treatment can restore the changes in H_2S metabolism (Yusuf, et al., 2005). Pancreatic H_2S level in Zucker diabetic fatty rats (a T2DM model) was significantly higher than that in non-diabetic Zucker fatty rats (Jia, et al., 2004). Furthermore, increased CSE activity and H_2S production from different tissues (pancreas and liver) in diabetic animals and humans have been observed in various laboratories (Veldman, et al., 2005; Wijekoon, et al., 2005;

Ratnam, et al., 2002; Hargrove, et al., 1989). DL-propargylglycine (PPG) as a CSE inhibitor significantly decreased H_2S production and improved plasma insulin level in Zucker diabetic fatty rats (Jia, et al., 2004). As the metabolic source of H_2S, cysteine was also found to be elevated in diabetic patients with diabetic nephropathy and in the STZ-treated rats (Hargrove, et al., 1989; Herrmann, et al., 2005). Moreover, *in vitro* study showed that H_2S and cysteine inhibit insulin secretion from islets and insulin-secreting β cell lines (INS-1E cells and MIN6 cells), and overexpression of CSE inhibits insulin release in INS-1E cells; in contrast, lowering of endogenous H_2S by PPG or CSE-targeted short interfering mRNA (CSE-siRNA) has the opposite effect (Yang, et al., 2005; Kaneko, et al., 2006). All of these evidences suggest a derangement of H_2S biosynthesis has occurred in diabetes (Yusuf, et al., 2005).

3.1 Inhibition of glucose metabolism and insulin release by H_2S

Treatment of pancreatic β cells with H_2S or its donor L-cysteine at physiological relevant concentrations decreased ATP content from glucose, which results from an immediate and more potent inhibition of glucose metabolism assessed by a fluorescence dye (Kaneko, et al., 2006). Even though L-cysteine slightly but significantly inhibited glucose induced mitochondrial membrane hyperpolarization, H_2S exerted this effect in a more potent way. Similarly, L-cysteine and NaHS have inhibitory effects on glucose-induced $[Ca^{2+}]$ oscillations from isolated mouse islets and β cells. It is also reported that H_2S induces a suspended animation-like state in laboratory mice by decreasing the metabolic rate, although the concentration of H_2S used here is far beyond physiological range (Blackstone, et al., 2005; Blackstone, et al., 2007).

Exogenously applied H_2S was demonstrated to inhibit insulin secretion by opening of K_{ATP} channels (Yang, et al., 2005; Ali, et al., 2007). Pancreatic islets isolated from CSE knockout mice display a better insulin secretion rate upon stimulation by high glucose (Yang, et al., 2011), which is related to the shortage of H_2S production. The interaction of H_2S and glucose metabolism in pancreatic β cells under pathological status is still unclear, and whether inhibition of H_2S biosynthesis represents a novel approach to the treatment of these conditions in diabetes, remains to be seen.

3.2 Proapoptotic and proinflammatory effect of H_2S

Exposure to H_2S has an inhibitory effect on mitogen-induced T-lymphocytes proliferation and induces apoptosis of polymorphonuclear (PMN) cells (Valitutti, et al., 1990; Mariggio, et al., 1998). Endogenous H_2S also plays a potential proinflammatory role in inflammatory conditions (Bhatia, et al., 2005; Li, et al., 2006). Previous studies from our lab have shown that overproduction of endogenous H_2S via up-regulating CSE expression leads to the inhibition of HEK-293 cell proliferation (Yang, et al., 2004a) and induction of human aorta smooth muscle cell (HASMC) apoptosis (Yang, et al., 2006). In addition, exogenously applied H_2S induces apoptosis of pancreatic acinar cells (Cao, et al., 2006), SMCs, human lung fibroblast cells, and inhibits proliferations of cardiac fibroblast cells (Liu, et al., 2011), rat pancreatic stellate cells (Schwer, et al., 2012) and human airway smooth muscle cells (Perry, et al., 2011), etc.

3.2.1 Apoptosis induced by H_2S in pancreatic acinar cells and β cells

Diabetes is a collection of clinical conditions due to absolute or relative insulin deficiency with a deficit of functional β cell mass (Mathis, et al., 2001). Any change in β

cell mass will be a reflection of an imbalance between cell proliferation and cell death (Scaglia, et al., 1997). Excessive loss of β cell mass composes one of the causes of diabetes, and apoptosis is regarded to be the main reason for β cell death in both T1DM and T2DM (Chandra, et al., 2001; Lipson, et al., 2006). Apoptotic cell death in pancreatic β-cells is involved in the pathogenesis of diabetes. Signals from death receptors and DNA damage have been widely accepted as being triggers of apoptosis in β-cells. Endoplasmic reticulum (ER) can sense and transduce apoptotic signals. ER stress-mediated apoptosis in β-cells plays an important role in the development of diabetes (Oyadomari, et al., 2002; Ozcan, et al., 2004). A very recent investigation supports the role of ER stress as a trigger for β-cell dysfunction and autoimmunity in type 1 diabetes (O'Sullivan-Murphy, et al., 2012).

Recent study from our laboratory demonstrated that exogenously applied H_2S or endogenously produced H_2S derived from overexpressed CSE can induce apoptosis of INS-1E cells via the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and up-regulated expression of endoplasmic reticulum (ER) stress-related genes, BiP, CHOP, and SREBP-1c. Taken together, it is suggested that CSE/H_2S system plays a novel role in regulating pancreatic functions under both physiological and diabetic condition by stimulating β cell apoptosis (Yang, et al., 2007b).

3.2.2 Proinflammatory effect of H_2S

Apart from the role of H_2S in cell survival or death and its pathophysiological relevance in cardiovascular and neuronal disorders, considerable interest exists in the significance of H_2S in inflammation. By using H_2S donors and specific inhibitors such as PPG, a number of investigations have provided evidence for both pro- and anti-

inflammatory role of H_2S. Active research is underway to elucidate the mechanisms of H_2S action in inflammation, including acute pancreatitis, sepsis, burn injuries and arthritis. Recent studies support a proinflammatory role of H_2S in animal models of hindpaw edema (Bhatia, et al., 2005; Li, et al., 2006), acute pancreatitis (Bhatia, et al., 2005), haemorrhagic shock, cecal ligation and puncture-induced sepsis (Zhang H, et al., 2006) and LPS-induced endotoxemia (Li, et al., 2005; Collin, et al., 2005). In these studies, tissue CSE expression is up-regulated, leading to increased H_2S biosynthesis, whereas inhibition of H_2S formation by PPG has been shown to display distinct anti-inflammatory activity (Collin, et al., 2005; Li, et al., 2006). In addition, exogenous H_2S itself causes lung inflammation in normal mice as evidenced by the elevation in lung myeloperoxidase (MPO) activity and plasma tumor necrosis factor-α (TNF-α) (Zhang H, et al., 2007). H_2S has also been shown to induce the synthesis of proinflammatory cytokines in human monocyte cells and potentiate interleukin-1β (IL-1β)-induced nitric oxide production in SMCs via enhancement of extracelluar signal-regualted kinase (ERK) activation (Li, et al., 2006; Zhi, et al., 2007).

T1DM is characterized as an inflammatory disease of the pancreatic islets in which a process of apoptosis is elicited in beta cells by interaction of activated T-cells and proinflammatory cytokines in the immune infiltrate (Kim, et al., 2007; Rabinovitch, et al., 1992). The causes of immune-mediated beta cell destruction which triggers T1DM include environmental factors, virus, diet and T1DM susceptible gene variants (Burkart, et al., 1993; Tschöpe, et al., 2005). Growing evidence has also pointed to a correlative and causative relationship between inflammation and insulin resistance/T2DM, and lifestyle modifications and medical treatments lowering the inflammatory state reduce

risk of future development of T2DM, suggesting that inflammation may play a role in the pathogenesis of type 2 diabetes (Otero, et al., 2006; Xu, et al., 2003; Hotamisligil, et al., 1993). The correlation of abnormal production of H_2S in beta cells and inflammation during the development of diabetes is not clarified, and whether inhibition of H_2S biosynthesis represents a novel approach to the treatment of inflammation in diabetes, remains to be known.

3.3 H_2S as an opener of ATP-sensitive K^+ channels (K_{ATP} channels)

K_{ATP} channels exist in a variety of tissues. H_2S has consistent effect to open K_{ATP} channels in VSMCs, gastro-intestinal SMCs, cardiomyocytes, neurons and pancreatic β-cells, thereby modulating vascular tone, gastrointestinal contractility, myocardial contractility, neurotransmission and insulin secretion. In addition to that, lines of evidence support that the processes of inflammation, nociception, pain and cell death are regulated by H_2S, and H_2S displays a beneficial (anti-inflammatory, anti-nociceptive and anti-apoptotic) effects via its stimulating effect on K_{ATP} channels (Tang, et al., 2010).

Opening of K_{ATP} channel by H_2S may underlie H_2S induced vasorelaxant effects in various vascular tissues, including isolated rat aorta (Zhao, et al., 2001), portal veins (Ali, et al., 2006), perfused rat mesenteric beds (Cheng, et al., 2004) and hepatic vascular beds (Fiorucci, et al., 2005b). This conclusion is largely based on the application of pharmacological antagonist of K_{ATP} channel, glibenclamide, to block the vasorelaxation induced by H_2S (Zhao, et al., 2001; Cheng, et al., 2004). Direct evidence was supplied by patch clamp recording showing that exogenous H_2S increases macroscopic or unitary K_{ATP} currents, and the effect is blocked by glibenclamide in isolated rat aorta and mesenteric SMC. Glibenclamide also abolished the hyperpolarization of cell membrane

induced by H_2S (Tang, et al., 2005; Zhao, et al., 2001; Cheng, et al., 2004). Moreover, other mechanisms cannot be excluded, since the vasorelaxation induced by H_2S is only partially mediated by K_{ATP} channels in rat aorta (Zhao, et al., 2001; Zhao, et al., 2002). Vasorelaxation induced by H_2S is endothelium-dependent and might be mediated by NO release and endothelium-derived hyperpolarizing factor (EDHF) as well (Zhao, et al., 2001; Cheng, et al., 2004). The endothelium-dependent vasorelaxation induced by H_2S shares many common traits with those of endothelium-derived hyperpolarizing factors (EDHF). It is assumed that H_2S might fulfil a role of EDHF and/or EDRF (Wang, 2009).

The importance of K_{ATP} channels in the regulation of glucose-induced insulin secretion from pancreatic β cells has been elaborated before (Chapter 2.1.3.1). H_2S is the first discovered endogenous gaseous opener of K_{ATP} channels in vascular smooth muscle cells (VSMCs). The activation of K_{ATP} channels by H_2S underlies many H_2S induced effects such as lowering blood pressure, protecting the heart from ischemia and reperfusion injury, and exerting anti-inflammatory, anti-nociceptive and anti-apoptotic effects. By analogy to the activatory effect of H_2S on K_{ATP} channels in VSMCs, H_2S also functions as an endogenous opener of K_{ATP} channels in INS-1E cells independent of activation of cytosolic second messengers (Zhao, et al., 2001; Zhao, et al., 2002; Yang, et al., 2005) to inhibit insulin secretion from pancreatic β cells. Additionally, basal K_{ATP} channel currents were significantly reduced by lowering endogenous H_2S level with CSE-siRNA transfection in INS-1E cells (Yang, et al., 2005). Interaction among H_2S and K_{ATP} channels in insulin-secreting cells may form a critical and potential mechanism for the fine control of insulin secretion from pancreatic β cells, however, their interaction during the development of diabetes remains to be known.

3.3.1 Molecular basis of K_{ATP} channels in pancreatic β cells

K_{ATP} channels are a group of hetero-octamer assembling of four pore-forming inwardly rectifying potassium subunits (Kir6.x) and four regulatory sulfonylurea receptors (SURx). These channels are inhibited by intracellular ATP and extracellular sulfonylureas, but stimulated by ADP and K_{ATP} channel openers (Nichols, 2006).

. K_{ATP} channels in cardiac myocytes and β-cells are inhibited by sulfonylureas at remarkably different concentrations. The reason is that these channels have a tissue specific design in SUR subunits with different affinity to sulfonylureas. β-cell/neuronal channels are known to be composed of SUR1, which has highest affinity for sulfonylureas, and $KIR_{6.2}$ as the inward rectifier. The cardiac K_{ATP} channels, however, are assembled from a lower affinity SUR_{2A}, and $KIR_{6.2}$, explaining the reason for the necessity of higher doses of sulfonylureas to inhibit cardiac channels. In contrast, K_{ATP} channels in smooth muscle cells consist of SUR_{2B} receptors and $KIR_{6.1}$ belonging to the lower conductance nucleotide diphosphate (NDP)-activated K^+ channels (Akrouh, et al., 2009).

The early discoveries pursued in β-cells discovered that glucose induced closure of K_{ATP} channels underlies the ionic mechanism of first-phase GSIS (Bennett, et al., 2010).

3.3.2 Mechanism of H_2S induced direct K_{ATP} activation

Pancreatic K_{ATP} channels play a major role in the regulation of insulin secretion in β cells. Electrophysiological study provides direct evidence that exogenous H_2S increases macroscopic or unitary K_{ATP} currents, which are blocked by glibenclamide in isolated rat

aortic and mesenteric arterial SMCs (Zhao, et al., 2001; Cheng, et al., 2004; Tang, et al., 2005). In HEK-293 cells transfected with rvKir6.1 and/or rvSUR1 genes, the stimulatory effect of H_2S on recombinant K_{ATP} channels was observed only when rvSUR1 subunit was co-expressed with $rvK_{IR}6.1$ gene, but not on expressed K_{ATP} channel with $rvK_{IR}6.1$ gene alone, indicating that rvSUR1 subunit of K_{ATP} channel complex is the target of H_2S (Jiang, et al., 2010). Among the replaced cysteine residues of rvSUR1 subunit by mutagenesis approach, only the replacement of extracellular C6S or C26S renders the loss of channel sensitivity to H_2S (Jiang, et al., 2010), demonstrating that these two extracellular cysteine residues of rvSUR1 subunit are the target amino acids of H_2S action. H_2S, as a simplest donor of –SH group, might induce sulfhydration of cysteine residues on target proteins and then enhance protein activity (Mustafa, et al., 2009).

However, it remains unclear whether H_2S induced activation of K_{ATP} channels resulted from S-sulfhydration of K_{ATP} channels via the formation of persulfides between H_2S and exposed free-SH groups of channel protein cysteine residues (Jiang, et al., 2010).

3.4 H_2S, a novel regulator of insulin resistance

The involvement of imbalance of endogenous H_2S production to the pathogenesis of diabetes has been discussed previously. As an insulin-sensitive organ, adipose tissue is critical for glucose homeostasis in the whole body by mediating glucose uptake and metabolism. White adipose tissue as an energy metabolism-active organ involves in glucose uptakes and takes up amino acids, including methionine, to convert into fatty acid (Feller, et al., 1963). It is reported that CSE expressed in rat adipose tissue is responsible for generating H_2S endogenously. Both rat adipocytes and pre-adipocytes

possess CSE/H_2S system, which was up-regulated with aging (Feng, et al., 2009). However, higher level of glucose was found to down-regulate the system in a dose- and time-dependent fashion. Both basal and insulin-stimulated glucose uptake of mature adipocytes was inhibited by H_2S, whereas application of CSE inhibitors improved the glucose uptake from adipocytes. It is suggested that the PI3-K but not K_{ATP} channel pathway underlies the mechanism of the inhibitory effect of H_2S on glucose uptake. Additionally, in fructose-induced diabetic rat model, up-regulated CSE/H_2S system was observed in adipose tissue, which was negatively correlated with glucose uptake (Feng, et al., 2009). A very recent study reported that H_2S enhanced renal gluconeogenesis by upregulating the gene expression level and gene transcriptional activity of peroxisome proliferator-activated receptor-γ (PGC)-1 α, a major regulator of gluconeogenesis by activating fructose-1,6-bisphosphatase (FBPase) and PEPCK (Untereiner, et al., 2011). Previous study from our lab also revealed that NaHS deteriorated glucose tolerance in mice and increased blood glucose level in nonfasting mice (Yang, et al., 2011). All of the findings above suggest that H_2S might fulfil the role of a novel insulin resistance regulator.

The involvement of H_2S in the pathogenesis of insulin resistance and metabolic syndrome has been reviewed in detail lately (Desai, et al., 2011). Since research on H_2S and the metabolic syndrome is still preliminary, it is difficult to get a conclusive mechanism of how H_2S is associated with the pathogenesis of, and/or protective mechanism against, the metabolic syndrome from varied reports.

Elevated blood glucose level (hyperglycemia) is one of the criteria to define metabolic syndrome (Alberti, et al., 2005). It will lead to hyperinsulinemia at the early

stage, then reduced insulin levels at the late stage if β cells fail to compensate eventually. The leptin receptor-mutated (fa/fa) Zucker diabetic fatty (ZDF) rats represent a typical genetic animal model of Type2 Diabetes (T2DM), featured by both hyperglycemia and insulin resistance. Plasma insulin level in ZDF rats is initially high but drops a lot after 7–10 weeks associated with disruption of islet morphology, β-cell degranulation, and increased β-cell death (Clark, et al., 1983). Overproduction of H_2S in the pancreatic islets of ZDF rats suggested a probable role of CSE/H_2S system in the development of T2DM and in insulin synthesis/release (Wu L, et al., 2009). The downstream of H_2S effect is the opening of K_{ATP} channel (Yang, et al., 2005), which plays a primary role in insulin secretion.

16-week ZDF rats display noticeably greater pancreatic CSE level and H_2S production but lower serum insulin level compared to the nondiabetic Zucker fatty (ZF) or Zucker lean (ZL) rats (Wu, et al., 2009). 8-week ZDF rats in the prediabetic stage had a higher serum insulin level than that of age-matched ZL and ZF rats. However, increased CSE expression in pancreatic islets from ZDF rats is already observable at this early stage. Administration of PPG to inhibit CSE activity improved glucose tolerance of ZDF rats, which should be due to improved insulin release associated with reduced pancreatic H_2S formation. Other parameters like blood glucose and HbA_{1c} level were also improved by PPG allocation in ZDF rats. The suppression of the impaired effect of H_2S by PPG may underlie the antidiabetic effect of PPG on pancreatic insulin release. Of note, non-specific effect of PPG independent of CSE inhibition cannot be excluded, since PPG was conducted only at one concentration owing to the high expense of animal models. Taken together, the relationship between plasma insulin, plasma glucose concentration,

pancreatic H_2S production and the stage (prediabetic or diabetic) of insulin resistance still needs to be elucidated using different rodent models of diabetes, even in diabetic patients (Wu, et al., 2009; Wang, 2012).

Hypothetically, structure of insulin molecule or insulin receptors on pancreatic β cells may be modified by H_2S via sulfhydration, leading to impaired insulin release and insulin resistance (Wang, 2012). Indeed, injection of the mice with NaHS has lead to an instant elevation in blood glucose, decreased plasma insulin, and deteriorated glucose tolerance in mice (Yang, et al., 2011).

All these data would suggest a potential pharmacological therapeutic approach to insulin resistance by inhibiting CSE/H_2S system in adipose and liver tissues.

4. Glucose regulation of gene expression

Nutrient regulated gene expression is an important adaption mechanism allowing species from yeast to mammals to survive on their intermittent food supplies. Glucose, as the most abundant nutrient and primary source of energy in most organisms, is an example of such. Regulatory mechanism has been developed by organisms including most prokaryotic and eukaryotic species to cope with a fluctuating level of glucose supply. Specifically in mammals, the response to glucose is more complicated considering the combined effects related to glucose metabolism itself and effects secondary to glucose-stimulated hormonal regulations, mainly glucose stimulated insulin secretion from pancreatic β cells and inhibition of glucagon secretion from pancreatic α cells (Girard, et al., 1997). Apart from the well-known role of insulin and glucagon in the regulation of gene expression, glucose has also been shown to be of importance in the transcriptional regulation of genes.

Multiple gene expressions are modulated by glucose on the transcriptional level, but mostly reported in liver cells such as L-type pyruvate kinase (L-PK), spot 14 (S14), aldolase B and fatty acid synthase (Liu, et al., 1993; Bergot, et al., 1992; Thompson, et al., 1991; Diaz Guerra, et al., 1993; Vaulont, et al., 1994; Decaux, et al., 1991; Giffhorn-Katz, et al., 1986). In most cases, glucose and insulin are both required to activate glycolytic and lipogenic genes in hepatocytes and the promoters of these genes contained a glucose responsive element (GRE).

A comprehensive study analyzed large-scale gene expression using Affymetrix microarrays and qRT-PCR from islets of normal rats (Wistar rats) and spontaneously diabetic Goto-Kakizaki (GK) rats after 48 h of culture at 3 or 20 mM glucose (Ghanaat-Pour, et al., 2007). Among the 2020 transcripts being investigated, 1033 were up-regulated and 987 were down-regulated in diabetic islets compared to control. A big proportion of these glucose responsive genes are involved in metabolism, signalling, transport, apoptosis, transcription, proliferation, and immune response (Ghanaat-Pour, et al., 2007). To be specific, several genes encoding glycolytic enzymes were up-regulated by high glucose in normal islets, such as glucose phosphate isomerase (Gpi), hexokinase 2 (Hk2) and Aldolase C, indicating an adaptive response of islets to hyperglycemia. In contrast, these glycolytic genes were down-regulated by high glucose in diabetic islets, suggesting impaired glycolytic signalling that may account for β-cell dysfunction in GK rats, which also confirmed the similar results obtained in islets from patients with type 2 diabetes (Gunton, et al. 2005). Numerous critical effectors of protein phosphorylation and exocytosis were also imbalanced in GK islets (Ghanaat-Pour, et al., 2007). AMPK, proposed as a novel β-cell glucose sensor (Leclerc & Rutter 2004), was shown to be

down-regulated in diabetic islets (Ghanaat-Pour, et al., 2007), which is consistent with reports of reduced AMPK activity in human islets from type 2 diabetic subjects (Del Guerra et al. 2005).

To explore the alterations of glucose regulated gene expression may provide evidence to the pathogenesis of human type 2 diabetes and should be of predictive and therapeutical value to confer β-cell protection against apoptosis, impaired regenerative capacity, and functional suppression occurring in diabetes.

4.1 Kinase cascade and role of MAPK

At least five different mammalian MAPK signalling modules including ERK 1/2, p38 MAPK, Jun N-terminal kinase (JNK), ERK3, and ERK5 participate in signal transduction pathways (Pearson, et al., 2001). They are activated by several extracellular factors and are involved in numerous cellular regulatory processes, such as gene expression, protein translation, protein stability, protein localization and enzyme activity, thus affecting various cellular endpoints including cell proliferation, differentiation, cell survival and cell death (Wada, et al., 2004; Turjanski, et al., 2007). Therefore, it is not surprising to see that MAPKs play a variety of roles in physiological processes, including embryogenesis, innate and adaptive immunity, metabolic homeostasis, cardiac function and neuronal plasticity, and the abnormalities in MAPK signalling are correlated with various pathological statuses, ranging from obesity⁄diabetes, rheumatoid arthritis, neuro-degenerative disorders to cancers (Kyriakis, et al., 2012; Rincon, et al., 2009; Dhillon, et al., 2007; Lawrence, et al., 2008).

Conventionally, the activation of ERKs is associated with survival responses, whereas p38 MAPK and JNK, belonging to the category of stress activated protein kinase

(SAPK), are reported to correlate with cell death. However, it is suggested that the actions of p38 MAPK are more than generalized stress-response. A more broad biological and physiological role of p38 MAPK has been realized (Schindler, et al., 2007). p38 MAPK is reported to mediate physiological factor(cytokines, hormones, and osmotic and heat shock)-regulated cellular inflammation, development, differentiation, proliferation, and survival (Nebreda, et al., 2000; Ono, et al., 2000; Brancho, et al., 2003). Extracellular signal-regulated protein kinases 1 and 2 (ERK1 and ERK2) has been well known as the terminal enzymes in a ubiquitous three-kinase cascade (Raf——MEK1, MEK2——ERK1, ERK2). ERK1 and ERK2 have been implicated not only cell growth and proliferation, but also in several inflammatory processes (Kracht, et al., 2002). In insulin secreting β cells, ERKs were demonstrated to be stimulated by secretory agonists (Frödin, et al., 1995). It has been previously identified that glucose can activate ERK1/2 and p38 MAPK pathway in insulin-secretion cell lines, whereas JNK were insensitive to glucose (Khoo, et al., 1997). The activation of ERK1/2 in pancreatic β cells has been supported by other studies (Briaud et al., 2003; Longuet et al., 2005) and it is shown that this activation remains in the cytoplasm and participates in the regulation of insulin secretion (Longuet et al., 2005).

4.2 Glucose regulates gene transcription in pancreatic β cells

Physiologically speaking, the most important property of pancreatic β cells is to store, synthesize and release insulin corresponding to the surrounding glucose level in order to maintain glucose homeostasis. Logically and indeed, glucose is a regulator of β cell. The β cells have been evolved to be delicately sensitive to glucose and respond to even small changes in glucose level in widely varying species. Multiple genes in

pancreatic β cells are demonstrated to be regulated by glucose in an acute or chronic way. Generally speaking, the acute effects of glucose within minutes usually rely on the allosteric or covalent modification of certain proteins, such as K_{ATP} channels modulated by the ratio of [ATP/ADP] (Detimary, et al., 1998) and small G-protein Rab-3a activated by GTP (Lang, et al., 1999). The consequence will be the closure of K_{ATP} channels leading to insulin release and GTP induced insulin vesicle budding and exocytosis respectively. In addition, glucose induced alteration of the gene expression encoding the effector or regulator proteins involved in the acute effect is the foundation for subacute (hours) and chronic (days) effect of glucose.

Several investigations have explored the identity of genes undergoing glucose regulation in pancreatic β cells (Flamez, et al., 2002; Webb, et al., 2000; Minami, et al., 2000; Ghanaat-Pour, et al., 2007). Glucose level has been shown to be of importance for the maintenance of β cell function (energy metabolism, transport, signalling pathway, insulin synthesis and secretion and protein synthesis/degradation), whereas prolonged exposure to high glucose environment results in insensitivity and eventual toxicity to β cells (Schuit, et al., 2002).

In pancreatic β cells, glucose is well known to be a key stimulator of insulin gene transcription. Glucose can induce two to five folds increase in insulin gene transcription in order to restore depleted insulin stores within the first hour after glucose stimulation. DNA sequence located around 4 kb upstream of transcription start site is responsible for glucose regulated insulin gene transcription (Steiner, et al., 1985). However, a 340 bp region proximal to the transcriptional start site is most critical and sufficient for glucose

regulated β cell-specific transcription (Fromont-Racine, et al., 1990; German, et al., 1994).

Glucose has to be metabolized to exert the regulatory function for gene transcription, which means not glucose itself, but glucose metabolite generated an intracellular signal after glucose enters insulin-sensitive tissues, including liver, adipose tissues, and pancreatic β cells. G-6-P, as the metabolite in the first step of glycolysis, is shown to be necessary for glucose-dependent gene regulation (either upregulated or downregulated). Indeed, lines of evidence suggested that G-6-P itself functions as a glucose sensitive signalling molecule not only in β cells, also in liver cells (Girard, et al., 1997; Goya, et al., 1999; Li, et al., 2010; Dentin, et al., 2012). It is demonstrated that G-6-P concentration is correlated closely with the glucose induced transcription of genes (Mourrieras, et al., 1997). However, the role of other glucose metabolites in mediating glucose signalling cannot be excluded (Girard, et al., 1997; Scott, et al., 1998; Jitrapakdee, et al., 2010; Wiederkehr, et al., 2012). Alternatively, the hexosamine biosynthetic pathway as a cellular "sensor" of energy availability mediated the transcriptional effects of glucose on gene expression (Sayeski, et al., 1996; Wang, et al., 1998).

4.3 Transcriptional factors of the Sp1 family are involved in glucose responsiveness

Mounting evidences demonstrate that Sp1 as a ubiquitously existed transcription factor may underlie a mechanism of glucose responsiveness (Daniel, et al., 1996a; Daniel, et al., 1996b; Chen, et al., 1998; Moreno-Aliaga, et al., 2007; Li, et al., 2008). Gene transcriptions of leptin, fatty acid synthase, ATP citrase-lyase and mouse vesicular glutamate transporter 2 were reported to be regulated by glucose via the Sp1 family members (Fukuda, et al., 1999a; Fukuda, et al., 1999b; Li, et al., 2008). Acetyl-CoA

carboxylase (ACC), as the critical enzyme for the biosynthesis of fatty acids, was the first gene found in adipocytes to be induced by glucose via the Sp1 binding activity (Daniel, et al., 1996a). Dephoshorylation but not the alteration of amount of Sp1 in the nucleus was responsible for this induction. The dephosphorylation of Sp1 was mediated by type 1 protein phosphatise, which was activated by glucose treatment (Daniel, et al., 1996b). The glucose-dependent dephosphorylation of Sp1 led to an enhanced binding activity to ACC promoter. Similar phenomenon was observed in the promoter activities of aldolase A and pyruvate kinase M2, which were increased by glucose via the dephosphorylation of Sp1 (Schäfer, et al., 1997). A key role of Sp1 in the transcriptional activation of the leptin gene promoter by glucose metabolism has also been explored (Moreno-Aliaga, et al., 2007).

5. Sp1 phosphorylation in gene regulation

Sp1 is the prototype of a family of zinc finger transcription factors, including at least four Sp1 transcription factors (Black, et al., 2001; Cook, et al., 1999). The activities of these transcription factors can be regulated at different levels, such as post-translational modification (e.g,phosphorylation and glycosylation) and intracellular translocation. Specifically for Sp1, the phosphorylation has led to altered DNA binding or promoter activation, involving in a variety of physiological processes including cell growth, differentiation, apoptosis, angiogenesis, and immune response (Tan, et al., 2009), suggesting an important role of the phosphorylation state of Sp1 in the regulation of cell physiology. Meanwhile, a recent investigation found that Sp1 phosphorylation by cyclin-dependent kinase 1 (CDK 1) /cyclin B1 represses its DNA-binding activity during mitosis in cancer cells, which promoted cancer-cell proliferation (Chuang, et al., 2012) and

implicated Sp1 in the pathological condition. Accumulated evidence has shown that Sp1 can be phosphorylated by many kinases and the phosphorylation can be affected by protein phosphatases.

5.1 Gene regulation by Sp1

GC (GGGGCGGGG) boxes as important *cis*-acting elements are known to be necessary for the transcriptional regulation of many genes ranging from housekeeping genes to tissue specific genes, viral and inducible genes (Philipsen, et al., 1999; Suske, et al., 1999; Bouwman, et al., 2002). Transcription factor Sp1 is a zinc finger protein that binds to GC-rich DNA sequence in both constitutively activated genes and inducible genes to regulate their expressions. Sp1 can also interact with cellular factors (Merika, et al., 1995; Datta, et al., 1995; Karlseder, et al., 1996; Perkins, et al., 1994; Lee, et al., 1993) and viral gene products (Trejo, et al., 1997; Krady, et al., 1995; Li, et al., 1991), suggesting protein protein interactions are underlying Sp1 mediated signal transduction in a cell and gene specific manner despite its ubiquitous existence. Additionally, posttranlational modification such as phosphorylation, glycosylation, acetylation, sumoylation, and ubiquitination as well as changes in the level of Sp1 can potently modulate activity of Sp1 (Saffer, et al., 1991). Until very recently, potential interplay between various modification forms of Sp1 has been demonstrated to exert an important role in cell cycle entry and mitosis (Wang, et al., 2011).

5.2 Pathways of Sp1 phosphorylation

Investigations have identified more and more kinases that phosphorylate Sp1 in a direct or indirect way, including DNA-dependent protein kinase (DNA-PK), PKA, PKC-ζ,

casein kinase II (CK-II), ERK, cyclin-dependent kinases (CDK), PP2A, GSK3β and some unknown kinases (Armstrong, et al., 1997; Chun, et al., 1998; Fojas de Borja, et al., 2001; Milanini-Mongiat, et al., 2002; Chen, et al., 2011; Chu, et al., 2012). Viral infection, growth factors, hormones, some drugs and mechanical stress are the triggers known to induce Sp1 phosphorylation (Chu, et al., 2012).

Putative phosphorylation sites were found in human Sp1 (NCBI accession number P08047). The residues include Ser, Thr, and Tyr (from NetPhos 2.0; data not shown) (Tan, et al., 2009), which have been confirmed by numerous studies (Chu, et al., 2012).

5.3 Phosphorylation sites of Sp1 correlating to functional changes

Ser59, Ser131, Thr453, Thr579, and Thr739 are the confirmed phosphorylation sites on Sp1. They are phosphorylated by ERK, CDK, DNA-PK and CK-II respectively (Armstrong, et al., 1997; Chun, et al., 1998; Fojas de Borja, et al., 2001; Milanini-Mongiat, et al., 2002; Zheng, et al., 2001). Considering the wide range of pathways, kinases or phosphatases that regulate Sp1 phosphorylation, one can expect complicated results to occur after Sp1 phosphorylation (Chu, et al., 2005). Indeed, it has long been noted that the phosphorylation of Sp1 may have positive or negative effects on Sp1 dependent DNA binding and promoter activation. Take glucose-responsive genes as an example, it has been reported that glucose activated adolase A gene in a rat hepatoma cell line via Sp1 dephosphorylation (Schäfer, et al., 1997), whereas glucose induced phosphorylation of Sp1 through MAPK enhanced Sp1 binding activity to the mVGLUT2 promoter in a mouse pancreatic β cell line, and dephosphorylation by protein phosphatase 1 dramatically decreased its binding activity to the same promoter (Li, et al., 2008). The

varied effect can be explained because of the cell-type and gene dependent role of Sp1 on gene transcription.

6. Rationale and hypotheses

The association of CSE/H_2S system with hyperglycemic and insulin resistant condition such as Type 1 and Type 2 diabetes mellitus has been intensively studied. CSE appears to be the predominant H_2S producing enzyme in the pancreas and liver (Wu, et al., 2009; Mani, et al., 2011). Increased CSE protein level in pancreas and liver tissues of diabetic animals, which in turn gives rise to enhanced H_2S production, has been linked to the development of diabetic symptoms such as decreased insulin secretion and impaired glucose uptake (Yusuf, et al., 2005; Yang, et al., 2005; Yang, et al., 2011; Wu, et al., 2009). Previous study from our lab showed that H_2S inhibited insulin secretion from INS-1E cell line and high glucose appears to decrease H_2S production in these pancreatic β cells (Yang, et al., 2005). Specifically for pancreatic β cells, glucose is the major physiological stimulus for the regulation of insulin secretion. Glucose has also been demonstrated to be critical in regulation of genes at both the transcriptional and translational level (Girard, et al., 1997; Towle, et al., 1997; Scott, et al., 1998).

Considering both the critical role of glucose on gene expression related to insulin secretion and effects of CSE/H_2S system on insulin secretion, we hypothesize that glucose may regulate CSE gene expression in pancreatic β cells and we want to explore the underlying mechanisms as well.

In terms of the relationship between glucose metabolism and H_2S, there has been evidence that both exogenous and endogenous H_2S can enhance renal gluconeogenesis by upregulating PGC-1α, a critical regulator of energy metabolism (Untereiner, et al., 2011).

Given that liver is a major part for gluconeogenesis, we hypothesize that H_2S may regulate hepatic glucose metabolism by influencing important enzyme activities of glucose metabolism, such as PEPCK and GK. Since reports from our lab showed the elevated serum H_2S level existed in ZDF rats which developed insulin resistance (Wu, et al., 2009), and PPG treatment improved serum insulin level of ZDF rats to that of their control littlemates, we therefore hypothesize CSE/H_2S system may also influence hepatic insulin resistance and insulin signalling pathway in liver cells.

In summary, the above observations lead us to hypothesize that the lack of H_2S from the β cells and liver cells of CSE knockout mice has protective effects against the development of diabetes. Administration of PPG by inhibiting endogenous CSE activity may have similar effects in those cells, including improved insulin secretion in β cells and improved glucose utilization in hepatocytes. Furthermore, glucose may modulate CSE gene expression in pancreatic β cells. Interplay between glucose and H_2S may be involved in the physiological and pathological regulation of glucose and insulin level.

7. Objectives and experimental approaches

I. *To investigate the relationships of high glucose, CSE gene expression and* H_2S *production in rat pancreatic β cells (INS-1E cells) and freshly isolated islets*.

We investigated whether high glucose had effects on CSE protein level and mRNA level in INS-1E cells. Glucose effect on CSE promoter activity was also examined by constructing a proximal Cse promoter vector containing specificity protein 1 (Sp1) consensus sequence. H_2S levels were measured in INS-1E cells cultured in the presence of high (20 mM) or low glucose (5 mM) for 24 h. As a functional parameter,

insulin release was detected at INS-1E cells after exposure to high or low glucose with or without PPG (5 mM).

II. *To test the effect of exogenous and endogenous H_2S on glucose uptake, glycogen storage, and glucose production (including the rate of gluconeogenesis and glycegenolysis) in liver cells and explore the underlying molecular mechanism.*

The role of H_2S on glucose utilization, glycogen synthesis, as well as gluconeogenesis and glycogenolysis in human hepatoma $HepG_2$ cells and primary mouse hepatocytes were investigated by a colorimetric method. Activities of glucose metabolic enzymes including GK and PEPCK were examined in $HepG_2$ cells in the presence of various doses of H_2S and PPG. Adenovirus-mediated CSE overexpression was used to increase endogenous H_2S production. Then the glycogen content was measured in these CSE overexpressed $HepG_2$ cells. Glycogen content was also compared between wild type mice and CSE knockout mice in both fasting and fed conditions. $HepG_2$ cell viabilities were determined after exposure to H_2S and PPG at different time points or after infections of adenovirus vectors using MTT and trypan blue methods, respectively.

III. *To determine the role of H_2S on hepatic insulin resistance and the alteration of the molecular markers in the insulin signalling pathway.*

Protein levels of phospho-AMPK and total AMPK were checked after incubation with physiological concentrations of H_2S and PPG by Western blots. AICAR was used as a stimulator of AMPK activity to see the reversibility of glucose uptake by H_2S. Insulin stimulated protein level of phospho-AKT was explored under treatment of H_2S, which reflects the strength of insulin signalling transduction.

CHAPTER 2

GENERAL METHODOLOGY

CELL CULTURE

Rat insulinoma INS-1E cells were kindly provided by Dr. C. B. Wollheim, Geneva, Switzerland. INS-1E cells contain numerous insulin granules and exhibit glucose stimulated insulin secretion (GSIS).

For experiments, passages between No. 50 and 80 were taken. The cells were routinely cultivated in RPMI 1640 medium without glucose (Sigma) supplemented with glucose (5 or 20 mM), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES), 10% fetal bovine serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin. The experiments were performed when the cells reached 70–80% confluence as previously described (Yang W, et al., 2005). Before treatment with different concentrations of glucose, INS-1E cells were preincubated overnight in RPMI 1640 medium containing 1% FBS and 5 mM glucose at 37°C in a humidified mixture of 5% CO_2 and 95% O_2 and then subjected to different concentrations of glucose or other compounds together with 10% FBS. Glucose stock solution was filtered and added directly to culture medium to reach the desired final concentration.

$HepG_2$ human hepatoma cell line was obtained from American Type Culture Collection (Manassas,VA) and maintained in basal glucose (5.5 mM) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/ml streptomycin and kept at 37 °C in a humidified atmosphere of 5% CO_2. Cells were grown upon reaching 70-80% confluence, and then preincubated in serum-free medium for 24 h before treatments.

ANIMAL PREPARATION

CSE KO mice were generated as previously described (Yang, et al., 2008). Twelve week old male CSE KO mice and age-matched male wild-type (WT) littermates on C57BL/6J × 129SvEv background were used. PCR-genotyping of CSE KO mice was performed using a three-primer assay in two reactions. 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. All animals were maintained on standard rodent chow and had free access to food and water.

MEASUREMENT OF ENDOGENOUS H_2S PRODUCTION IN THE MEDIUM AND H_2S PRODUCTION RATE

H_2S production rate was measured as previously described (Stipanuk & Beck, 1982) with modifications, which has been routinely used in our laboratory (Zhao, et al., 2001, 2003; Cheng, et al., 2004; Yang, et al., 2008). Briefly, cultured cells reaching 80% confluent were collected and homogenized in 50 mM ice-cold potassium phosphate buffer pH 6.8. The reaction mixture contained (mM): 100 potassium phosphate buffer pH 7.4, 10 L-cysteine, 2 pyridoxal 5′-phosphate, and 10% (w/v) homogenate. Cryovial test tubes (2 ml) were used as the centre wells, each containing 0.5 ml 1% zinc acetate as trapping solution and a filter paper 2 cm×2.5 cm to increase air: liquid contacting surface. Reaction was performed in a 25 ml Erlenmeyer flask (Pyrex, USA). The flasks containing the reaction mixture and centre wells were flushed with N_2 before being sealed with a double layer of Parafilm. Reaction was initiated by transferring the flasks from ice to a 37°C shaking water bath. After incubating at 37°C for 90 min, 0.5 ml of

50% trichloroacetic acid was added to stop the reaction. The flasks were sealed again and incubated at 37°C for another 60 min to ensure a complete trapping of H_2S released from the mixture. Contents of the centre wells were then transferred to test tubes, each containing 3.5 ml of water. Subsequently, 0.5 ml of 20 mM *N*, *N*-dimethyl-*p*-phenylenediamine sulphate (DPD) in 7.2 M HCl was added immediately followed by addition of 0.5 ml 30 mM $FeCl_3$ in 1.2 M HCl. Absorbance of the resulting solution at 670 nm was measured 20 min later with a spectrophotometer. H_2S content was calculated against the calibration curve of standard H_2S solutions.

To measure H_2S concentration in the medium, 200 μl of culture media from each treatment were collected and added to microcentrifuge tubes containing zinc acetate (1% w/v, 600 μl) to trap H_2S. After 5 min, the reaction was terminated by adding 400 μl of DPD (20 μM in 7.2 M HCl) and 400 μl of $FeCl_3$ (30 mM in 1.2 M HCl). After the mixture was kept in the dark for 20 min, 300 μl of trichloroacetic acid (10% w/v) was added to precipitate any protein that might be present in the culture media. Subsequently, the mixture was centrifuged at 10,000×g for 10 min. H_2S in the sampled culture media interacts with N,N-dimethyl-p-phenylenediamine sulfate to form methylene blue, and the absorbance of the resulting solution was determined at 670 nm (Yang, et al., 2007). H_2S concentration in the culture media was calculated against the calibration curve of standard H_2S solutions.

WESTERN BLOTS

Whole cell extracts were prepared as described previously (Yang W, et al., 2005). Briefly, cultured cells were harvested and lysed in a lysis buffer (EDTA 0.5 M; Tris-Cl 1 M, pH 7.4; sucrose 0.3 M; antipain hydrochloride 1 μg/ml; benzamidine hydrochloride

hydrate 1 M; leupeptin hemisulphate 1 μg/ml; 1,10-phenanthroline monohydrate 1 M; pepstatin A 1 μM; plenylmethylsulphonyl fluoride 0.1 mM, and iodoacetamide 1 mM). Extracts were separated by centrifugation at 14 000 g for 15 min at 4°C. SDS-PAGE and Western blot analysis were performed as previously described (Yang, et al.,2004a). Briefly, equal amount of proteins were boiled in 1 × SDS sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue) and resolved on a 10% SDS-PAGE gel, and transferred onto nitrocellulose membranes (Pall corporation, Pensacola, US). Dilutions for the primary antibodies were 1: 1000 for CSE, and 1: 5000 for β-actin. HRP-conjugated secondary antibody was used at 1: 5000. Immunoreactions were visualized by enhanced chemiluminescence (ECL) and exposed to X-ray film (Kodak Scientific Imaging film). Membranes were stripped by incubating in a buffer containing 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl (pH 6.8).

CELL VIABILITY ASSAY

MTT method and Trypan blue assay were used to determine cell viability in INS-1E cells and $HepG_2$ cells respectively. MTT assay was employed to determine whether high glucose had cytotoxic effect on INS-1E cells or $HepG_2$ cells. In brief, cells were seeded into 96-well plates at a density of 10^4 cells per well for 24 h. Detailed experimental stages were as follows: 0.5% (5 mg/ml) stock MTT solution was prepared by dissolving 100 mg MTT (Sigma) in 20 ml PBS under a sterile and dark atmosphere. Solution was then filtered with a 0.22-μm filter and debris was removed. This solution could be kept for a month in the dark at 4°C. Culture mediums containing no treatment agents were used as control. After incubation with high or basal glucose RPMI-1640 medium or different doses of H_2S or PPG, 20 μl (5mg/ml) MTT was added to each well

to a final volume of 200 μL. The plates were wrapped by aluminium foil and incubated for 4 h at 37°C incubator. The absorbance of formazan products at 570nm was measured in a FLUOstar OPTIMA microplate spectrophotometer (BMG LABTECH, Offenburg, Germany). The cells incubated with control medium were considered 100% viable.

Trypan blue method was used to detect cell viability of $HepG_2$ cells after infection of adenovirus vectors. Briefly, cells were resuspended in 1 ml PBS (10^5cells/ml). 0.4% trypan blue and cell suspension (dilution of cells) were mixed in 1:1 ratio and incubated for 3 min at room temperature. Around 20 μl of the trypan blue/cell mixture was applied to a hemacytometer to do cell counting. The unstained (viable) and stained (nonviable) cells were counted separately in the hemacytometer to obtain the total number of viable cells. Cell viability was expressed as a percentage of cells excluding trypan blue out of the total cells.

PREPARATION OF INTACT RODENT PANCREATIC ISLETS

Both rat and mice pancreatic islets were isolated in different studies. Male SD rats (8-12w) were received from Charles River Laboratories Canada. (Senneville, QC). These animals were housed at 21 °C with 12-h light/dark cycles. SD rats were anaesthetized by intraperitoneal injection of ketamine (90 mg/kg body weight) and xylazine (50 mg/kg body weight). For mouse studies, 2-3 month-old mice (CSE-WT and KO) were anesthetized with CO_2. The abdominal wall was then opened. The pancreas was distended by injection of 1 ml of Krebs-Ringer Bicarbonate (KRB) solution (PH=7.4) (mM) (129 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 5 $NaHCO_3$, 1.2 $MgCl_2$, 10 HEPES, 2.5 $CaCl_2$) containing 0.5~1 mg/ml Collagenase type V (Sigma, Oakville, Ontario) and 0.5~1 ul/ml DNase I (Sigma, Oakville, Ontario), and BSA (0.5 mg/ml) through a cannula inserted in

the common bile duct. The pancreas was then removed carefully and transferred to a 50 mL Falcon tube with above enzyme solution, and incubated for 5~10 minutes in a water bath at 37°C. Following this digestion procedure, clean islets of Langerhans were hand-picked under a dissect microscope after 3 time centrifuge washout (at 1500 rpm for 3 min at 4°C) with Ca^{2+}/Mg^{2+}-free KRB solution. Isolated islets were cultured in RPMI 1640 media supplemented with 10% FBS and 5 mM glucose at a 37°C humidified mixture of 5% CO_2 and 95% O_2.

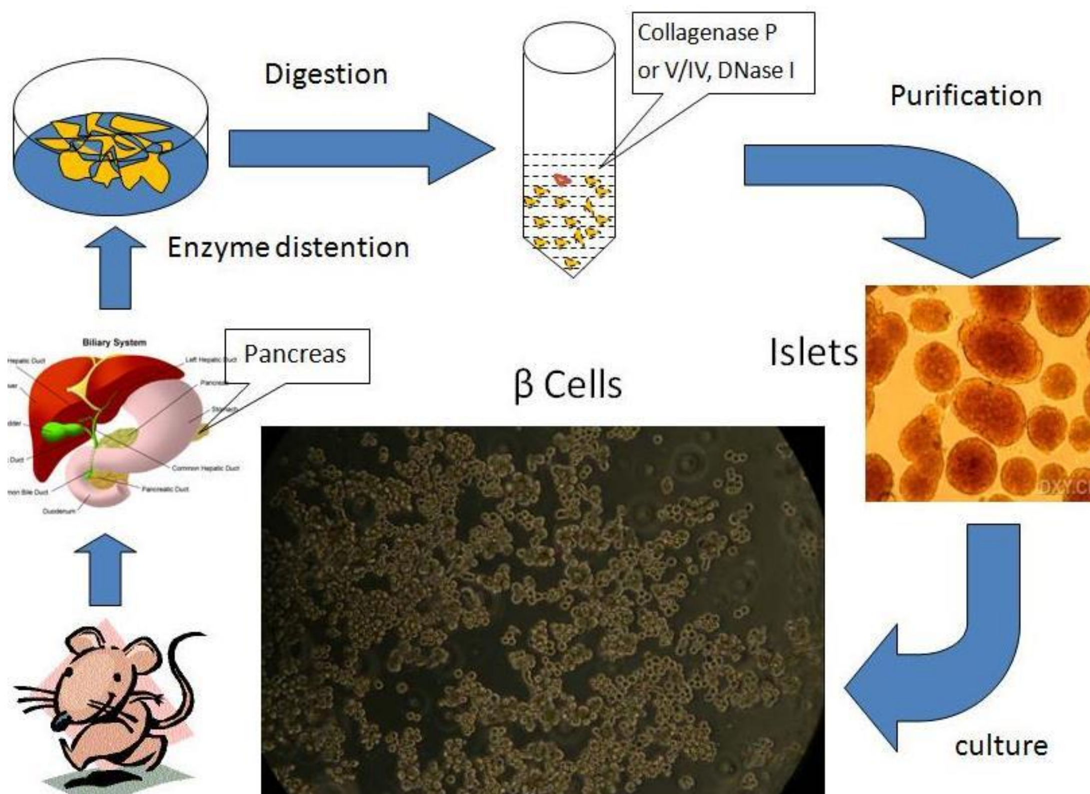


Figure 2-1. Flow chart of the islet isolation experiments

HEPATOCYTE PREPARATION

Hepatocytes were isolated from both CSE KO and WT mice as described before (Klaunig, et al., 1981) with slight modification. In brief, livers of 8-12 week old mice were perfused through the inferior vena cava with a buffer consisting of (in mM) 140 NaCl, 2.6 KCl, 0.28 Na_2HPO_4, 5 glucose, and 10 HEPES (pH 7.4). The perfusion was first for 5 min with the buffer supplemented with 0.5 mM EGTA and then for 10 min with the buffer containing 5 mM $CaCl_2$ and 100 U/ml collagenase type IV (Worthington, Lakewood, NJ). All the solutions were prewarmed at 37°C incubator. The isolated hepatocytes were filtered on nylon mesh (100 μm pore size), and selected by centrifugation in a 36% Percoll isodensity gradient and then seeded at 7.5×10^4 cells/per well in collagen coated 24 well plates in DMEM (5.5 mM glucose) containing 10% fetal bovine serum. The viability of hepatocytes was measured by Trypan blue staining. The viability of all preparations were above 95%.

DETERMINATION OF mRNA LEVEL BY REAL-TIME PCR

INS-1E cells were harvested and the total RNA was isolated using TriReagent. First-strand cDNA was prepared by reverse transcription using M-MuLV reverse transcriptase and random hexamer primers from a ProtoScript II RT-PCR Kit (New England Biolabs, Pickering, ON, Canada) according to the manufacturer's protocol. Control samples containing no reverse transcriptase were used to safeguard for genomic DNA contamination. The primers of rat *Cse* (GenBank accession number AY032875) were 5'-AGCGATCACACCACA-GACCAAG-3'ı (sense, position 432–453) and 5'-ATCAGCACCCAGAGCCAAAGG-3' (antisense, position 589–609). These primers

produced a product of 178 bp. Quantum RNA β-actin internal standards were purchased from Ambion (Foster City, CA, USA), which produce a product of 295 bp. Real-time PCR was performed in an iCycler IQ 5 apparatus (Bio-Rad, Mississauga, ON, Canada) associated with the iCycler optical system software (version 3.1) using SYBR Green PCR Relative mRNA quantification was calculated by using the arithmetic formula "$2^{-\Delta\Delta CT}$" (Yang, et al., 2005; Yang, et al., 2007b), where ΔCT is the difference between the threshold cycle of a given target cDNA and an endogenous reference β-actin cDNA. Based on the calculated ΔCT value, the target mRNA level in the treated group was subsequently expressed as the percentage of that in the control group.

SHORT INTERFERING RNA (siRNA) TRANSFECTION

Pre-designed Sp1-targeted siRNA (Sp1-siRNA) and control siRNA were purchased from Santa Cruz. INS-1E cells were seeded in six-well plates at a density of 1×10^5 cells per well in the presence of 5 mM glucose. Transfection of siRNA into INS-1E cells was achieved using Lipofectamine 2000 Transfection Reagent (Invitrogen, Burlington, ON, USA). Briefly, the cells were transfected with 20 nM Sp1-siRNA or control-siRNA (non-targeting siRNA) in Opti-MEM I culture medium (Invitrogen) without antibiotics for 4 h. Fresh normal growth medium including antibiotics was then added, and the cells were incubated for another 44 h.

MEASUREMENT OF INSULIN SECRETION FROM ISOLATED PANCREATIC ISLETS

For insulin release determination, freshly isolated islets (10 for each batch) were washed and pre-incubated with glucose-free Krebs–Ringer-bicarbonate medium (pH 7.4)

containing (mM): 135 NaCl, 3.6 KCl, 5 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$, 10 Hepes, plus 0.1% BSA in 24-well plates (Yang W, et al., 2005). After 30 min pre-incubation, cells were incubated for 30 min at 37 °C in the presence of different concentrations of tested chemicals (glucose, NaHS, STZ, PPG, sodium pyruvate or 2-deoxy-D-glucose). Where sodium salts of pyruvate were added, the Na^+ content of the KRB was correspondingly decreased. At the end of each incubation period, the medium was collected and centrifuged for 10 min at 1500 rpm to remove islet debris. The supernatant was immediately stored at −20 °C until insulin determination using the mouse insulin ELISA kit (Mercodia AB, Sylveniusgatan, Uppsala, Sweden). After insulin measurement, the islets were scraped from 24 well plates on the ice into the centrifuge tube and then measure protein concentration using Bradford Reagent (Sigma).

ADENOVIRUS-MEDIATED CSE OVEREXPRESSION IN $HEPG_2$ CELLS

Recombinant CSE adenovirus vector (Ad-CSE) was previously constructed in our lab (Yang W, et al., 2005). The recombinant adenovirus encoding bacterial β-galactosidase (Ad-LacZ) derived from the same vector was used as a control. For adenoviral infection, subconfluent $HepG_2$ cells were incubated with Ad-CSE or Ad-LacZ in serum-free media. After 4 h of incubation, medium was removed, and cells were incubated in appropriate media for 48 h. The transfection efficiency of adenoviral vector in $HepG_2$ cells was determined by Western blotting of CSE protein. At MOI (multiplicity of infection) = 100, a great increase of CSE expression was seen in Western blotting without significant loss in cell viability. Subsequent experiments were performed at MOI of 100. To determine the cell viability after CSE overexpression, all cells were collected

by trypsinization followed by centrifugation after transfection. Cell viability was expressed as a percentage of cells excluding trypan blue out of the total cells.

MEASUREMENT OF ENZYME ACTIVITY

PEPCK activity assay

PEPCK activity was determined for cells lysed in a solution containing 50 mM TEA (pH 7.4), 300 mM sucrose, 1 mM EDTA, and 0.1% Triton X-100 using three freeze-thaw cycles and brief sonication. The conversion of oxaloacetate to PEP was monitored by coupling the reaction to the pyruvate kinase (PK)-lactate dehydrogenase (LDH)-coupled enzyme reaction. Briefly, the PEP produced from the PEPCK reaction will be converted to pyruvate by PK, and that pyruvate is reduced by LDH to produce lactate. During the LDH reaction, NADH is converted to NAD^+, resulting in a decrease in the absorbance at 340 nm. Again, 50 µg of cell lysate was added to buffer containing 10 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 20 mM KCl, 5 mM ATP, 10 mM oxaloacetic acid (OAA), 0.18 mM NADH, and 2 U PK and 16 U LDH (both from Sigma). The absorbance at 340 nm was monitored for 15 min at 25°C.

This method has been summarized in the following formula (Figure 2-2).

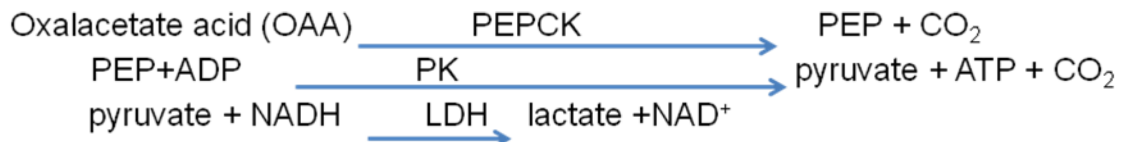


Figure 2-2. Coupled enzyme assay of PEPCK activity

GK activity assay

Enzyme activity of glucokinase (GK) was measured following a protocol on Sigma's website.

http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Enzyme_Assay/glucokinase.Par.0001.File.dat/glucokinase.pdf.

For each assay, enzymatic activity was determined by the conversion of NADH to NAD^+ measured by the change in absorbance at 340 nm over time. Enzyme activity was measured in a multicell spectrophotometer (Fisher Scientific, Ottawa, Ontario) and was expressed as per mili- international unit per minute per milligram (mIU/min/mg) of protein. Results were normalized by protein concentration determined by Bradford method (Sigma).

MATERIALS AND DATA ANALYSIS

Chemicals were all obtained from Sigma (Oakville, ON, Canada) unless otherwise mentioned. Unless otherwise specified, "glucose" refers to D-glucose in this communication. The data are expressed as mean ± SEM from at least three or four independent experiments. Statistical analyses were performed using Student's *t* test on paired data or one-way ANOVA. Statistical significance was considered at $p<0.05$.

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CHAPTER 3

RAT PANCREATIC LEVEL OF CYSTATHIONINE GAMMA-LYASE IS REGULATED BY GLUCOSE LEVEL VIA SP1 PHOSPHORYLATION

Ling Zhang[1], Guangdong Yang[2], Guanghua Tang[3], Lingyun Wu[3], Rui Wang[1*]

[1]Department of Biology, Lakehead University, Thunder Bay, Ontario, Canada

[2] The School of Kinesiology, Lakehead University, Thunder Bay, Ontario, Canada

[3]Department of Pharmacology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

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ABSTRACT

Aims/hypothesis Cystathionine γ-lyase (CSE) catalyses the endogenous production of hydrogen sulfide (H_2S) in pancreatic β cells, and H_2S has been shown to inhibit insulin release from these cells. As altered pancreatic H_2S production modulated by glucose has been previously shown, we hypothesised that the *Cse* gene could be regulated by glucose level in insulin-secreting cells.

Methods The effects of glucose on CSE protein level and mRNA level were analysed in INS-1E cells. Glucose effect on *Cse* promoter activity was tested by constructing a proximal *Cse* promoter vector including specificity protein 1 (*Sp1*) consensus sequence.

Results High glucose (20 mM) inhibited H_2S production in INS-1E cells and freshly isolated rat pancreatic islets. *Cse* mRNA expression, CSE activity and protein abundance were also profoundly reduced by high glucose. The involvement of SP1 in basal and high-glucose-regulated CSE production was demonstrated. *Sp1*-knockdown abolished a large portion of CSE production at basal glucose. Phosphorylation of SP1 stimulated by high glucose was inhibited by p38 mitogen-activated protein kinase (MAPK) inhibitors SB203580 and SB202190. After blocking p38 MAPK phosphorylation, the inhibitive effects of high glucose on CSE protein production and promoter activity in INS-1E cells were also virtually abolished.

Conclusions/interpretation Glucose stimulates the phosphorylation of SP1 via p38 MAPK activation, which leads to decreased *Cse* promoter activity and subsequent downregulation of *Cse* gene expression. Inhibited H_2S production through glucose-mediated CSE activity and production alterations may be involved in the fine control of glucose-induced insulin secretion.

Keywords: CSE, Glucose, H_2S, INS-1E cells, SP1

Abbreviations: CBS, Cystathionine β-synthase; CSE, Cystathionine γ-lyase; K_{ATP} channels, ATP-sensitive K^+ channels; MA, Mithromycin A; MAPK, Mitogen-activated protein kinase; PPG, DL-propargylglycine; SP1, Specificity protein-1

INTRODUCTION

Study of H_2S has gained momentum in recent years as its role as an important gasotransmitter with multifaceted biological significance has become recognised. Gasotransmitters are a family of endogenous gaseous signalling molecules involved in multi-level regulation of physiological and pathological functions (Wang, 2002). Following the identification of nitric oxide (NO) and carbon monoxide (CO) as the first two gasotransmitters, mounting evidence has demonstrated that H_2S is the third one.

H_2S is produced in various types of cells via the enzymatic function of two enzymes: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Wang, 2002). CSE has been shown to be the predominant H_2S-generating enzyme in pancreatic β cells (Yang, et al., 2005) and freshly isolated rat islets (Wu, et al., 2009).

Activation of ATP-sensitive K^+ (K_{ATP}) channels is demonstrated to be responsible for the inhibitory effect of H_2S on glucose-induced insulin release from INS-1E (Yang, et al., 2005) and HIT-T15 cell lines (Ali, et al.,2007). As opening of K_{ATP} channels leads to β cell membrane hyperpolarisation, insulin release from pancreatic islets would be inhibited because of reduced Ca^{2+} influx. Exogenously applied H_2S or overproduction of CSE has been reported to induce apoptosis of INS-1E cells, suggesting an inhibitory

effect of H_2S on insulin production by reduction of β cell mass (Yang, et al., 2005). Glucose level has been seen to regulate various gene expressions in β cells such as *Glut2* (also known as *Slc2a2*) (Waeber, et al., 1996), acetyl-CoA carboxylase (*Acc* [also known as *Acaca*]) (Brun, et al., 1993), and connexin 36 (Allagnat, et al., 2005). Previous research suggested a suppressive role of glucose on endogenous H_2S production in INS-1E cells (Yang, et al., 2005). Considering the critical role of glucose level on gene regulation in pancreatic β cells and the effect of H_2S on insulin secretion, we planned to investigate if the production of CSE is regulated by glucose level in insulin-secreting cells and the underlying mechanism. Our results indicated that CSE production is inhibited by glucose at high concentrations. We further demonstrated that glucose-induced downregulation of CSE production requires SP1 and p38 mitogen-activated protein kinase (MAPK) phosphorylation. The effect of high glucose on CSE production may constitute a novel regulatory mechanism for the fine control of insulin secretion from pancreatic β cells.

METHODS

Cell culture

INS-1E cells were cultured as previously described by Yang *et al*. and used between passages 50 and 80 (Yang, et al., 2005). The experiments were performed when cultured cells reached 70-80% confluence. Before treatment with different concentrations of glucose, INS-1E cells were pre-incubated overnight in RPMI 1640 medium containing 1% FBS and 5 mM glucose at 37°C in a humidified mixture of 5% CO_2 and 95% O_2

(vol./vol.) and then subjected to different concentrations of glucose or other compounds together with 10% FBS.

Preparation of intact islets

Pancreatic islets were isolated from male Sprague Dawley rats (8-12 weeks) by the collagenase digestion method. The solution used for the isolation was KRB supplemented with 0.5–1 mg/ml collagenase type V, 0.5–1 μl/ml DNase I and 0.5 mg/ml bovine serum albumin. 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 Use Committee of Lakehead University, ON, Canada.

Measurement of endogenous H_2S production

H_2S production rate was measured as previously described (Zhao, et al., 2001; Cheng, et al., 2004; Yang, et al., 2008). Briefly, INS-1E cells were incubated with either 5 or 20 mM glucose for 24 h, then collected and lysed in 50 mM ice-cold potassium phosphate buffer (pH 6.8). The cell lysates were first incubated with L-cysteine (10 mM) for 90 min at 37°C, and then trichloroacetic acid was added to stop the reaction. The level of Methylene Blue generated by the interaction of H_2S with *N*,*N*-dimethyl-*p*-phenylenediamine sulfate was determined at 670 nm with a FLUOstar OPTIMA microplate spectrophotometer (BMG LABTECH, Offenburg, Germany). H_2S content in the culture medium was measured as previously described by Yang et al. (Yang, et al., 2007).

Western blots

Cultured cells were harvested and lysed in a Tris-EDTA sucrose lysis buffer plus protease inhibitors as previously described by Yang et al. (Yang, et al., 2005). Protein extracts were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Pall Corporation, Pensacola, FL, USA) (Yang, et al., 2004b). The primary antibodies used were: anti-CSE antibody (Abnova, Taiwan, Republic of China), anti-SP1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho SP1 (threonine 453) (Abcam, Cambridge, MA, USA), anti-phospho-p38 MAPK, anti-p38 MAPK (Cell Signalling Technology, Beverly, MA, USA) and β-actin (Sigma). Immunoreactions were visualised by ECL Western Blotting System (GE Healthcare, Amersham, UK). Densitometric quantification was performed using Alpha Digi Doctor software (Richardson, TX, USA) and normalised against the quantity of β-actin.

Short interfering RNA (siRNA) transfection

Pre-designed *Sp1*-targeted siRNA (*Sp1*-siRNA) and control siRNA were purchased from Santa Cruz. INS-1E cells were seeded in six-well plates at a density of 1×10^5 cells per well in the presence of 5 mM glucose. Transfection of siRNA into INS-1E cells was achieved using Lipofectamine 2000 Transfection Reagent (Invitrogen, Burlington, ON, USA). Briefly, the cells were transfected with 20 nM *Sp1*-siRNA or control-siRNA in Opti-MEM I culture medium (Invitrogen) without antibiotics for 4 h. Fresh normal growth medium was then added, and the cells were incubated for another 44 h.

Determination of mRNA level by real-time PCR

INS-1E cells were harvested and the total RNA was isolated using TriReagent. First-strand cDNA was prepared by reverse transcription using M-MuLV reverse transcriptase and random hexamer primers from a ProtoScript II RT-PCR Kit (New England Biolabs, Pickering, ON, Canada) according to the manufacturer's protocol. The primers of rat *Cse* (GenBank accession number AY032875) were 5'-AGCGATCACACCACA-GACCAAG-3' and 5'-ATCAGCACCCAGAGCCAAAGG-3'. Quantum RNA β-actin internal standards were purchased from Ambion (Foster City, CA, USA). Real-time PCR was performed in an iCycler IQ 5 apparatus (Bio-Rad, Mississauga, ON, Canada) associated with the iCycler optical system software (version 3.1) using SYBR Green PCR Master Mix and relative mRNA quantification was calculated as described previously (Yang, et al., 2005; Yang, et al., 2007b).

Cloning of mouse Cse promoter region and construction of reporter plasmids

The 172 bp genomic DNA fragment upstream from the transcriptional start site (-149 to +23) of the *Cse* gene, containing canonical TATA and CAAT boxes and the SP1 site, was isolated by PCR from mouse-tail genomic DNA (Ishii, et al., 2004). Briefly, the small fragment was amplified using 1.5 μg mouse genomic DNA as template in a 20 μl volume reaction with 5 pmol/μl of each primer. The sequences of the primers used were as follows: F-*Cse*-*Sp1* (KpnI) (-149/-134), 5'-CGGTACCTCTGTGCCACTGGGAG-3'; R-*Cse*-*Sp1* (HindIII) (+7/+23), 5'-GAAGCTTGAGTGCGAGGTGTTGCT-3'. The underlined sequence is the restriction site for KpnI or HindIII. The cloned fragments were subcloned into the promoterless expression vector pGL3 basic (Promega, Madison, WI, USA) to obtain the reporter plasmid pGL3 (-149/+23)-*Cse*-Prom-Luc. Another plasmid was constructed, which was derived from the pGL3 (-149/+23)-*Cse*-Prom-Luc, and

contained a mutated SP1 site. The SP1 response element (5'-GAGGCGGGGC-3') was mutated into (5'-GA*TT*CGGGGC-3') by using QuikChange Site-Directed Mutagenesis Kit (Stratagene, Mississauga, ON), with oligonucleotide (-144 to -117) 5'-GCCACTGGGA*TT*CGGGGCAGGAACGATC-3' and its complementary oligonucleotide according to the manufacturer's recommendations. The mutations were underlined and in italic letters. All plasmid constructs were verified by DNA sequencing.

Transient transfection and luciferase assay

INS-1E cells were transfected with 900 ng of the reporter plasmid pGL3 (-149/+23)-*Cse*-Prom-Luc DNA (*CSE-Sp1* vector), mutated-*Sp1* promoter or pGL3-basic vector, mixed with 100 ng pRL-TK vector (Promega) as an internal control using Lipofectamine 2000. Six hours after the transfection, the OptiMEM medium was replaced by RPMI 1640 medium with 5 mM or 20 mM glucose in the presence or absence of 10 μM p38 MAPK inhibitors SB203580, SB202190 or their non-active analogue SB202474 and incubated for another 24 h before harvesting with passive lysis buffer (Promega). Samples of the lysates were assayed for luciferase activities in a Fluostar Luminometer (BMG LABTECH, Germany) using the Dual-Luciferase Reporter Assay System (Promega). Both firefly and Renilla luciferase activities were measured as luminescence intensities and the promoter activity was expressed as ratios between firefly and Renilla luciferase activities.

Measurement of insulin secretion from INS-1E cells

INS-1E cells were washed and pre-incubated with glucose-free KRB (pH 7.4) plus 0.1% BSA in 24 well plates. After 30 min pre-incubation, cells were incubated for

another 30 min at 37 °C in the presence of different glucose concentrations with or without 5 mM DL-propargylglycine (PPG), 20 mM sodium pyruvate or 20 mM 2-deoxy-D-glucose (2-DG). Where sodium salts of pyruvate were added, the Na^+ content of the KRB was correspondingly decreased. At the end of each incubation period, the medium was collected and immediately stored at −20°C until insulin determination was completed by using the rat insulin ELISA kit (Mercodia AB, Sylveniusgatan, Uppsala, Sweden).

Materials and data analysis

Chemicals were all obtained from Sigma (Oakville, ON, Canada) unless otherwise mentioned. Unless otherwise specified, 'glucose' refers to D-glucose in this communication. The data are expressed as mean ± SEM from at least three independent experiments. Statistical analyses were performed using Student's *t* test on paired data or one-way ANOVA. Statistical significance was considered at $p<0.05$.

RESULTS

High glucose suppressed H_2S release from INS-1E cells and pancreatic islets

High glucose (20 mM) lowered H_2S content of the culture media by 42.1 ± 2.8 % (n=6, p<0.05) for INS-1E cells (Figure 3-1A) and 76.4 ± 1.8 % (n=3, p<0.05) for freshly isolated rat pancreatic islets (Figure 3-1B) compared with that treated with 5 mM glucose. High glucose also decreased H_2S production rate in INS-1E cells (n=4; p<0.05) (Figure 3-1C).

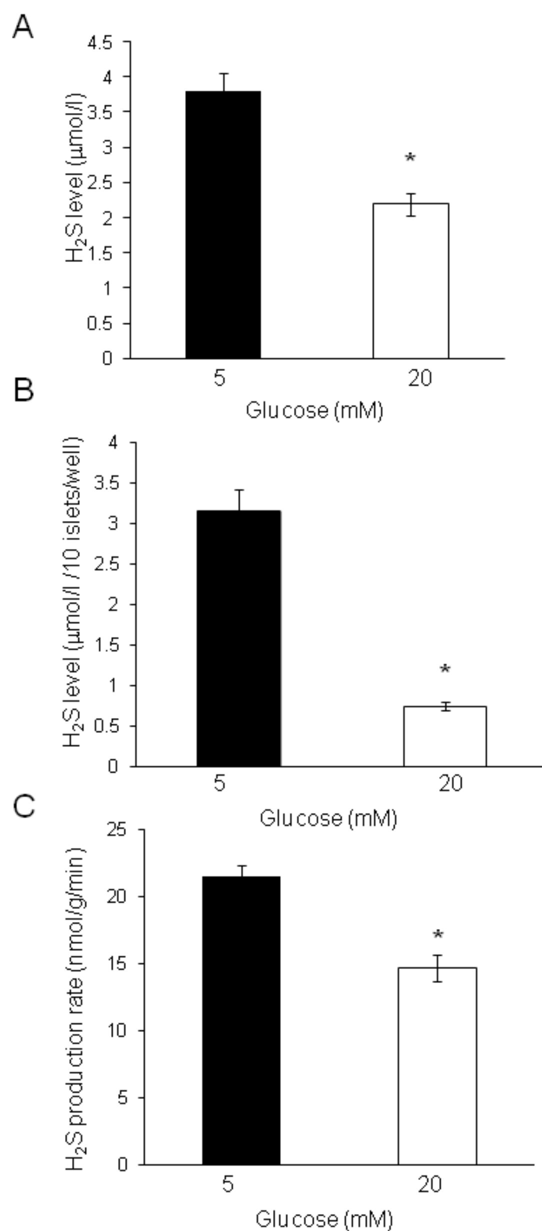


Figure 3-1. High glucose inhibited H_2S production from INS-1E cells and freshly isolated rat islets. INS-1E cells and freshly isolated rat islets were cultured overnight in 5 mM glucose medium with 1% FBS, and then changed to 5 or 20 mM glucose medium with 10% FBS for another 24 h. High glucose significantly inhibited H_2S production in the culture medium for INS-1E cells (**A**) and pancreatic rat islets (**B**). H_2S production rate in INS-1E cells (**C**) was also significantly decreased by high glucose. Data in (**A**) and (**B**) were from at least three independent experiments; data in (**C**) were from four independent experiments. $^{*}p<0.05$

High glucose decreased Cse mRNA expression and CSE protein production in INS-1E cells

After incubation with high glucose for 24 h, CSE protein production was significantly decreased (Figure 3-2A). *Cse* mRNA level in INS-1E cells treated with 20 mM glucose was 36.5 ± 9.9% of that with 5 mM glucose (Figure 3-2B). To test if the decreased *Cse* gene expression occurred at the transcriptional level, INS-1E cells pre-incubated for 24 h in 5 or 20 mM glucose medium were then exposed to actinomycin D (5 μM) for 0.5 or 2 h (Figure 3-2C). Inhibition of RNA synthesis with actinomycin D in the presence of 20 mM glucose did not lead to further reduction in the *Cse* mRNA level compared with 5 mM glucose incubation, suggesting that glucose may not affect *Cse* mRNA stability. Therefore, it repressed the expression of *Cse* mRNA at the level of transcription.

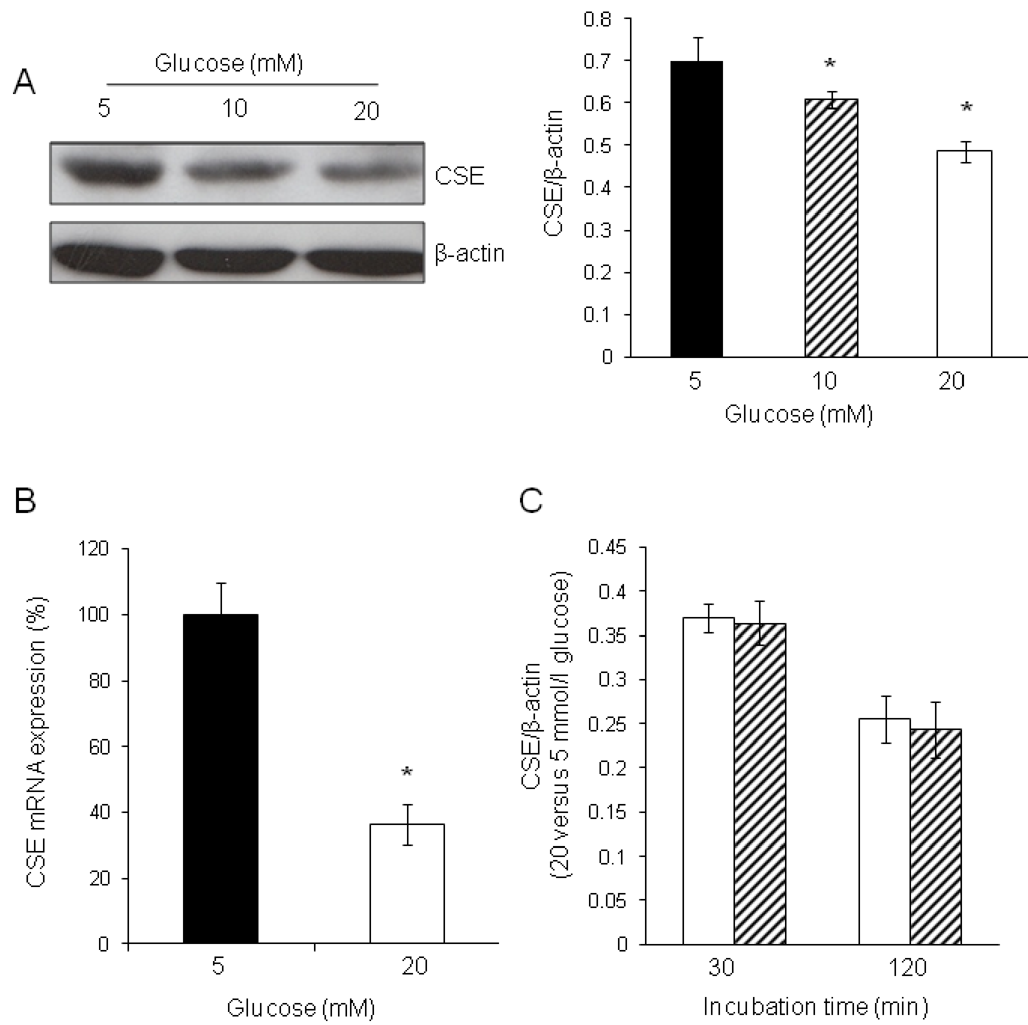


Figure 3-2. High glucose downregulated *Cse* mRNA and CSE protein expression in INS-1E cells. After INS-1E cells were incubated with the indicated concentration of glucose for 24 h, the cells were collected and subjected to western blots (**A**) and real-time PCR (**B**) analyses. (**C**) Glucose had no effect on the stability of the *Cse* transcript. INS-1E cells were pre-incubated with 5 or 20 mM glucose for 24 h, and then treated in the absence (white bars) or presence of 5 μM actinomycin D (shaded bars) for 0.5 or 2 h. The data were normalised to β-actin production and are presented relative to production at 5 mM glucose. In (**A**) and (**B**) the data are from four independent experiments; in (**C**) the data are from three independent experiments. $^{*}p$<0.05

Effects of pharmacological treatments on the expression of CSE

Western blots results showed that 20 mM 2-DG mimicked the inhibitive effect of 20 mM glucose on CSE production in INS-1E cells (Figure 3-3A) and rat islets (Figure 3-3B). Pyruvate at 20 mM significantly decreased CSE production in both INS-1E cells (Figure 3-3A) and isolated rat islets (Figure 3-3B), whereas 20 mM L-glucose and mannitol were ineffective. To explore whether β cell depolarisation affects glucose-induced downregulation of CSE production, INS-1E cells were incubated with 5 mM glucose in the presence of KCl (20 mM). KCl treatment did not alter CSE production (Figure 3-3A).

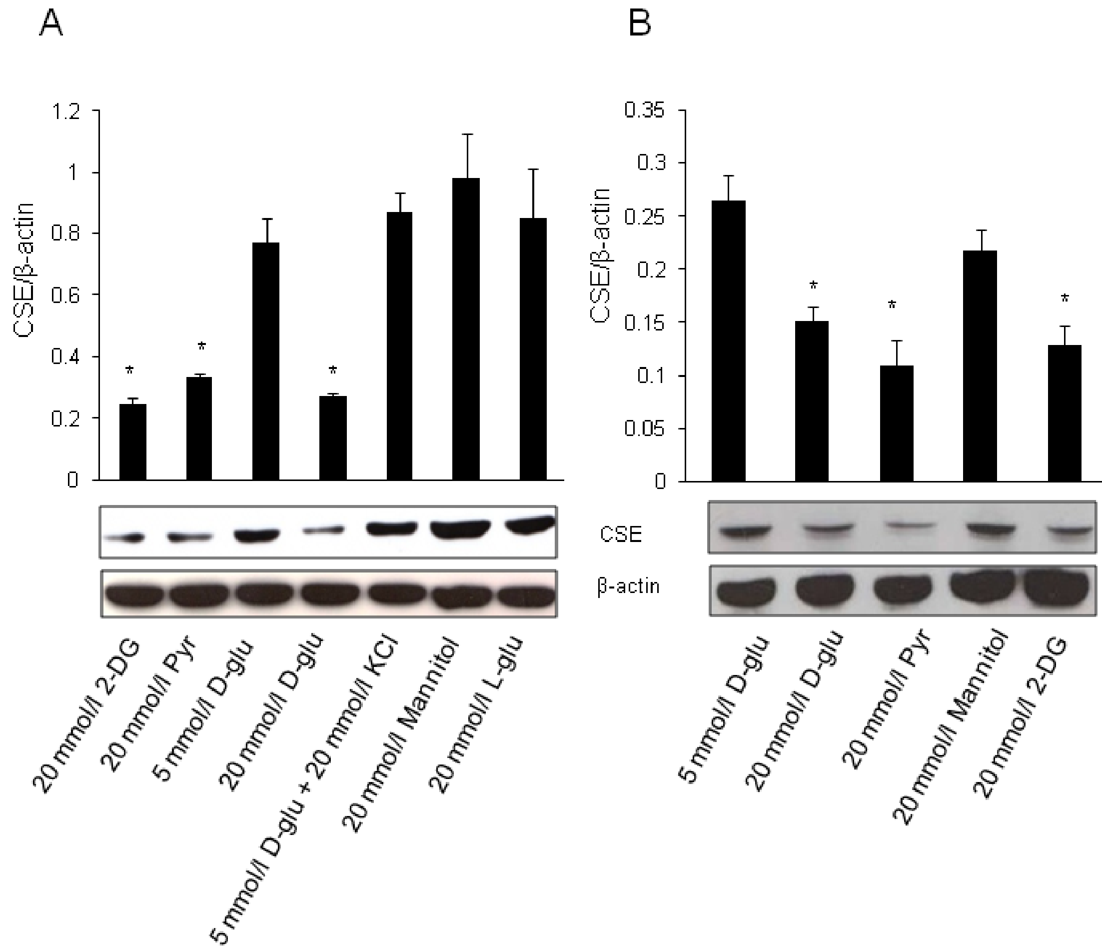


Figure 3-3. Effect of pharmacological treatments on the expression of CSE. INS-1E cells (**A**) and pancreatic rat islets (**B**) were incubated with 5 or 20 mM D-glucose, 20 mM pyruvate, 20 mM 2-DG, 20 mM KCl in the presence of 5 mM D-glucose, 20 mM mannitol or 20 mM L-glucose for 24 h, respectively. After that, the cells or islets were collected and subjected to western blot analysis. The data are from three independent experiments. *p<0.05 vs 5 mM D-glucose. Glu, glucose; Pyr, pyruvate

High-glucose-modulated CSE expression requires the phosphorylation of p38 MAPK and SP1

The phosphorylation of p38 MAPK in INS-1E cells was evident within 15 min of high glucose treatment and peaked at 1 h (Figure 3-4A). High-glucose-induced phosphorylation of SP1 protein without changing the amount of total SP1 protein (Figure 3-4B). SB203580 and SB202190 (p38 MAPK inhibitor) but not SB202474 at 10 μM abolished the inhibitive effect of high glucose on CSE production. Moreover, inhibition of the SP1 binding to the GC boxes of promoter by 100 nM mithromycin A (MA) induced a greater decrease of CSE production (Figure 3-4C).

Figure 3-4. Glucose-stimulated phosphorylation of p38 MAPK and SP1. (**A**) Glucose induced phosphorylation of p38 MAPK. INS-1E cells were incubated with glucose at 5 mM or 20 mM for the indicated time. After that, the cells were collected and subjected to western blots analysis. $^{*}p<0.05$ vs 5 mM glucose treatment group at the same time point. (**B**) Glucose stimulated SP1 phosphorylation. INS-1E cells were pretreated with 5 mM glucose RPMI-1640 medium containing 1% FBS overnight and then incubated with 5 mM or 20 mM glucose for 24 h. After that, the cells were collected and subjected to western blot analysis. $^{*}p<0.05$. (**C**) The p38 MAPK-SP1 pathway mediated the effect of glucose on CSE production. INS-1E cells were pretreated with or without SB203580, SB202190 or SB202474 (control reagent) for 1 h, and then incubated with glucose at 5 mM or 20 mM for 24 h in the presence or absence of 100 nM MA. $^{*}p<0.01$ vs 5 mM glucose alone group; $^{\#}p<0.01$ vs 5 mM glucose with SB202474 group. The data are from three independent experiments

Role of p38 MAPK in glucose-induced phosphorylation of SP1

To confirm the observation that p38 MAPK is involved in SP1-mediated glucose deactivation of CSE, we examined the effects of p38 MAPK inhibitors on glucose-induced change in production of phospho and total SP1. Pretreatment with two specific p38 MAPK inhibitors, SB203580 and SB202190 (both 10 μM), drastically decreased glucose-induced phosphorylation of SP1 without changing the amount of total SP1 compared with the control reagent SB202474, DMSO (1%) or high glucose alone (Figure 3-5A). Furthermore, the effects of *Sp1*-specific siRNA on *Cse* expression were determined at protein levels (Figure 3-5B). Transfection of *Sp1*-specific siRNA significantly reduced CSE production. These results indicated an important role of SP1 in CSE production.

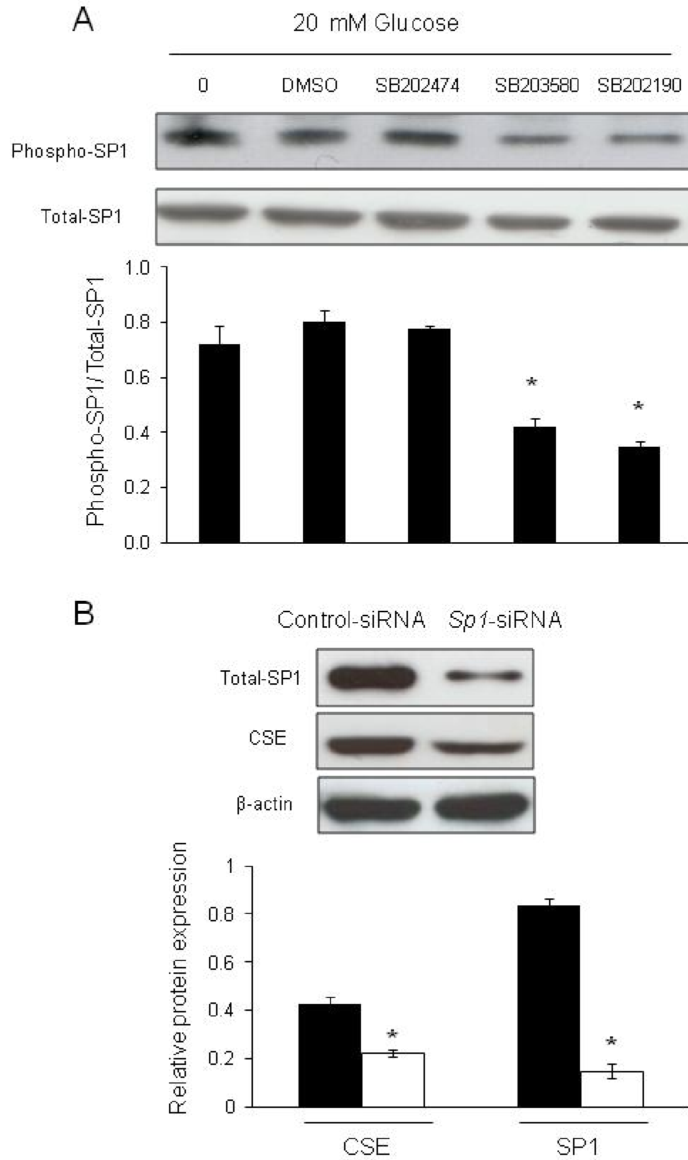


Figure 3-5. Role of SP1 in glucose-induced regulation of CSE. INS-1E cells were cultured in 5 mM glucose RPMI-1640 medium containing 1% FBS overnight, and were changed on the next day to fresh medium (5 mM glucose), pretreated with or without 0.1% DMSO (vehicle), 10 μM SB202474, SB203580 or SB202190 for 1 h, respectively, and then incubated with 20 mM glucose for an additional 24 h. Production of SP1 and phosphorylated SP1 was detected by western blot using specific antibodies. (**A)** p38 MAPK inhibitors SB203580 and SB202190 but not non-active analogue SB202474 decreases high-glucose-induced SP1 phosphorylation in INS-1E cells without changing total SP1 production. $^{*}p<0.05$ vs control at 20 mM glucose alone. (**B**) INS-1E cells were transfected with either control siRNA (black bars) or *Sp1*-siRNA (white bars) for 48 h at 5 mM glucose RPMI-1640 medium. Western blot analysis showed successful *Sp1* knockdown and reduced CSE production in INS-1E cell lines. The data were normalised and are presented as ratio to β-actin production. The data are from three independent experiments. $^{*}p<0.05$

p38 MAPK-SP1 signalling pathway mediated high-glucose-induced decrease of Cse promoter activity

Transfection of INS-1E cells with *Cse* core promoter (*Cse-Sp1* promoter) resulted in a 39-fold increase in luciferase activation in the presence of 5 mM glucose (n=5; *p<0.01 vs cells transfected with the promoterless pGL3 basic vector). However, *Cse-Sp1* promoter activity was inhibited significantly by high glucose (20 mM).

Mutating the consensus SP1 binding site completely abolished *Cse* core promoter (*Cse-Sp1* promoter) activity (Figure 3-6A). By inhibiting p38 MAPK, SB202190 and SB203580 completely abolished the inhibitory effect of 20 mM glucose on *Cse* promoter activity in the presence or absence of 10 μM SB202474 (Figure3-6B).

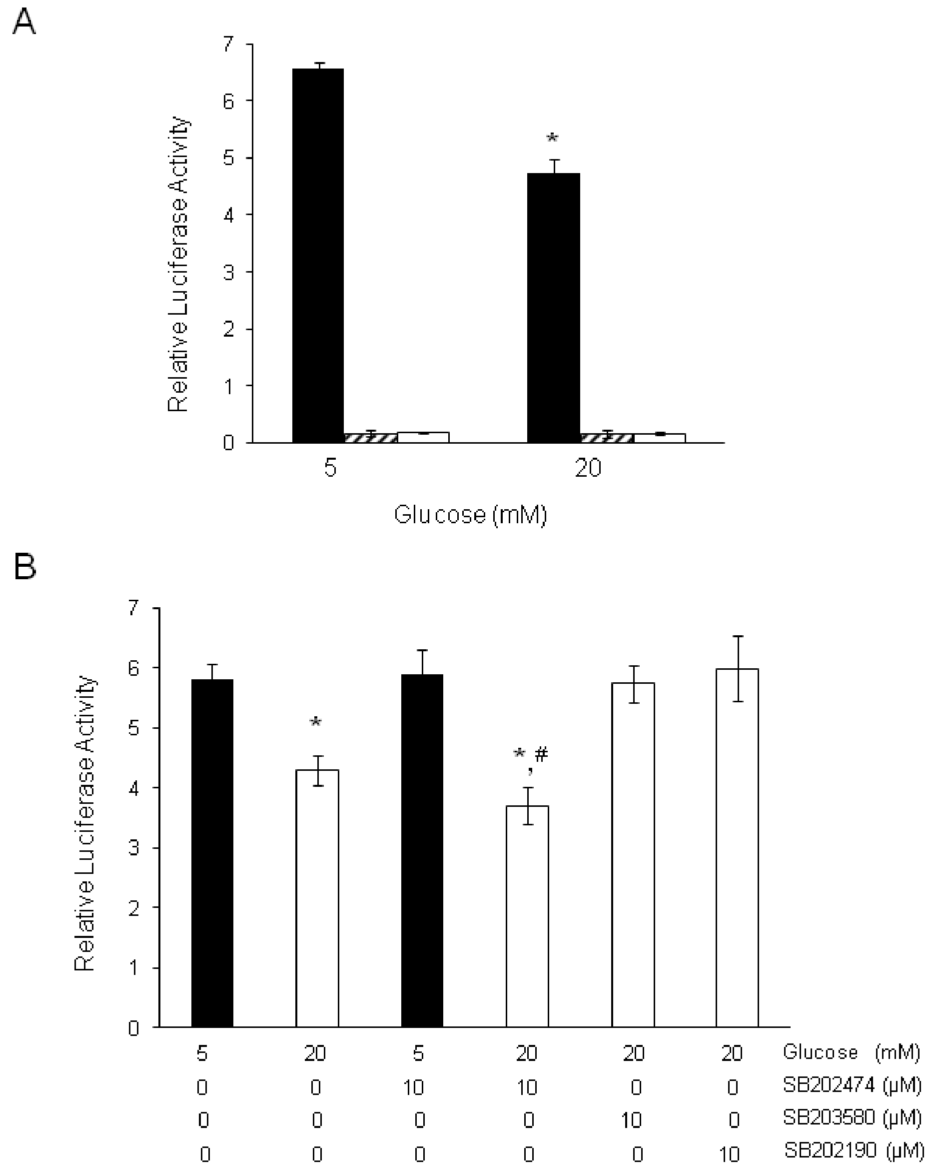
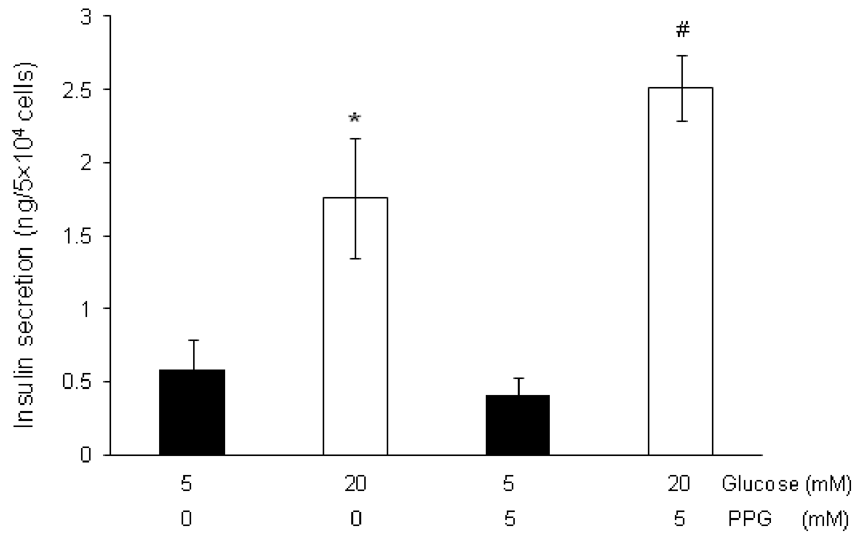


Figure 3-6. Effect of glucose on mouse CSE promoter activity. (A) High glucose attenuated CSE promoter activity. INS-1E cells were co-transfected with reporter plasmids *Cse-Sp1* vector (black bars), pGL3-basic vector (shaded bars) or mutated-*Sp1* construct (white bars) together with pRL-TK vector for 6 h, then incubated at 5 or 20 mM glucose RPMI-1640 medium for an additional 24 h. Relative luciferase activity was normalised with Renilla luciferase from pRL-TK vector. The data were from five independent experiments. $^{*}p<0.01$ vs 5 mM glucose alone group. (B) SB203580 and SB202190 but not SB202474 reversed glucose-reduced CSE promoter activity. INS-1E cells were transfected with *Cse-Sp1* vector in the presence or absence of the indicated concentration of glucose or inhibitors for 24 h. Relative luciferase activity was normalised with Renilla luciferase from pRL-TK vector. The data were from four independent experiments. $^{*}p<0.01$ vs 5 mM glucose alone group; $^{\#}p<0.01$ vs 5 mM glucose plus SB202474 group

PPG, but not pyruvate or 2-DG, enhances high glucose-induced insulin secretion

High glucose (20 mM) stimulated insulin secretion from INS-1E cells by approximately threefold compared with the basal glucose (5 mM). After treatment with 5 mM PPG to inhibit CSE activity, insulin secretion at basal glucose was not altered. However, high-glucose-stimulated insulin secretion was slightly but significantly increased (Figure 3-7A). Conditions of 20 mM 2-DG alone or in the presence of 5 mM glucose did not stimulate insulin secretion. However, in the presence of 20 mM glucose, 2-DG significantly inhibited glucose-induced insulin secretion. Pyruvate (20 mM) had no effect on insulin release in the presence or absence of glucose within 30 min of application (Figure 3-7B).

A



B

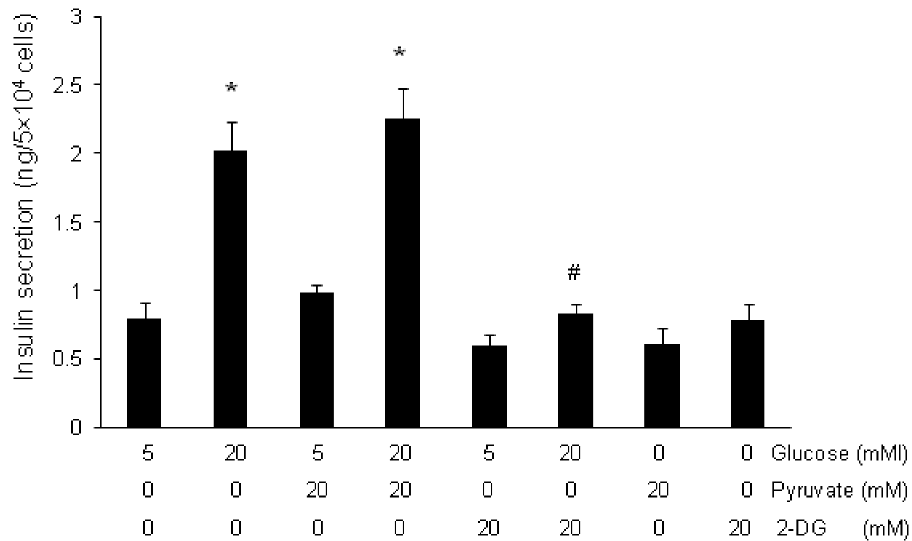


Figure 3-7. Inhibition of CSE activation enhanced glucose-induced insulin secretion. After 30 min pre-incubation with glucose-free KRB, INS-1E cells were incubated for another 30 min at 37°C in the presence of different glucose concentrations with or without 5 mM PPG (**A**), 20 mM pyruvate or 2-DG (**B**). Insulin level in the culture media was measured by ELISA. The data were from four independent experiments. $^{*}p<0.05$ vs 5 mM glucose alone group. $^{\#}p<0.05$ vs 20 mM glucose alone group

DISCUSSION

Endogenous H_2S level in pancreatic beta cells plays a critical role in regulating insulin release. H_2S has been shown to inhibit insulin secretion from insulin-secreting cell lines (INS-1E, MIN6 and HIT-T15) and isolated islets (Yang, et al., 2005; Ali, et al., 2007; Kaneko, et al., 2006; Yusuf, et al., 2005). H_2S has also been linked to glucose metabolism and insulin resistance by interaction with methylglyoxal, an intermediate of glucose metabolism (Chang, et al., 2010) and by inhibiting basal and insulin-stimulated glucose uptake in adipocytes (Feng, et al., 2009).

Pancreatic production of H_2S is regulated by CSE and/or CBS (Yang, et al., 2005; Ali, et al., 2007; Kaneko, et al., 2006; Yusuf, et al., 2005). *Cse* gene knockdown by *Cse*-siRNA largely eliminated H_2S production from INS-1E cells (Yang, et al., 2005). We reported significant production levels of CSE in rat pancreatic islets, but *Cbs* mRNA expression was extremely low (Wu, et al., 2009). PPG, as a specific CSE inhibitor, drastically reduced H_2S production rate to near zero in pancreatic islets from both Zucker diabetic fatty and Zucker fatty rats. Taken these together, it is believed that CSE is the main enzyme for H_2S production in rat pancreatic beta cells and the INS-1E cell line.

High glucose is capable of regulating the expression of many genes in pancreatic beta cells (Schuit, et al., 2002). For example, *Glut2* (Waeber, et al., 1996) and *Acc* (Brun, et al., 1993) were upregulated by glucose, whereas *Cx36* (also known as *Gjd2*) (Allagnat, et al., 2005), *Pparα* (also known as *Ppara*) (Roduit, et al., 2000) and those encoding the sulfonylurea receptor 1/inwardly rectifying K^+ channel 6.2 (*Sur*/*Kir6.2* [also known as *Abcc8*/*Kcnj11*]) (Moritz, et al., 2001) were downregulated by high glucose. We have previously demonstrated that endogenous H_2S production rate in INS-1E cells was

modulated by different glucose levels (Yang, et al., 2005). The underlying molecular mechanism for the interaction of high glucose and H_2S production, however, had been unclear. In this study, we found that high glucose inhibited CSE activity represented by decreased H_2S production rate within 2 h (data not shown). Also for the first time, we demonstrated CSE production was repressed by high glucose. Therefore, high glucose would both downregulate CSE production and inhibit CSE activity. The latter may be a relatively rapid mechanism for mediating glucose-induced insulin secretion.

We further investigated whether this inhibitory effect of glucose on CSE production is specifically related to cellular metabolism and use of glucose. L-glucose, an analogue of glucose that cannot enter beta cell (Vanderford, et al., 2007), had no effect on CSE protein abundance. The inhibitory effect of 20 mM glucose on CSE production is also unlikely to be due to osmolality change as 20 mM mannitol did not alter CSE production. Another analogue of glucose, 2-DG, can be taken up by beta cells through GLUT2 transporters and phosphorylated by glucokinase but cannot be metabolised beyond the level of glucose 6-phosphate in the glycolytic or pentose-phosphate pathway (Girad, et al., 1997). Interestingly, 2-DG inhibited CSE production (Figure 3). This phenomenon suggests that glucose 6-phosphate may be key to the glucose-induced downregulation of CSE production. Brun et al. (Brun, et al., 1993) reported previously that a wide variety of metabolisable nutrients were incapable of inducing *Acc* mRNA in the INS-1 cell line. However, 2-DG was capable of inducing *Acc* mRNA, suggesting that glucose does not have to be metabolised beyond glucose 6-phosphate in the glycolytic pathway to induce production of this gene product. Further studies are merited to determine the direct effect of glucose 6-phosphate on CSE production to confirm this

hypothesis. Pyruvate also significantly decreased CSE protein production even more than high glucose did in rat islets (Figure 3-3B). This result indicates that pyruvate, a product of a CSE-catalysed reaction, may inhibit CSE production as a product-based feedback control mechanism.

As 2-DG and pyruvate decreased the production of CSE, it would be expected that the consequently lowered endogenous H_2S level might promote insulin release, the same effect as offered by PPG (Figure 3-7A). However, as the application of 2-DG and pyruvate in the insulin release experiments was limited to 30 min, CSE protein level would not be changed within this short period of time. We also found that 2-DG and pyruvate alone had no effect on H_2S production rate in INS-1E cells, which reflects CSE activity (data not shown). Taken together, the effects of 2-DG and pyruvate on insulin release (Figure 3-7B), if any, could not be ascribed to changed CSE production or activity. Indeed, 20 mM 2-DG significantly inhibited the insulin release stimulated by 20 mM glucose. Similar results had been reported previously that 2-DG at 17 mM decreased insulin release from rat pancreas (Gagliardino, et al., 1966). The underlying mechanism for this interaction of 2-DG and glucose may be related to the competition of 2-DG with glucose for transportation into the cells (Kipnis, et al., 1959) and for reaction with glucokinase or hexokinase within the cell (Nakada, et al., 1956; Landau, et al., 1958; Wick, et al., 1957).

Glucose metabolism stimulates insulin secretion by closing K_{ATP} channels and subsequent calcium influx, eventually leading to exocytosis of insulin granules. To examine if glucose-induced depolarisation of beta cells is responsible for CSE downregulation, INS-1E cells were treated with 20 mM KCl in the presence of basal

glucose. This treatment did not alter CSE production (Figure 3-3A). Similar results were obtained from freshly isolated islets under the same treatments (Figure 3-3B). These results suggested that Ca^{2+} influx caused insulin secretion was not implicated in glucose-repressed CSE production.

The human *CSE* and rodent *Cse* genes have been cloned before (Ishii, et al., 2004; Lu, et al., 1992). The core promoter of mouse *Cse* contains several putative transcriptional factor-binding sites, including myeloid zinc finger protein 1 (MZF-1) and SP1 (Ishii, et al., 2004). Deletion of *Mzf*-1 (in Cos-7 and HEK-293 cells) or *Sp1* consensus sequence (in HEK-293 cells) from *Cse* promoter significantly decreased the promoter activity, suggesting the involvement of these factors in the basal transcriptional activity of *Cse*. Bioinformatics analysis of the promoter region of the mouse and rat *Cse* also revealed the presence of a potential *Sp1* consensus sequence (5'-GAGGCGGGGC-3') located within the -149/+23 region of the mouse *Cse* promoter and at -182/-173 region in the rat *Cse* promoter. Previous investigation found (-137 to +18) sequence conferred the highest promoter activity in HEK-293 cells using the promoter deletion and mutation method (Ishii, et al., 2004). Given that the use of mouse *Cse* promoter has been examined thoroughly, and SP1 is a ubiquitous transcription factor abundantly expressed in different species with a highly conservative consensus sequence of ‘GAGGCGGGGC’, we generated a pGL3 (-149/+23)-*Cse*-promoter expression vector (*Cse-Sp1* vector) cloned upstream to luciferase and used it to transfect INS-1E cells. We demonstrated that SP1 is a crucial transactivator for *Cse* gene expression because transfection of INS-1E cells with this *Cse* core promoter containing the *Sp1* consensus sequence (*Cse-Sp1* vector) resulted in a 39-fold increase in luciferase activity compared with promoterless vector (Figure 3-

6A). MA binds to GC-rich regions in chromatin and interferes with the transcription of genes bearing GC-rich motifs in their promoter and also known as a major SP1 inhibitor, as SP1 recognises GC-rich sequences (Blume, et al., 1991). It drastically decreased CSE production in the presence of either 5 mM or 20 mM glucose (Figure 3-4C). *Sp1* knockdown also abolished a large portion of CSE protein production (Figure 3-5B), suggesting a critical role of SP1 in CSE production. A single *Sp1* site mutation of *Cse-Sp1* vector completely abolished the promoter activity of *Cse* (Figure 3-5A), again suggesting the importance of *Sp1* in the basal transcriptional activity of *Cse*. High-glucose treatment significantly attenuated SP1 transactivation, which provided evidence that a glucose-responsive element is located within the proximal portion of the mouse *Cse* promoter (Figure 3-6 A, B).

Several reports have suggested the ubiquitously produced transcription factor SP1 may provide a mechanism for glucose responsiveness (Li, et al., 2008; Schäfer, et al., 1997). Changes in SP1 phosphorylation result in either facilitation or suppression of DNA binding, promoter activation (Schäfer, et al., 1997; Rohlff, et al., 1997; Merchant, et al.,1999) and gene transcription (Lam, et al.,2003; Ge, et al., 2003; Ko, et al., 2003). SP1 protein can be phosphorylated at various sites by different kinases including protein kinase A, protein kinase C, MAPK, casein kinases 1 and 2, and calmodulin kinases (Chu, et al., 2003). It was previously reported that glucose can de-phosphorylate SP1 and increase promoter binding activity on the *Acc* gene in adipose tissue (Daniel, et al., 1996). The phosphorylation status of SP1 appears to be cell- and gene-type dependent, which is also a major factor in mediating the effects of extracellular stimuli such as insulin (Samson, et al., 2002).

In the present study, we examined possible signal-transduction pathways related to SP1 phosphorylation in high-glucose-induced downregulation of *Cse* expression. Exposure to high glucose has been reported to lead to the activation of various MAPK cascades in pancreatic beta cell lines (Frodin, et al., 1995; Khoo, et al., 1997). While p38 MAPK phosphorylation became significant 15 min after high-glucose treatment (Figure 3-4A), the decreased *Cse* mRNA and protein levels were detected 24 h later. Whether the downregulation of *Cse* mRNA expression actually occurred immediately after p38 MAPK phosphorylation was not tested. It is reasoned that, however, p38 MAPK phosphorylation functions as a trigger and the decreased CSE production followed. This effect of high glucose is relatively specific as no significant difference was found in phospho-p44/42 MAPK level between 5 mM and 20 mM glucose treatments (data not shown). Inhibition of p38 MAPK phosphorylation by SB203580 or SB202190 at 20 mM glucose significantly decreased SP1 phosphorylation without changing the amount of total SP1 protein (Figure 3-5A), indicating that high glucose phosphorylates the existing SP1 protein through p38 MAPK. SB203580 and SB202190 but not SB202474 also completely reversed the inhibitory effect of high glucose on CSE production (Figure 3-4C). Moreover, inhibition of SP1 phosphorylation by SB203580 or SB202190 abolished the inhibitive effect of glucose to CSE promoter activity (Figure 3-6B). These novel observations suggest that high glucose increases phosphorylation of SP1 via p38 MAPK activation in INS-1E cells, resulting in a decreased CSE promoter activity and reduced *Cse* transcription.

Kaneko et al. (Kaneko, et al., 2009) reported glucose-induced H_2S production by observing stimulated CSE production after 18 h incubation with high glucose in mouse

islets and MIN6 cells. The discrepancies between the present study and the findings of Kaneko et al. may be resolved by considering that H_2S level is not directly assayed in their study and glucose level in the pre-incubation before treatment was not indicated. The discrepancy may also be related to different responses of different cell types or animal species to H_2S and other experimental conditions. The reported effects of CSE/H_2S on the survival/apoptosis of pancreatic beta cells are not consistent either (Yang, et al., 2007; Kaneko, et al., 2009). We did not find any reduced cell viability after incubation with high glucose for 24 h compared with basal glucose (data not shown). Our observation was supported by another study in which high glucose promoted pancreatic islet beta cell survival through phosphoinositide-3-kinase/Akt signalling at 24 h (Srinivasan, et al., 2002).

Our study did not directly test whether the phosphorylation of SP1 at the Thr453 leads to reduced SP-1 binding to the *Cse* promoter region. A reduced transcriptional or promoter activity is not always accompanied by reduced DNA binding (Chu, et al., 2005). Transcription activity could be regulated through changes in the interaction between SP1 and other regulatory factors as a result of SP1 phosphorylation. Thus, altered *Cse* promoter activity associated with SP1 phosphorylation might be related to other transcription factors, whose activity has been modified via the phosphorylation of SP1.

In conclusion, we report a novel transcriptional mechanism of glucose-induced downregulation of CSE in which SP1 phosphorylation by p38 MAPK acts as one molecular link between glucose level and CSE production as outlined in Figure 3-8. A delicate interplay between glucose level and CSE/H_2S in pancreatic beta cells would

potentially underlie the regulation of glucose metabolism and insulin secretion under both physiological and pathological situations.

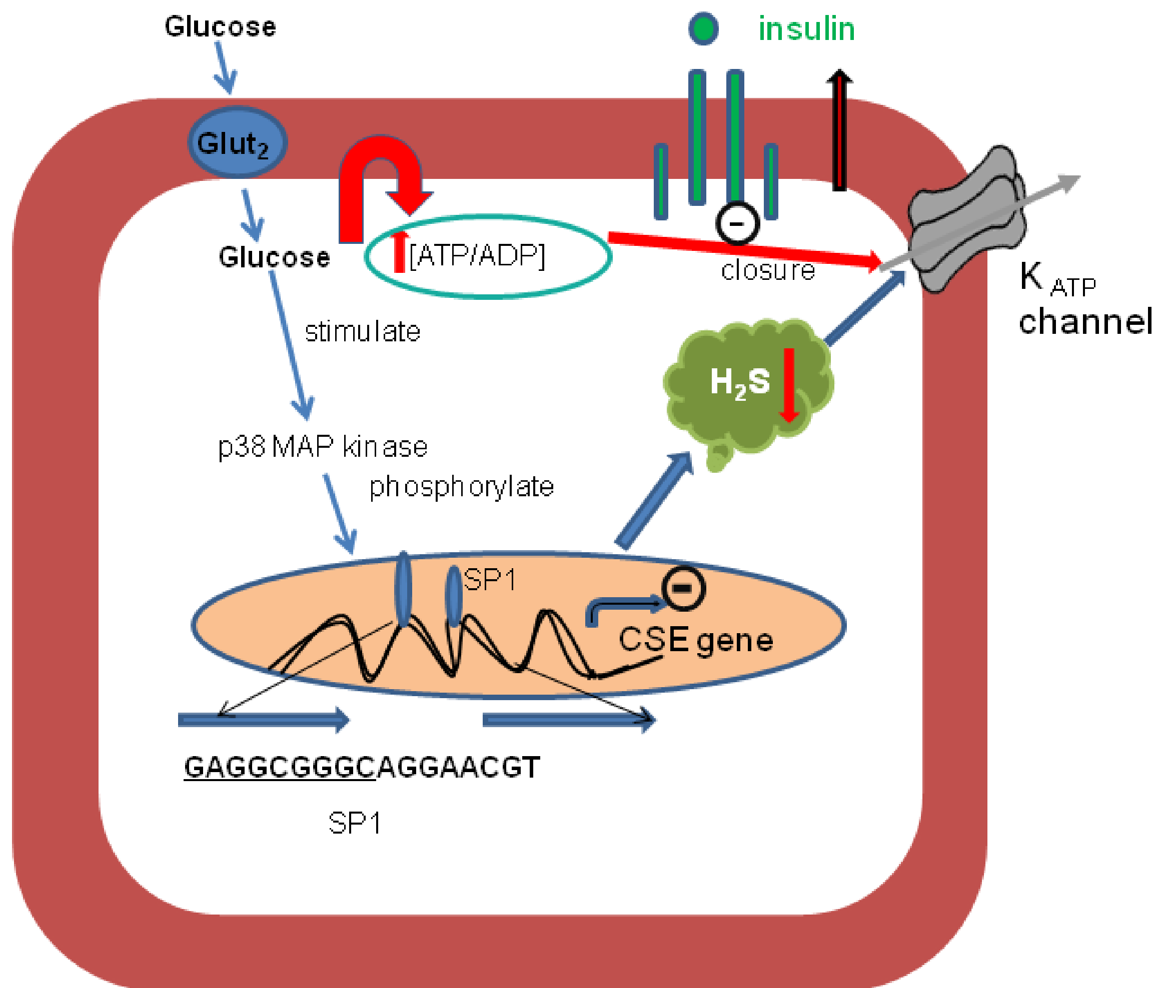


Figure 3-8. Schematic signal transduction pathways underlying glucose-repressed CSE production

CHAPTER 4

HYDROGEN SULFIDE IMPAIRS GLUCOSE UTILIZATION AND INCREASES GLUCONEOGENESIS IN HEPATOCYTES

Ling Zhang[1], Guangdong Yang[2], Ashley Untereiner[1], Youngjun Ju[1], Lingyun Wu[3], Rui Wang[1*]

[1]Department of Biology, Lakehead University, Thunder Bay, Ontario, Canada

[2] The School of Kinesiology, Lakehead University, Thunder Bay, Ontario, Canada

[3] Department of Health Sciences, Lakehead University, Thunder Bay, Ontario, Canada

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ABSTRACT

Mounting evidence has established hydrogen sulfide (H_2S) as an important gasotransmitter with multifaceted physiological functions. The aim of the present study was to investigate the role of H_2S on glucose utilization, glycogen synthesis, as well as gluconeogenesis in both $HepG_2$ cells and primary mouse hepatocytes. Incubation with NaHS (a H_2S donor) impaired glucose uptake and glycogen storage in $HepG_2$ cells via decreasing glucokinase activity. Adenovirus-mediated cystathionine gamma-lyase (CSE) overexpression increased endogenous H_2S production and lowered glycogen content in $HepG_2$ cells. Glycogen content was significantly higher in liver tissues from CSE knockout (KO) mice compared to that from wild type (WT) mice in fed condition. Glucose consumption was less in primarily cultured hepatocytes isolated from WT mice than those from CSE KO mice, but more glucose was produced by hepatocytes via gluconeogenesis and glycogenolysis pathways in WT mice than in CSE KO mice. NaHS treatment reduced the phosphorylation of AMPK, whereas stimulation of AMPK by 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside reversed H_2S-impaired glucose uptake. H_2S increased glucose production was likely through increased phosphoenolpyruvate carboxykinase (PEPCK) activity. In addition, insulin at the physiological range inhibited CSE expression, and H_2S decreased insulin-stimulated phosphorylation of Akt in $HepG_2$ cells. CSE expression was, however, increased in insulin resistant state induced by exposing cells to high levels of insulin (500 nM) and glucose (33 mM) for 24 hours. Taken together, these data suggest that the interaction of H_2S and insulin in liver plays a pivotal role in regulating insulin sensitivity and glucose metabolism.

Key words: AMPK; Akt; CSE; Gluconeogenesis; Glycogenolysis; H_2S; $HepG_2$ cells; Insulin resistance.

Abbreviations

2-NBDG,2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose;
AICAR, 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside;
AMPK, 5'-AMP-activated protein kinase;
CSE, cystathionine gamma-lyase;
Dex, dexamethasone;
GK, glucokinase;
IR, insulin resistance;
KO, knockout;
PEPCK, phosphoenolpyruvate carboxykinase;
PPG, DL-propargylglycine;
WT, wild-type.

INTRODUCTION

The liver has a central role in maintaining glucose homeostasis. When plasma glucose and insulin levels are high, the liver takes up glucose, replenishes depleted glycogen stores, and synthesizes fatty acids (Pilkis, et al., 1992). Type 2 diabetes is associated with the inability of hepatic control of glucose homeostasis. The defects of insulin to trigger downstream actions in liver are defined as hepatic insulin resistance, including impaired glycogen synthesis, and failure to suppress endogenous glucose production (Kim, et al., 2003).

H_2S, a gasotransmitter, has been implicated in the pathogenesis of diabetes. Gasotransmitters are a group of gaseous signalling molecules, which are generated and metabolized in our body. Nitric oxide (NO), carbon monoxide (CO), and H_2S are three known gasotransmitters (Wang, 2012). H_2S formation in pancreases of Zucker diabetic fatty rats was significantly higher than that in non-diabetic Zucker fatty rats (Wu, et al., 2009). Compared with non-diabetic animals, streptozotocin-induced diabetic rats had higher H_2S production rate in liver tissues (Yusuf, et al., 2005). In addition, H_2S has been reported to regulate glucose metabolism by interaction with methylglyoxal, an intermediate of glucose metabolism (Chang, et al., 2010) and is a novel insulin resistance regulator (Feng, et al., 2009). Increased hepatic cystathionine gamma-lyase (CSE) activity and content in diabetic animals have been observed in different laboratories (Wijekioon, et al., 2005; Hargrove, et al., 1989). CSE has been demonstrated to be the predominant H_2S producing enzyme in rodent liver tissues (Kabil, et al., 2011; Mani, et al., 2011). Given the importance of liver for the pathogenesis of diabetes and altered endogenous hepatic production of H_2S in diabetes, it becomes imperative to understand

whether glucose metabolism in liver cells is regulated by H_2S and to explore the underlying signalling cascade.

The present study was to determine whether H_2S affects glucose metabolism and insulin signalling in the $HepG_2$ human hepatoma cell line and primary hepatocytes isolated from wild-type (WT) and CSE knockout (KO) mice. To test this hypothesis, we examined the basal and insulin stimulated glucose consumption and glucose production by measuring the glucose concentration in the medium after treatment with NaHS (a H_2S donor) or DL-propargylglycine (PPG, a CSE inhibitor). Glycogen content was also compared under fed and fasting conditions between CSE KO and WT mice.

MATERIALS AND METHODS

Animal preparation

CSE KO mice were generated as previously described (Yang, et al., 2008). Twelve week old male CSE KO mice and age-matched male WT littermates on C57BL/6J × 129SvEv background were used. PCR-genotyping of CSE KO mice was performed using a three-primer assay in two reactions. 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. All animals were maintained on standard rodent chow and had free access to food and water.

Cell culture and treatments

HepG2 human hepatoma cell line was obtained from American Type Culture Collection (Manassas,VA) and maintained in low glucose (5.5 mM) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/ml streptomycin and kept at 37 °C in a humidified atmosphere of 5% CO_2. Cells were grown upon reaching 70-80% confluence, and then preincubated in serum-free medium for 24 h before treatments. The effects of NaHS and PPG on cell viability were assessed as the ability of the cells to reduce 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT). For these assays, HepG2 cells were seeded in 96 well plates, maintained with 5.5 mM glucose DMEM medium and treated with NaHS or PPG at different doses.

Hepatocyte preparation

Hepatocytes were isolated from both CSE KO and WT mice as described before (Klaunig, et al., 1981) with slight modification. In brief, livers of 8-12 week old mice were perfused through the inferior vena cava with a buffer consisting of (in mM) 140 NaCl, 2.6 KCl, 0.28 Na_2HPO_4, 5 glucose, and 10 HEPES (pH 7.4). The perfusion was first for 5 min with the buffer supplemented with 0.5 mM EGTA and then for 10 min with the buffer containing 5 mM $CaCl_2$ and 100 U/ml collagenase type IV (Worthington, Lakewood, NJ). All the solutions were prewarmed at 37°C incubator. The isolated hepatocytes were filtered on nylon mesh (100 μm pore size), and selected by centrifugation in a 36% Percoll isodensity gradient and then seeded at 7.5×10^4 cells/per well in collagen coated 24 well plates in DMEM (5.5 mM glucose) containing 10% fetal

bovine serum. The viability of hepatocytes was measured by Trypan blue staining. The viability of all preparations was above 95%.

Adenovirus-mediated CSE overexpression in $HepG_2$ cells

Recombinant CSE adenovirus vector (Ad-CSE) was constructed as previously described (Yang, et al., 2005). The recombinant adenovirus encoding bacterial β-galactosidase (Ad-LacZ) derived from the same vector was used as a control. For adenoviral infection, subconfluent $HepG_2$ cells were incubated with Ad-CSE or Ad-LacZ in serum-free media. After 4 h of incubation, media was removed, and cells were incubated in appropriate media for 48 h. The transfection efficiency of adenoviral vector in $HepG_2$ cells was determined by Western blotting of CSE protein. At MOI (multiplicity of infection) = 100, a great increase of CSE expression was seen in Western blotting without significant loss in cell viability compared to Ad-LacZ transfected vector control. Subsequent experiments were performed at MOI of 100. To determine the cell viability after CSE overexpression, all cells were collected by trypsinization followed by centrifugation after transfection. Cell viability was expressed as a percentage of cells excluding trypan blue out of the total cells.

Measurement of glucose consumption and glucose uptake

$HepG_2$ cells or primary hepatocytes from WT or CSE KO mice were plated onto 24-well tissue culture plates at a concentration of 7.5×10^4 cells per well with some wells left blank and maintained in low glucose-DMEM (5.5 mM) medium. Cells were starved in serum-free low glucose-DMEM medium for 24 h, and then subjected to various

concentrations of glucose in the presence of NaHS, and/or insulin (10 nM) for 6 or 24 h. After treatments, cell medium was collected for glucose measurement. Glucose concentration was determined using a glucose assay kit II (Biovision, Mountain view, CA), since it is designed to be unaffected by reducing substances such as H_2S, which is different from other suppliers offering oxidase-based kits (http://www.biovision.com/glucose-assay-kit-ii-3972.html). Glucose level of the wells with cells was subtracted from that of the blank wells at the same treatment to obtain the amount of glucose consumption (Yin, et al., 2002).

Glucose uptake was determined by a nonradioactive method using a new fluorescent analog of D-glucose, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) (Yoshioka, et al., 1996). Different from the above glucose consumption method, this method detects transient glucose uptake which can be saturated in a short time. 2-NBDG was obtained from Molecular Probes (Invitrogen, Burlington, ON). $HepG_2$ cells were plated at 1×10^4/well in 96-well plates and used at subconfluence after 24 h preincubation. For experiments, culture medium was removed from each well and replaced with 100 μL of glucose-free DMEM medium in the absence or presence of 10 μM 2-NBDG with or without NaHS or PPG at the indicated concentrations for 1 hour. After treatment, the cells were washed in a modified balanced salt solution (MBSS) to stop the reaction before fluorescence analysis. MBSS (pH 7.4) has an osmolarity of 300 mOsm/L and contains (mM): 140 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 3.3 $NaHCO_3$, 2.0 $CaCl_2$, 10 HEPES, 5.5 glucose (O'Neil, et al., 2005). Fluorescence intensity was detected by a FLUOstar OPTIMA microplate spectrophotometer (BMG LABTech, Offenburg, Germany) at 485 nm excitation and 544 nm emission wavelengths.

Measurement of glycogen content

$HepG_2$ cells were maintained in culture for 24 h after media change, and then exposed to NaHS and/or insulin (10 nM) or PPG for 6 h in medium glucose-DMEM (11.1 mM glucose) media. In another experiment, the cells were transfected with Ad-CSE or Ad-LacZ as a control vector for 48 h. After that, the cells were collected to measure glycogen content. For animal experiments, a liver biopsy was taken from WT or CSE KO mice under fed, 6 h fasting or overnight (16 h) fasting conditions. After laparotomy, a 1- to 2-g piece of the liver was removed and immediately frozen in liquid N_2 and then stored at -80°C. Protein concentration was analyzed by Bradford reagent. Glycogen content was determined using a glycogen assay kit according to the manufacturer's protocol (Biovision, Mountain view, CA) and normalized to total protein concentration determined from whole-cell lysates. In this method, glucose is the sole substrate for glycogen synthesis.

Hepatic glucose production

The effect of exogenous H_2S on hepatic glucose production was measured by incubating $HepG_2$ cells in 24-well plates at a density of 7.5×10^4/well at 37 °C in a humidified atmosphere (5% CO_2). To see the effect of endogenous H_2S on glucose production, primary hepatocytes from WT and CSE KO mice were incubated at the same condition as $HepG_2$ cells. After 24 h of pre-culture, the $HepG_2$ cells were treated with or without NaHS or PPG in serum-free low glucose medium (5.5 mM) for 6 h. $HepG_2$ cells or primary hepatocytes from WT and CSE KO mice were washed twice with phosphate

buffered saline (PBS) to remove glucose and then incubated for 3 h in glucose production assay medium (glucose and phenol red-free DMEM medium containing 2 mM sodium pyruvate, 20 mM sodium lactate, pH 7.4) stimulated by dibutyl-cAMP (100 μM) and dexamethasone (50 nM). Three hundred microliter medium was sampled for measurement of glucose concentration. Glucose concentration was normalized with cellular protein concentration. The above method can represent the rate of gluconeogenesis since the substrates were supplied.

The effect of H_2S on hepatic glycogenolysis was measured *in vitro* in $HepG_2$ cells and primary hepatocytes from WT and CSE-KO mice. Briefly, $HepG_2$ cells or primary hepatocytes were pre-cultured in 24-well plates with high glucose-DMEM (25 mM) for 24 hours. Next day, cells were washed twice by PBS, and then incubated with 1 mL DMEM in the absence of glucose, glutamine and pyruvate but containing 0.24 mM 3-isobutyl-1-methylxanthine (IBMX) in the presence or absence of NaHS (10 μM), PPG (1 mM) or glucagon (10 nM). IBMX was dissolved in dimethyl sulfoxide (DMSO) to a concentration in the medium that did not interfere with cell viability (maximally 0.1%, v/v). Glycogenolysis was estimated by the rate of glucose appearance in the incubation as no gluconeogenic substrates were added. Glucose content in the incubation medium was determined at 0, 10, 20 and 30 min of incubation. The supernatant was removed, and cells were lysed to measure protein concentration by Bradford reagent. The glucose content of the supernatant in the medium was measured using a glucose assay kit II (Biovision, Mountain view, CA). Glucose concentrations were normalized to cellular protein concentration. Different from the above assay measuring the rate of

gluconeogenesis from substrates, the assay of glycogenolysis measures glycogen breakdown from endogenous glycogen store within 30 mins.

Establishment of insulin resistant $HepG_2$ cell model

To develop a model of insulin resistance, $HepG_2$ cells were incubated with 500 nM insulin for 24 h with 33 mM glucose-DMEM medium. In the normal control group, 5.5 mM glucose-DMEM medium was used to culture cells for 24 h. The cells in the normal control group and model group were washed twice with PBS, then treated with or without insulin (10 nM) for 24 h at medium glucose-DMEM (11.1 mM). In another group, the insulin resistant model cells were washed and incubated with PPG (1 mM) in medium glucose-DMEM (11.1 mM) for 24 h. Glucose consumption (with or without insulin stimulation), which represents the amount of glucose depleted from the culture medium (11.1 mM glucose) within 24 h, were determined to prove the successful establishment of insulin resistant cell model.

Measurement of endogenous H_2S production

H_2S production rate was measured as previously described, which has been routinely used in our laboratory (Zhao, et al., 2001; Cheng, et al., 2004). Briefly, cell lysates were incubated with L-cysteine (10 mM) for 90 min at 37°C water bath, and then trichloroacetic acid (TCA) was added to stop the reaction. The level of methylene blue generated by the reaction of H_2S with 20 mM N,N-dimethyl-p-phenylenediamine sulphate (DPD) was determined at 670 nm with a multicell spectrophotometer (Fisher Scientific, Ottawa, ON).

Western blotting

Cultured cells were harvested and lysed in a Tris-EDTA sucrose lysis buffer plus protease inhibitors as previously described (Yang, et al., 2005). Protein extracts were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Pall Corporation, Pensacola, USA). Protein samples were analyzed with antibodies against CSE (Abnova, Taiwan), β-actin (Sigma), Phospho-Akt(Ser473), Akt, Phospho-AMPKα (Thr172), AMPKα (Cell Signaling Technology Inc., Beverly, MA). Immunoreactions were visualized with a chemiluminescence reagent (GE healthcare, UK) and exposed to X-ray films (Amersham Hyperfilm™ ECL, GE healthcare). Each blot shown in the figures is representative of at least four experiments. Protein quantification was performed by Alpha Digi Doctor software (Richardson, TX, USA), and the intensity values were normalized to the quantity of β-actin.

Enzyme assays

The enzymatic activities of phosphoenolpyruvate carboxykinase (PEPCK) were determined by spectrophotometric assays as previously described (Chambers, et al., 2010). Enzyme activity of glucokinase (GK) was measured following a protocol in Sigma's website http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Enzyme_Assay/glucokinase.Par.0001.File.dat/glucokinase.pdf. Briefly, for each assay, enzymatic activity was determined by the conversion of NADH to NAD^+ measured by the change in absorbance at 340 nm over time. Enzyme activity was measured in a multicell spectrophotometer (Fisher

Scientific, Ottawa, ON) and was expressed as per mili- international unit per minute per milligram (mIU/min/mg) of protein. Results were normalized by protein concentration determined by Bradford method.

Materials and data analysis

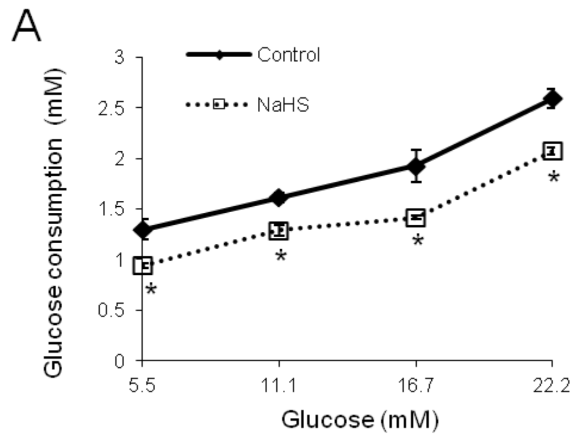
Chemicals were all obtained from Sigma-Aldrich (Oakville, ON) unless otherwise mentioned. The data were expressed as mean ± SEM from at least four independent experiments. Statistical analyses were performed using Student's t test for paired data or one-way ANOVA. Statistical significance was considered at $p<0.05$.

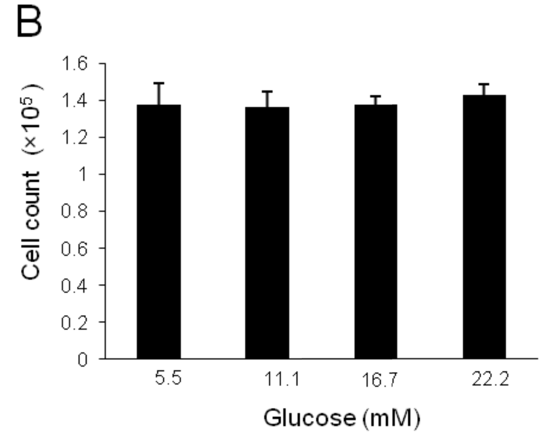
RESULTS

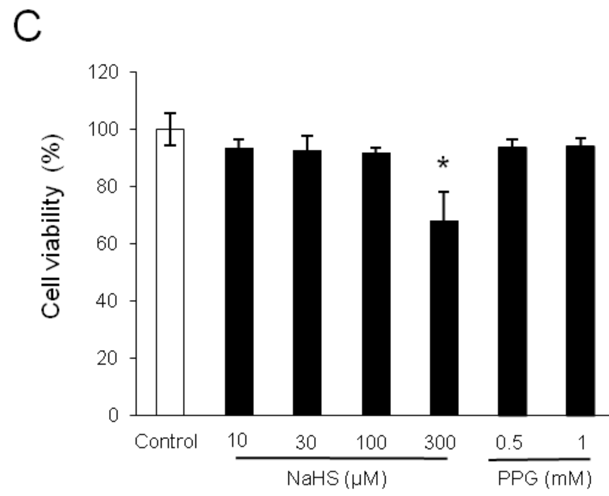
Effects of H_2S on glucose consumption

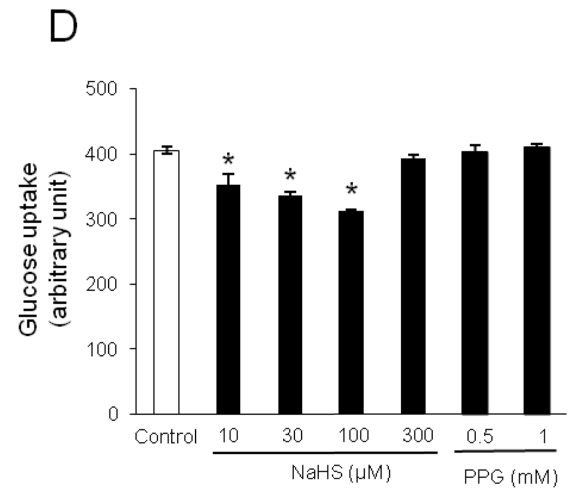
The presence of glucose stimulated glucose consumption in $HepG_2$ cells in a dose-dependent manner (Fig. 4-1A), and this effect does not appear to be due to the alteration of cell number (Fig. 4-1B). When the glucose concentration was elevated from 5.5 mM to 22.2 mM, glucose consumption was increased from 1.3 mM to 2.59 mM (Fig. 4-1A). Incubation of $HepG_2$ cells with 100 μM NaHS for 24 h significantly decreased glucose consumption by 20-30% ($p<0.05$) (Fig. 4-1A). The effect of NaHS was not due to a decrease in cell number. At the concentration between 10 to 100 μM, NaHS had no effect on cell viability measured by MTT assay. However, 300 μM NaHS started to decrease cell viability (Fig. 4-1C). Similarly, NaHS at 10, 30, 100 μM significantly

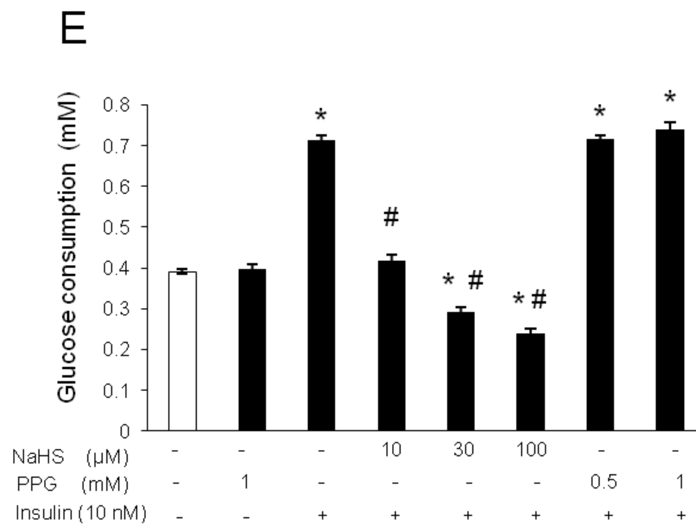
decreased 2-NBDG uptake, but not at 300 μM, which is probably far beyond physiological range and has toxic effect (Fig. 4-1D). NaHS also significantly reduced insulin-stimulated glucose consumption (Fig.4-1E). PPG at 1 mM did not show any effect on glucose uptake (Fig. 4-1D) and glucose consumption (Fig. 4-1E) at the basal level. Insulin stimulated-glucose consumption was not altered by PPG either (Fig. 4-1E). Primary hepatocytes from CSE-KO mice exhibited a two-fold increase in glucose consumption rate (25.52 ± 2.22 μM/mg protein) compared to that from WT mice (12.18 ± 0.97 μM/mg protein) at 24 hours (Fig.4-1F).


A
Control
NaHS
Glucose consumption (mM)
Glucose (mM)
5.5
11.1
16.7
22.2


B
Cell count (×10⁵)
Glucose (mM)
5.5
11.1
16.7
22.2


C
Cell viability (%)
Control
10
30
100
300
0.5
1
NaHS (μM)
PPG (mM)


D
Glucose uptake
(arbitrary unit)
Control
10
30
100
300
0.5
1
NaHS (μM)
PPG (mM)


E
Glucose consumption (mM)
NaHS (μM)
PPG (mM)
Insulin (10 nM)

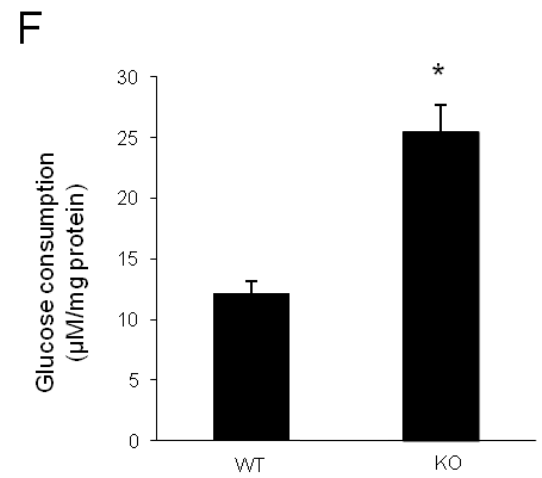

F
Glucose consumption
(μM/mg protein)
WT
KO

Figure 4-1. Effects of H_2S on glucose consumption and uptake in $HepG_2$ cells and hepatocytes.

(A) Effects of NaHS on glucose consumption at various levels of glucose (5.5, 11.1, 16.7, and 22.2 mM) in $HepG_2$ cells. Glucose consumption was measured after 24 h incubation with or without 100 μM NaHS. n=4 in each condition. * $p<0.05$ versus control. (B) Effect of glucose on cell proliferation. $HepG_2$ cells were plated at 6-well plate in the density of 10^5/well and cultured in vitro under varying doses of glucose for 24 h. Cell numbers were counted after trypsinization to reflect cell growth within 24 h. n=4. (C) Cell viability with NaHS or PPG treatment for 24 h. $HepG_2$ cells were seeded in 96 well plates, maintained with 5.5 mM glucose DMEM medium overnight upon reaching 70-80% confluence, and then treated with NaHS or PPG at different concentrations in serum-free medium for 24 h. Cell viability was assessed as the ability of the cells to reduce MTT. n=5. * $p<0.05$ versus control. (D) Changes in glucose uptake in $HepG_2$ cells. Glucose uptake was measured by incubating cells with 2-NBDG (10 μM) in the absence or presence of NaHS or PPG at indicated concentrations for 1 h. n=4. * $p<0.05$. (E) Effects of NaHS or PPG on insulin stimulated-glucose consumption. $HepG_2$ cells were incubated with different doses of NaHS or PPG for 6 h at medium glucose DMEM (11.1 mM) with 10 nM insulin stimulation. Glucose consumption was then measured as described in Methods. n=4. * $p<0.05$ versus control. # $p<0.05$ versus insulin alone group. (F) Glucose consumption over 24 hours by hepatocytes of CSE-KO or WT mice. n=4, * $p<0.05$.

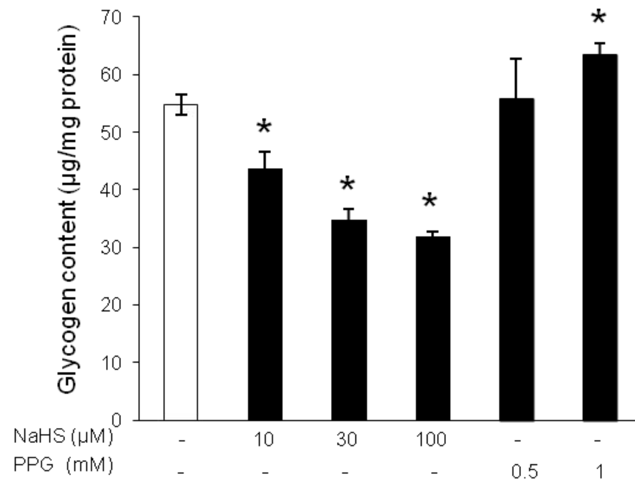
Effects of H_2S on glycogen storage

Exposing $HepG_2$ cells to 10, 30 and 100 μM NaHS for 6 h at medium glucose concentration (11.1 mM) resulted in a dose-dependent reduction in glycogen content (up to 42%) relative to control values (Fig. 4-2A), which remained constant until 24 h without or with insulin (Supplemental Fig. 4-1A and 4-1B, respectively). PPG at 1 mM significantly improved glycogen storage (Fig. 4-2A).

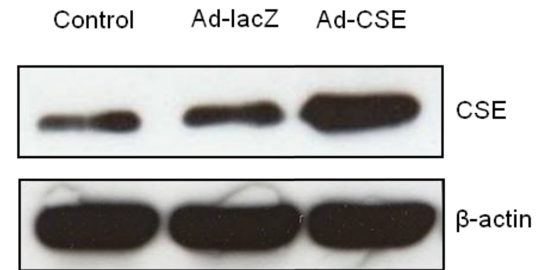
We overexpressed CSE gene in $HepG_2$ cells by using a highly effective replication-deficient adenovirus expression system. CSE expression in Ad-CSE transfected cells was apparently higher than that in Ad-LacZ transfected cells or control cells (Fig. 4-2B). The most significant expression was observed in cells after 48 h infection with Ad-CSE at 100 MOI (Fig. 4-2B). Cells infected with Ad-CSE at 100 MOI also exhibited a marked increase in CSE activity as reflected by H_2S production rate (27.0 ± 5.2 nmol/g/min) compared with Ad-LacZ infected cells (13.9 ± 1.0 nmol/g/min, $p<0.05$) or control cells (14.7 ± 0.8 nmol/g/min, $p<0.05$) (Fig. 4-2C). These results indicated that infection with Ad-CSE drastically enhanced the amount of functional CSE protein in the transfected $HepG_2$ cells. Cell viability was not significantly different between Ad-CSE and Ad-LacZ transfected cells in the range of 25-100 MOI. However, adenoviral infection significantly decreased cell viability at 100 MOI compared with the cells without transfection (0 MOI) (Fig. 4-2D). As shown in Fig. 4-2E, CSE overexpression significantly reduced glycogen storage in $HepG_2$ cells in comparison with the control cells or cells transfected with Ad-LacZ. In addition, under both fed and 6 h fasting conditions, liver glycogen content was significantly higher in CSE KO mice

compared to that in WT mice (Fig. 4-2F). After overnight fast, the glycogen level in both WT and CSE KO mice were essentially depleted, no difference was found between those groups (Fig. 4-2F).

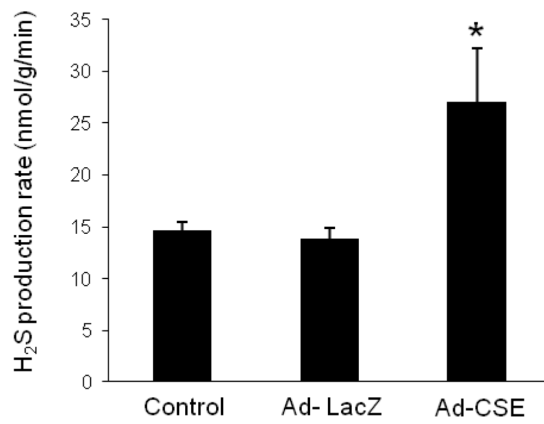
A



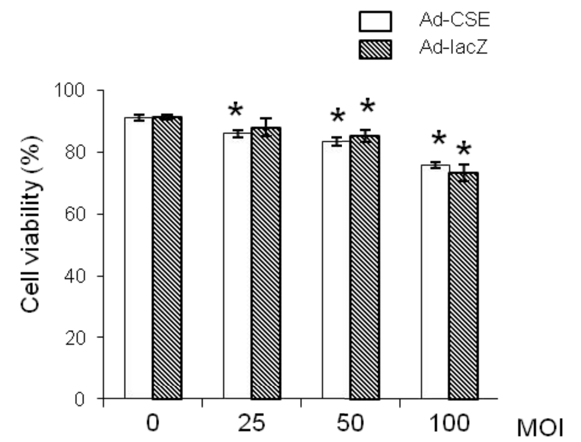
B



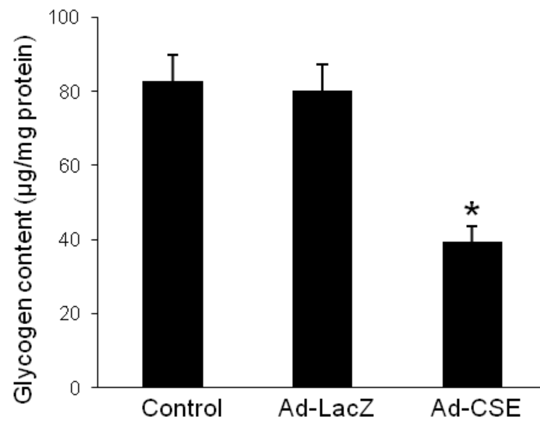
C



D



E



F

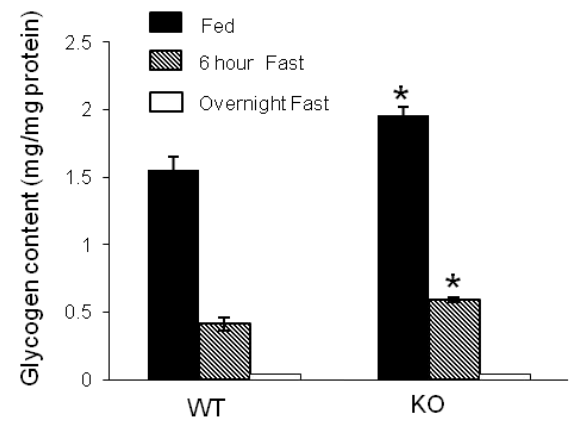
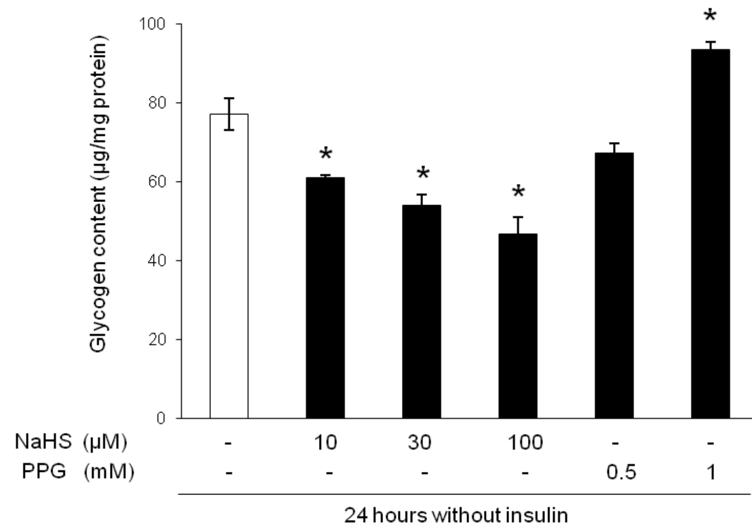
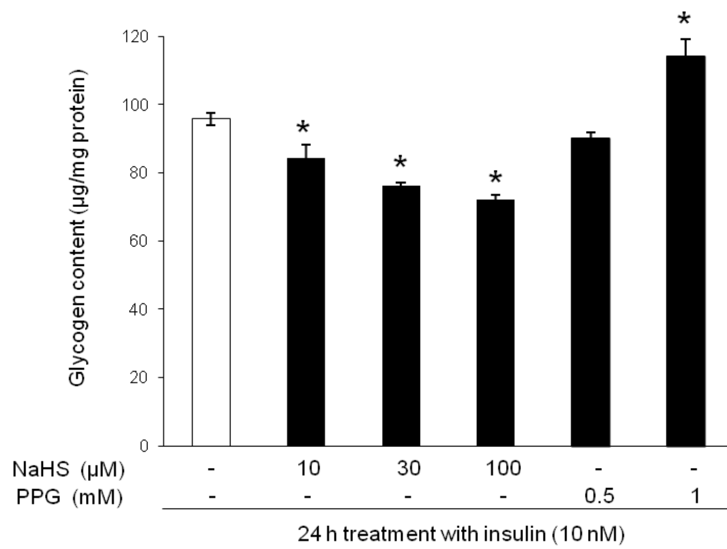


Figure 4-2. Effects of exogenous and endogenous H_2S on glycogen storage.
(A) Effects of increasing concentrations of NaHS or PPG on $HepG_2$ glycogen content. The cells were maintained in culture for 24 h after media change, then exposed to NaHS or PPG for 6 h at medium glucose DMEM (11.1 mM) and washed. Glycogen was extracted and quantitated as described in Methods. n=4, * $p<0.05$ versus control. (B) Ad-CSE mediated overexpression of CSE protein. $HepG_2$ cells were transfected either with the control adenovirus Ad-LacZ or Ad-CSE at 100 MOI for 48 h. Cells were collected and subjected to Western blotting. n=4. (C) Increased H_2S production rate in $HepG_2$ cells by overexpressing CSE via adenovirus. n=4, * $p<0.05$ versus control or Ad-LacZ transfected cells. (D) Cell viability after infection of adenovirus at different MOI. Various MOI were used to optimize the best transfection condition. To determine the cell viability after transfection, cells were collected by trypsinization followed by centrifugation after transfection. Cell viability was expressed as a percentage of cells excluding trypan blue out of the total cells. n=4. * $p<0.05$ versus control (0 MOI). (E) Decreased glycogen content in CSE-overexpressed $HepG_2$ cells. Glycogen was extracted and quantitated as described in Methods. n=4, * $p<0.05$ versus control or Ad-LacZ-transfected $HepG_2$ cells. (F) Glycogen content in liver tissues of WT and CSE KO mice. Glycogen content was measured in liver of 12 week-old mice (n=5-6) under fed, 6 hour fasting or overnight fasting conditions. * $p<0.05$ versus wild type.

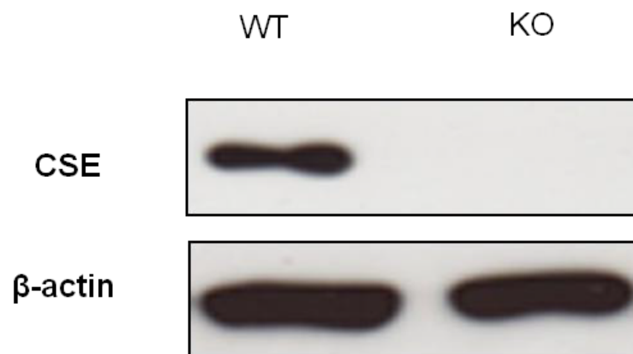
A



B



Supplemental Figure 4-1. Effects of increasing concentration of NaHS or PPG on $HepG_2$ glycogen content.
The cells were maintained in culture for 24 h after media change, then exposed to NaHS or PPG for 24 h without (A) or with 10 nM insulin stimulation (B) at medium-glucose DMEM (11.1 mM). Glycogen was measured as described in Methods. n=4, * $p<0.05$ versus control.



Supplemental Figure 4-2. The absence of CSE protein expression in primary hepatocytes isolated from CSE KO mice. n=4 mice in each group.

Involvement of AMPK activation in H_2S-impaired glucose consumption

Exposure of $HepG_2$ cells to NaHS for 6 h inhibited the phosphorylation of AMPK in the absence of insulin, whereas PPG at 1 mM improved the phosphorylation of AMPK (Fig. 4-3A). Application of AICAR (an AMPK agonist) stimulated AMPK activity (Muoio, et al., 1999), which led to the reversal of H_2S-impaired glucose consumption (Fig. 4-3B).

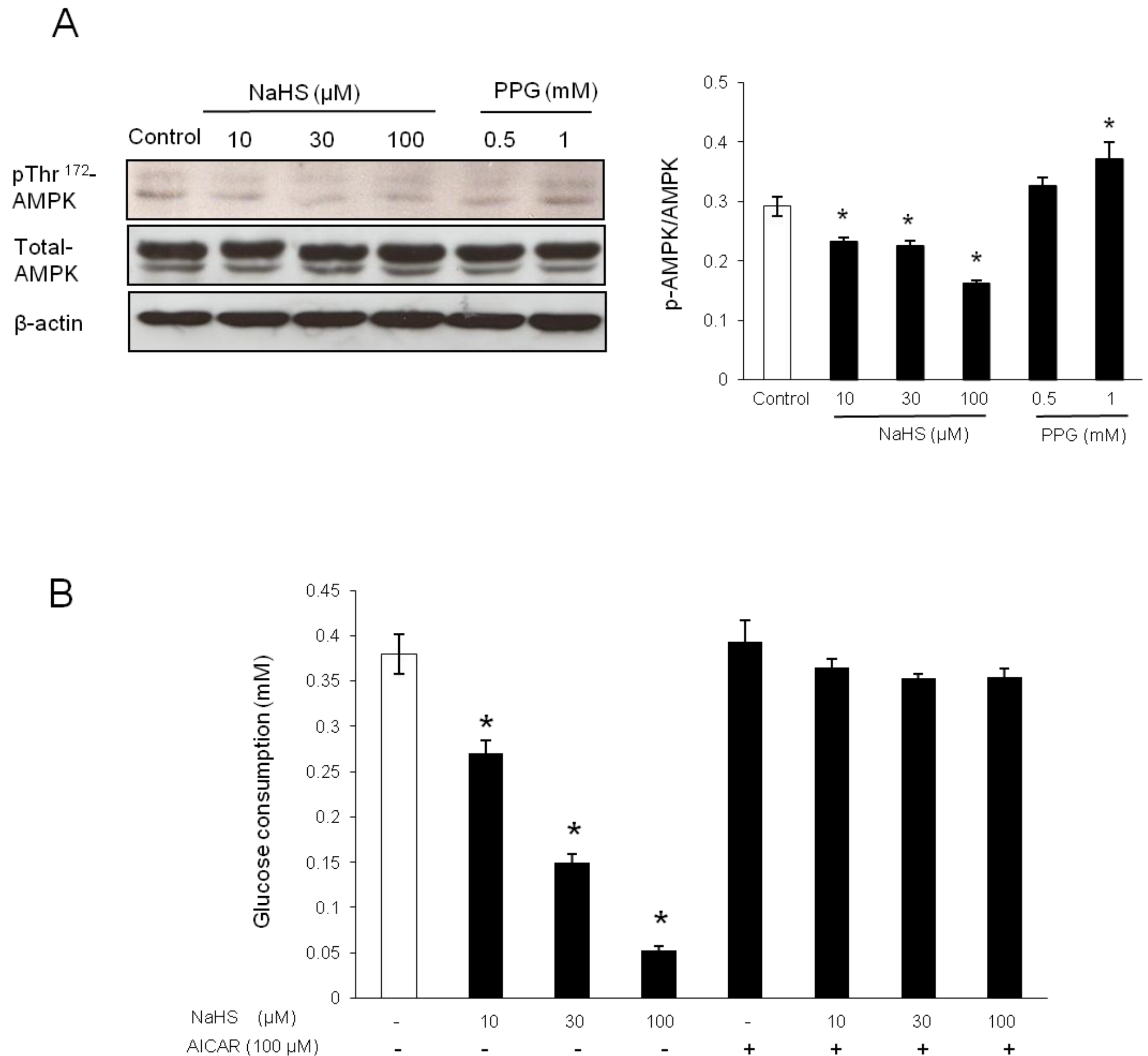
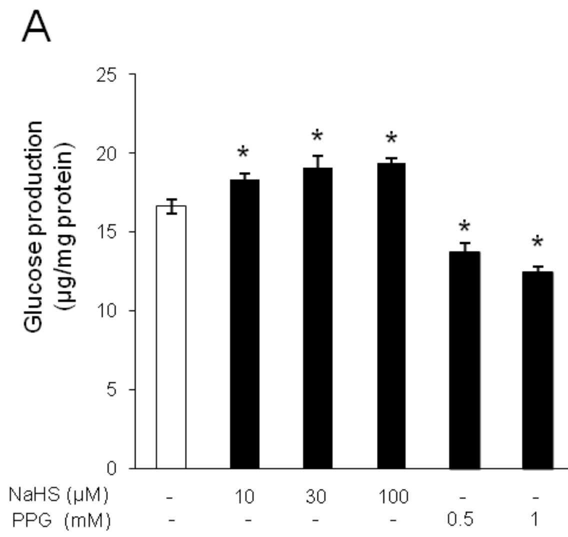


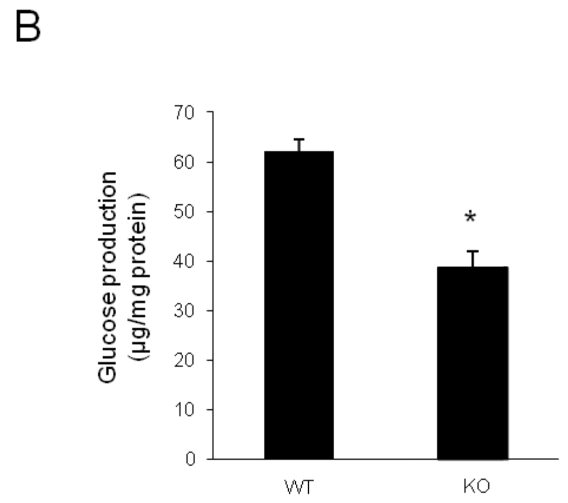
Figure 4-3. The involvement of AMPK phosphorylation in H_2S-impaired glucose consumption. (A) Altered levels of phospho-AMPK were induced by increasing concentrations of NaHS or PPG. $HepG_2$ cells were incubated with NaHS or PPG at different concentrations for 6 h. After that, cells were collected and subjected to Western blotting analysis. n=4. * $p<0.05$ versus control group. (B) Impaired glucose consumption by H_2S was reversed by AICAR. $HepG_2$ cells were pre-exposed to various concentrations of NaHS for 0.5 h and then treated with or without 100 μM AICAR for 6 h. Medium was collected at the beginning and after incubation to measure glucose level using a glucose-assay kit. Glucose consumption rate was calculated based on the values. n=4. * $p<0.05$.

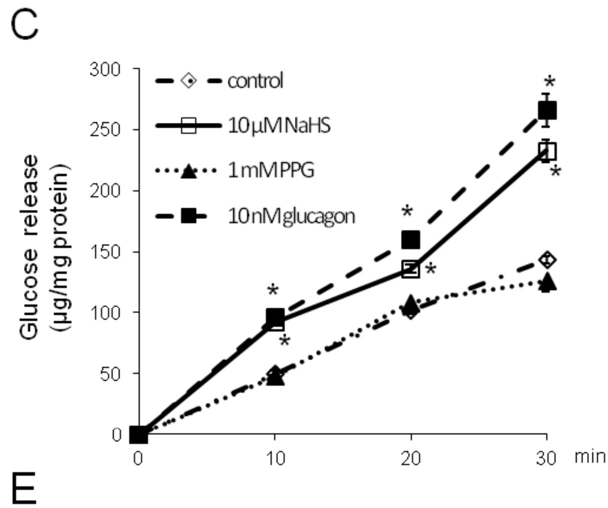
H_2S-mediated changes in PEPCK and GK activity on glucose generation

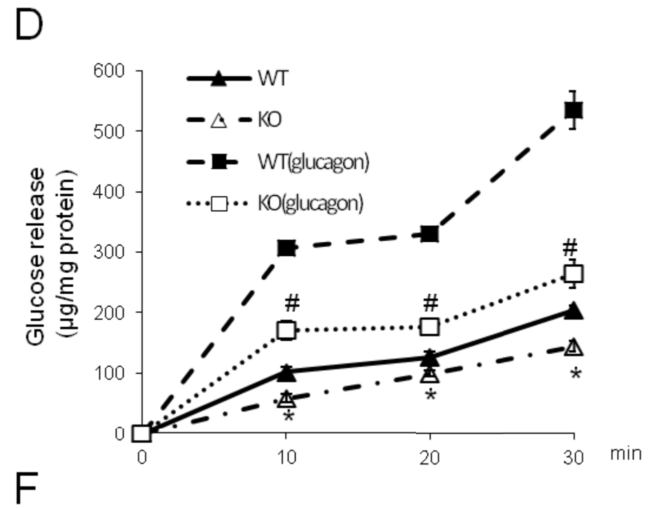
Exposure to H_2S significantly enhanced glucose production from substrates (pyruvate and lactate) in $HepG_2$ cells (Fig. 4-4A), which represents the rate of gluconeogenesis. Similarly, hepatocytes from CSE KO mice had a significantly decreased (23.18 ± 4.27 %) glucose production from substrates compared with WT mice (Fig.4-4B), due to markedly lower endogenous H_2S production in liver tissues from CSE-KO mice (Mani, et al., 2011). NaHS rapidly increased IBMX-stimulated glycogenolysis (measured by glucose release through glycogen breakdown) within 30 min (Fig. 4-4C). The rate of glucose release (glycogenolysis) from these cells at basal state was 143.6 ± 2.9 μg/mg protein/30 min, and increased to 266.0 ± 13.2 μg/mg protein/30 min with 10 nM glucagon stimulation (Fig. 4-4C). After treatment with 1 mM PPG, the glucose release (126.1 ± 8.3 nmol/mg protein/30 min) was comparable to control. However, 10 μM NaHS treatment significantly elevated glucose release at all the time points tested within 30 min compared to control state, which is comparable to glucagon treated cells (Fig. 4-4C). Similarly, the rate of glucose release is higher in hepatocytes from WT mice than that from CSE KO mice in the presence or absence of 10 nM glucagon (Fig. 4-1D).

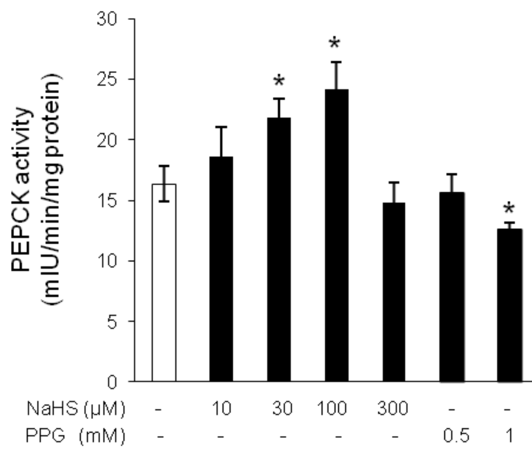
Treatment of $HepG_2$ cells with NaHS (below 300 μM) for 6 h significantly enhanced PEPCK activity (Fig. 4-4E), but reduced GK activity (Fig. 4-4F) in comparison to untreated cells, whereas PPG treatment imposed the opposite effect.


A
Glucose production (µg/mg protein)
NaHS (µM) - 10 30 100 - -
PPG (mM) - - - - 0.5 1


B
Glucose production (µg/mg protein)
WT
KO


C
control
10µMNaHS
1mMPPG
10nMglucagon
Glucose release (µg/mg protein)
min


D
WT
KO
WT(glucagon)
KO(glucagon)
Glucose release (µg/mg protein)
min


E
PEPCK activity (mIU/min/mg protein)
NaHS (µM) - 10 30 100 300 - -
PPG (mM) - - - - - 0.5 1

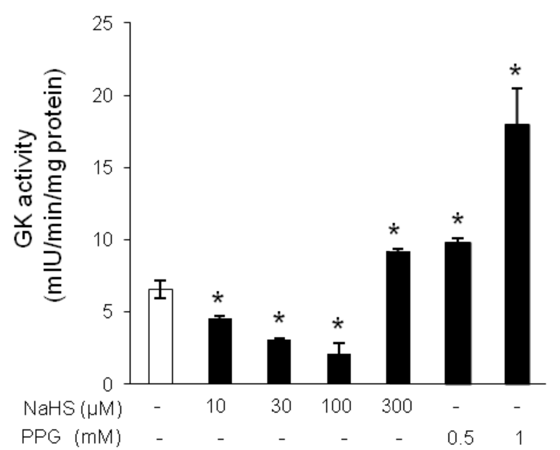

F
GK activity (mIU/min/mg protein)
NaHS (µM) - 10 30 100 300 - -
PPG (mM) - - - - - 0.5 1

Figure 4-4. H_2S-mediated glucose production and altered PEPCK and GK activities in hepatocytes.

(A) Effects of NaHS and PPG on glucose production. $HepG_2$ cells were treated with NaHS or PPG for 6 h, washed twice with PBS, then incubated in glucose-free DMEM (pH 7.4), supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate in the presence of Dex/cAMP. Glucose concentration was measured in extracellular medium and values were normalized with protein concentration. n=4, * $p<0.05$ versus control. (B) Glucose production from substrates via gluconeogenesis in primary hepatocytes from WT and CSE-KO mice. n=4, * $p<0.05$. (C) Effects of H_2S, PPG or glucagon treatments for 30 mins on the rate of glucose release via glycogenolysis in $HepG_2$ cells. * $p<0.05$ versus control. (D) The rate of glucose release via glycogenolysis in hepatocytes from WT and CSE-KO mice in the presence or absence of glucagon. n=4, * $p<0.05$ versus WT hepatocytes in the absence of glucagon; # $p<0.05$ versus WT hepatocytes with glucagon. (E, F) Enzymatic activities of PEPCK and GK under different treatment conditions. $HepG_2$ cells were treated with various concentrations of NaHS or PPG for 6 h, and then the cells were harvested to quantify enzyme activity. PEPCK and GK activities were determined by using a continuous spectrophotometric method. n=4. * $p<0.05$ versus control.

H_2S induces insulin resistance in $HepG_2$ cells

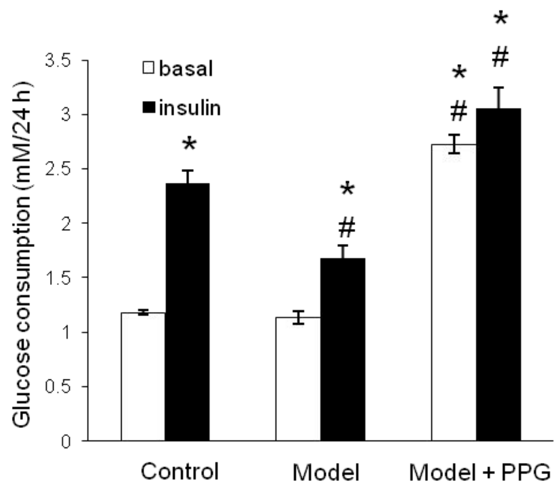
An insulin resistant cell model was established by incubating cells with high glucose (33 mM) and high insulin (500 nM) for 24 h. Glucose consumption was determined with or without insulin stimulation. Impaired insulin sensitivity in model cells was confirmed by significantly reduced insulin-stimulated glucose consumption during 24 hours (Fig. 4-5A). Application of PPG at 1 mM markedly elevated glucose consumption in the insulin resistant model cells with or without insulin stimulation (Fig. 4-5A).

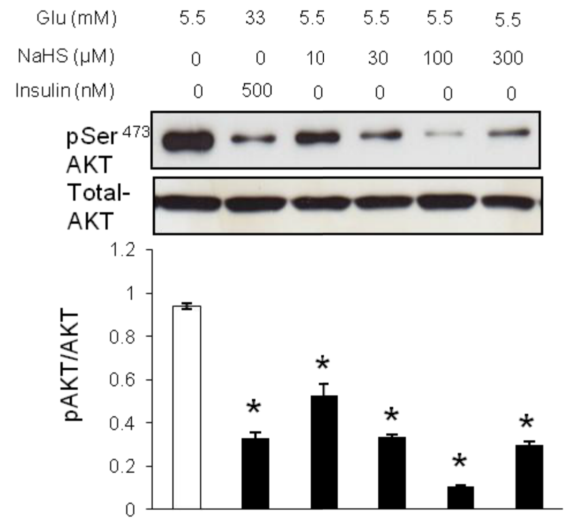
Upon acute 100 nM insulin stimulation for 10 mins, the treatment of the cells in the state of hyperglycemia (33 mM) and hyperinsulinemia (500 nM) for 24 h clearly blocked the insulin-induced phosphorylation of Akt at Ser-473 (Fig. 4-5B). In the range of 10-100 μM, NaHS induced a dose-dependent decrease in insulin-stimulated Akt phosphorylation compared to cells preincubated in low glucose (5.5 mM) with no insulin. NaHS at 300 μM was beyond the dose-dependent range, but still induced an impaired phosphorylation of Akt (Fig. 4-5B).

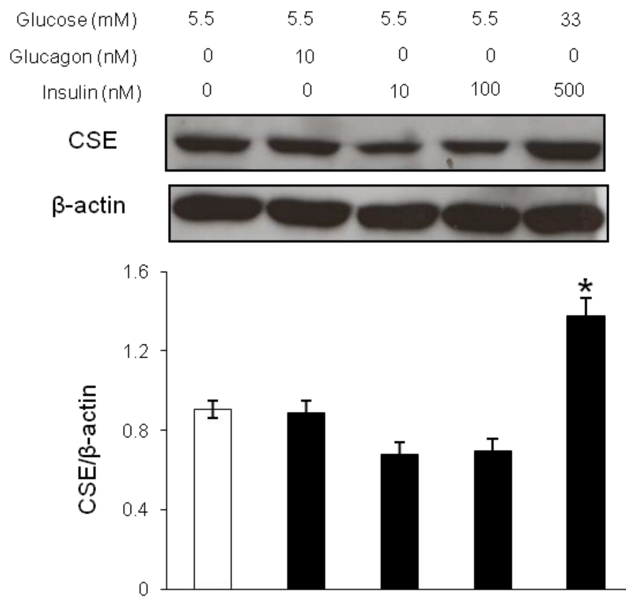
Hormonal regulation of CSE expression and activity in $HepG_2$ cells

Insulin and its counter regulatory hormones such as glucagon work synergistically to keep blood glucose concentrations normal. To evaluate whether physiological concentration of insulin and glucagon exert a direct and specific effect on CSE expression and activity in liver cells, we treated $HepG_2$ cells with insulin (10 nM and 100

nM) and glucagon (10 nM) at low glucose-DMEM (5.5 mM) for 24 h. CSE expression was also checked under hyperglycemia (33 mM) and high insulin (500 nM) induced insulin resistance state. As shown in Fig. 4-5C, physiological concentration of insulin decreased CSE expression, whereas glucagon has no effect to CSE protein level. CSE expression was enhanced in the insulin-resistant state (Fig. 4-5C). Additionally, CSE enzyme activity reflected by H_2S production rate was elevated in $HepG_2$ cells when stimulated with 10 nM glucagon for 24 h and lowered by 10 nM insulin for 24 h (Fig. 4-5D).


A
basal
insulin
Glucose consumption (mM/24 h)
3.5
3
2.5
2
1.5
1
0.5
0
Control
Model
Model + PPG


B
Glu (mM) 5.5 33 5.5 5.5 5.5 5.5
NaHS (μM) 0 0 10 30 100 300
Insulin (nM) 0 500 0 0 0 0
pSer 473 AKT
Total-AKT
pAKT/AKT
1.2
1
0.8
0.6
0.4
0.2
0


C
Glucose (mM) 5.5 5.5 5.5 5.5 33
Glucagon (nM) 0 10 0 0 0
Insulin (nM) 0 0 10 100 500
CSE
β-actin
CSE/β-actin
1.6
1.2
0.8
0.4
0

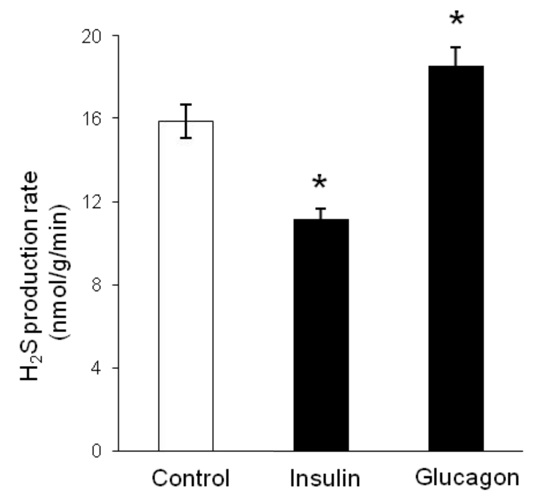

D
H2S production rate (nmol/g/min)
20
16
12
8
4
0
Control
Insulin
Glucagon

Figure 4-5. The effects of PPG and NaHS on insulin-resistance state of $HepG_2$ cells (A) Amelioration of impaired glucose consumption by PPG (1 mM) in high glucose and high insulin-induced insulin-resistant $HepG_2$ cells. Incubation of $HepG_2$ cells with 33 mM glucose and 500 nM insulin for 24 h was used to establish an insulin resistant cell model. Glucose consumption rate at 11.1 mM glucose medium during 24 h was then measured with or without 10 nM insulin stimulation using a glucose assay kit II. n=4. * $p<0.05$ versus low glucose alone group. # $p<0.05$ versus insulin alone group. (B) Phospho-AKT and total AKT expression in $HepG_2$ cells under various treatments. $HepG_2$ cells were pre-incubated with various doses of NaHS for 24 h, or exposed to high glucose (33 mM) and high insulin (500 nM) for 24 h to induce insulin resistance. After treatments, cells were challenged by 100 nM insulin for 10 min. Cell lysates were collected and phospho-Akt and total Akt were detected by Western blotting. n=4, * $p<0.05$ versus control cells. (C) Hormonal regulation of CSE expression. $HepG_2$ cells were incubated with physiological concentrations of insulin or glucagon for 24 h, or treated with 33 mM glucose and 500 nM insulin for 24 h to induce insulin resistance. Cell lysates were collected for Western blotting analysis of CSE and β-actin protein. n=4, * $p<0.05$. (D) Altered H_2S production rate by insulin and glucagon. $HepG_2$ cells were incubated with 10 nM insulin or 10 nM glucagon for 24 h, and then H_2S production rate in the cell lysates was measured by methylene blue method. n=4, * $p<0.05$ versus control cells.

DISCUSSION

Strong experimental evidence implicates H_2S as a pathophysiological factor in the pathogenesis of type 1 and type 2 diabetes and their complications observed in *in vitro* and *in vivo*. Our previous study showed that H_2S inhibited insulin secretion from islets and insulin-secreting β-cell lines (INS-1E cells) (Yang W, et al., 2005). Elevated H_2S level in pancreas (Wu, et al., 2009) and liver (Yusuf, et al., 2005) has been seen in different diabetic models. Furthermore, H_2S is endogenously produced in adipose tissues and inhibits glucose uptake by mature adipocytes (Feng, et al., 2009). CSE/H_2S system was found to be up-regulated in the adipose tissues of fructose-induced insulin resistant rat model (Feng, et al., 2009). Adipose tissue has been traditionally considered as a major secretory and endocrine organ which mediates insulin resistance through a variety of factors released from adipose cells (Trujillo, et al., 2006). These results strongly suggest that H_2S might be a novel insulin resistance regulator. Considering the important role of the liver in glucose disposal and glucose output upon fasting, which all contribute to insulin resistance, it becomes imperative to find the link between H_2S and liver glucose metabolism. In the present study, $HepG_2$ cells were adopted to establish a hepatic insulin resistance cell model as reported in the literature (Zhang, et al., 2009; Lee, et al., 2010). It has to be noticed that, however, the *in vitro* cellular model used in our study only partially mimics high glucose- and high-insulin induced insulin resistance. The one-day treatment of the cells with high levels of glucose and insulin would unlikely replicate the true situation of chronic insulin resistance *in vivo*. The sophisticated homeostatic control of insulin and glucose metabolisms in the whole body involves both genetic and non-

genetic factors with the participation of multiple organs and systems. As such, our data should be cautiously interpreted as per the role of H_2S as a regulator of insulin resistance.

The importance of the liver in the glucose homeostasis is well known before (Pilkis, et al., 1992), but the endogenous factors governing the utilization of glucose by the liver remain unclear. By using a colorimetric method (Zhang Z, et al., 2011), our results indicate H_2S is a novel endogenous inhibitor for glucose consumption in liver. NaHS as low as 10 μM significantly lowered glucose consumption at the basal and insulin-stimulated state. The underlying mechanism behind this might be associated with H_2S-induced decreased GK activity in liver cells as observed (Fig. 4-4F). Normally, glucose enters hepatocytes by facilitated diffusion in an insulin independent way mainly via GLUT2 transporters (Pessin, et al., 1992). In non-diabetic animals, GLUT2 provides glucose to hepatocytes much greater than the rate of phosphorylation by GK. Thus, GK became key in the regulation of glucose utilization by hepatocytes. Indeed, it is proved that GK is rate limiting enzyme for glucose utilization in the liver by using transgenic mice model (Ferre, et al., 1996). Interestingly, PPG did not affect basal glucose uptake and glucose consumption in the absence of high glucose and high insulin treatment, but it significantly enhanced basal glucose consumption after exposure to high glucose and high insulin for one day to establish insulin resistant cell model. Considering that CSE was overexpressed in the insulin resistant-model cells, the inhibitory effects of PPG on CSE activity and H_2S production would be more obvious in these cells.

Glucose is stored in the liver in the form of glycogen and this process can be facilitated by insulin. In our study, glycogen content in $HepG_2$ cells was observed to be significantly diminished by exposure to H_2S (both exogenous and endogenous) without being affected by loss of cell viability. In our assay, we measured absolute glycogen

levels in $HepG_2$ cells using a colorimetric method with glucose as sole substrate; however, many studies have determined the rate of glycogen synthesis by incorporation of radioactive glucose. Due to our purpose, the radioisotope method was not adopted, as it does not consider effects of agents on endogenous glycogen stores. In the present study, the reduction of glycogen content by H_2S might be attributed to both decreased glucose consumption and increased glycogenolysis. Most importantly, improved glycogen storage was observed in the liver of CSE knockout mice in both fed and 6 hour fasting conditions (Fig. 4-2F), supporting our hypothesis that glycogen content is elevated in the presence of inadequate H_2S.

Gluconeogenesis is the process of glucose generation especially in the liver from non-carbohydrate carbon substrates such as lactate, glycerol, and gluconeogenic amino acids. In our study, pyruvate and lactate were added as substrates to produce glucose and extracellular glucose production was measured from cell culture medium (Fig. 4-4A). The increased glucose production by H_2S is mainly attributed to elevated gluconeogenesis from substrates in the above assay. Interestingly, both WT and CSE KO mice were essentially depleted of glycogen upon fasting overnight (Fig. 4-2F). No compensatory effect was observed. In our previous study, we found that NaHS increased blood glucose levels only in non-fasting mice and in a rapid fashion (Yang, et al., 2011), which fits the properties of glycogenolysis. Meanwhile, it is classically known that glycogen can be compensated through gluconeogenesis mainly in the liver upon long term fasting. Therefore, it is assumed that H_2S may play a bigger role in glycogenolysis than gluconeogenesis, which led to the induction of instant blood glucose elevation by H_2S only seen in fed mice, but not in overnight fasting mice (Yang, et al., 2011).

Altered glucose metabolism and impaired insulin sensitivity are two hallmarks for diabetes mellitus. Our results indicate that physiological concentration of H_2S significantly impairs basal and insulin-stimulated glucose uptake and glycogen storage, but enhances gluconeogenesis and glycogenolysis in $HepG_2$ cells, all of which suggests a state of insulin resistance. The activation of Akt via PI3-kinase is well known to be a central step in insulin signalling. Insulin stimulated phosphorylation of Akt at serine 473 was remarkably diminished in the H_2S-treated cells (Fig. 4-5B). These results in $HepG_2$ cells suggest that H_2S plays a role in the hepatic regulation of glucose and glycogen metabolism dependent on the PI3-kinase/Akt signalling pathway. Upstream or downstream of this signalling pathway (e.g., IRS-1, PI3-K, and glycogen synthase kinase-3) might also be affected by H_2S, which merits further investigation. We observed that insulin at physiological concentration (10 nM, 100 nM) downregulated CSE protein expression (Fig. 4-5C); however, in the high glucose and high insulin induced insulin-resistance state, this down-regulatory response is impaired, resulting in induced CSE overexpression (Fig. 4-5C). In the insulin resistant cell model, we found that insulin-stimulated glucose consumption was impaired but the basal glucose consumption remained unchanged (Fig. 4-5A). Insulin resistance is a state in which a given concentration of insulin produces a less-than-expected biological effect. Therefore, insulin at physiological level may generate little effect on glucose consumption under insulin resistance status.

The AMP-activated protein kinase α2 catalytic subunit controls whole-body insulin sensitivity (Viollet B, et al., 2003). These impairing effects of H_2S have also been linked to AMPK inhibition, since the decreased expression of phospho-AMPK was

observed after H_2S treatment (Fig. 4-3A) and AICAR (an AMPK activation reagent) completely reversed NaHS impaired glucose consumption (Fig. 4-3B). Moreover, CSE overexpression by adenovirus displayed impaired glycogen storage (Fig. 4-2E). CSE was demonstrated to be the predominant enzyme for H_2S generation in liver cells, considering that H_2S production is remarkably less in the liver of CSE KO mice (Mani, et al., 2011). Previous study from our lab also revealed that NaHS deteriorated glucose tolerance in mice and increased blood glucose level in nonfasting mice (Yang, et al., 2011). PPG, a CSE inhibitor, significantly improved insulin-stimulated glucose consumption in the insulin resistant $HepG_2$ cell model (Fig. 4-5A). All these data suggest that CSE should be an important potential target for regulating insulin sensitivity.

It has been reported that liver glycogen stores gradually increase during fetal development and are pivotal for neonatal survival until gluconeogenesis is fully functional in the newborn liver (Girard, et al., 1992). It is worth noting that development of CSE activity in human is dependent on the postnatal age, increasing rapidly after birth and reaching mature levels at about 3 months of age (Zlotkin, et al., 1982; Heinonen, 1973). It was recently shown that CSE can be induced in human fetal liver by agents such as dibutyryl cAMP, glucagon or dexamethasone (Heinonen, 1973) and protein synthesis is required for the increased activity. All of these data strongly indicate the correlation between CSE and gluconeogenesis. Due to the early discovery, none of them is able to link this phenomenon to H_2S production. In this study, we found H_2S can induce hepatic gluconeogenesis, which may have a physiological role in fetal development, but exerts a pathological role during diabetes. A very recent study supported our notion. This laboratory reported H_2S enhanced renal gluconeogenesis by

upregulating the gene expression level and gene transcriptional activity of peroxisome proliferator-activated receptor-γ (PGC)-1 α, a major regulator of gluconeogenesis by activating fructose-1,6-bisphosphatase (FBPase-1) and PEPCK. And the activity of FBPase-1 is downregulated significantly in the kidneys of CSE KO mice (Untereiner, et al., 2011).

Insulin and glucagon are two important hormones for maintaining glucose homeostasis. Physiological concentration of insulin can inhibit CSE expression and H_2S production rate in $HepG_2$ cells, whereas glucagon (10 nM) increased H_2S production rate (Fig. 4-5C, 4-5D). Glucagon is a well-known potent stimulator of hepatic gluconeogenesis via the activation of cAMP formation. The addition of 10 nM glucagon increases H_2S production rate (Fig. 4-5D), and H_2S then enhances hepatic gluconeogenesis and glycogenolysis, suggesting that H_2S may mediate glucagon effect. Indeed, CSE-KO mice displayed an impaired rate of glucagon stimulated glycogenolysis (Fig. 4-4D). CSE expression was not altered by glucagon at 10 nM, indicating glucagon may only affect CSE activity by post-translational modification.

PEPCK is the key rate-limiting enzyme in hepatic gluconeogenesis. It has been well established that GK is the major glucose-sensing enzyme in multiple metabolically active organs, such as liver, brain and pancreas (Schuit, et al., 2001). In liver, GK activity is the rate-limiting enzyme for glucose utilization and glycogen synthesis (Ferre, et al., 1996). We have observed that increased PEPCK activity, but decreased GK activity were induced by H_2S at the physiological range. It is hypothesized that H_2S enhances gluconeogenesis pathway but shuts down glycolysis pathway at the same time. It has been known that activation of AMPK downregulates PEPCK gene expression via

the phosphorylation of a novel transcription factor referred to as AREBP (Inoue, et al., 2006). And glucose-6-phosphatase and PEPCK genes are both downregulated by activation of AMPK (Lochhead, et al., 2000). Therefore, we speculate that H_2S upregulated-PEPCK activity may go through decreased AMPK phophorylation.

Of note, H_2S at concentration higher than physiological related level (above 100 μM) fails to inhibit glucose uptake, and it even significantly increases GK activity. This may be related to the toxicity and pro-apoptotic effect of H_2S at high concentrations (Yang, et al., 2006). Moreover, higher level of NaHS (donor of H_2S) at 0.5 mM has been shown to deplete glutathione in isolated hepatocytes and inhibit ATP synthesis, leading to cytotoxicity (Truong, et al., 2006). This observation is consistent with decreased cell viability in the presence of NaHS at concentrations of 300 μM and above.

Importantly, multiple evidences point to the role of CSE as the dominant H_2S producing enzyme in the liver (Kabil, et al., 2011; Mani, et al., 2011). CSE protein levels were shown to be 60 times higher than cystathionine-β-synthase (CBS) in murine liver. It is estimated that only 3% of H_2S production was attributed to CBS in liver at physiologically relevant concentrations of substrate through transsulfuration pathway (Kabil, et al., 2011). To examine the effect of endogenous H_2S on hepatic glucose metabolism, primary hepatocytes were isolated from WT and CSE-KO mice. CSE gene deficiency plays a critical role in β cell function (Yang, et al., 2011). We found previously that CSE KO mice exhibited a delayed onset of diabetes status and a lower diabetic incidence after challenged with STZ compared to WT mice. Improved islet insulin release was also seen in CSE KO mice compared to WT mice (Yang, et al., 2011).

Our current work further confirms that CSE/H_2S system is involved in glucose metabolism in hepatocytes.

In summary, H_2S downregulated glucose uptake and glycogen storage was mediated by decreased AMPK activation. At the mean while, enhanced activity of gluconeogenic enzyme PEPCK was responsible for H_2S increased glucose production (Fig. 4-6). The total effect of decreased glucose input and enhanced glucose output will contribute to hyperglycemia, which is a major symptom of insulin resistance. Insulin resistance, manifested by decreased glucose peripheral uptake, increased hepatic glucose production, diminished insulin secretion and reduced insulin sensitivity, has been well established to play a central role in the development of type 2 diabetes (Guillausseau, et al., 2008). All our data suggest that H_2S may play an important role in hepatic insulin resistance, and is further involved in the pathogenesis of type 2 diabetes. Inhibition of hepatic H_2S biosynthesis or CSE activity may emerge as a potential organ-specific approach to protect against the development of insulin resistance.

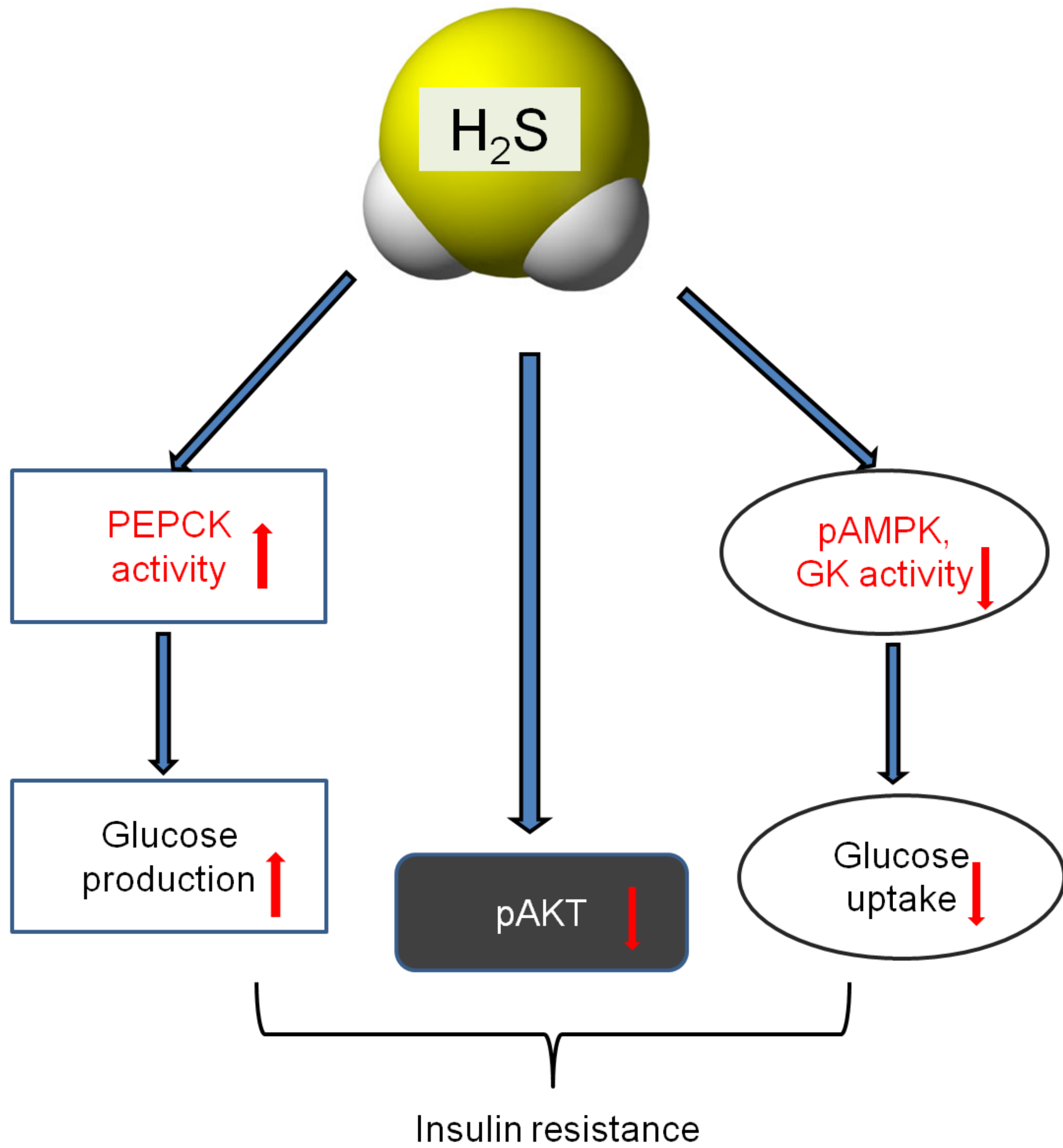


Figure 4-6. Schematic mechanism of H_2S-induced insulin resistance.

CHAPTER 5

Discussion and Conclusion

GENERAL DISCUSSION

Since the discussion related to specific results has been given in chapter 3-4, the general discussion of the results, therefore, is presented in this chapter.

The involvement of H_2S in pathophysiology and development of type 1 diabetes (T1DM) (Yang, et al., 2011) and type 2 diabetes (T2DM) (Wu, et al., 2009) was widely investigated in our lab. Our results implicated H_2S overproduction as a causative factor in the pathogenesis of β-cell death in T1DM (Yang, et al., 2011) and the development of insulin resistance in T2DM (Wu, et al., 2009). H_2S from adipose tissue has been postulated to contribute to the pathogenesis of insulin resistance (Feng, et al., 2009). However, very little is known about the regulatory mechanisms of CSE gene, a major H_2S-producing enzyme in β cells as well as some other tissues (such as liver) important for glucose homeostasis, during the development of diabetes.

In the present study, we demonstrated that H_2S release was markedly inhibited when pancreatic β cell lines and freshly isolated rat pancreatic islets were incubated with high glucose compared to those treated with basal glucose. High glucose not only decreased CSE activity, but also significantly reduced CSE mRNA and protein expression. Pyruvate and 2-deoxy-D-glucose, as glucose metabolites, similarly down-regulated CSE protein expression. However, the inhibitive effect of high glucose on CSE expression was not mimicked by its analogue (L-glucose), osmosis control (mannitol) or membrane depolarization reagent (KCl).

To further explore the mechanism, we demonstrated the involvement of a Sp1 binding sequence in glucose regulated CSE activity, which is located in the core CSE

promoter. It is concluded that glucose stimulates the phosphorylation of Sp1 via p38 MAPK activation, which leads to decreased binding of Sp1 to the CSE promoter and down-regulation of the CSE gene expression.

We also tested the effect and functions of CSE/H_2S system to hepatic glucose metabolism. CSE is demonstrated to be the dominant H_2S producing enzyme in the liver. In the present study, we found that H_2S significantly impaired glucose uptake and glycogen storage (from glucose as the sole substrate) at basal or insulin stimulated condition in $HepG_2$ cells, which might be mediated by decreased glucokinase (GK) activity. Adenovirus-mediated CSE overexpression increased endogenous H_2S production and lowered glycogen synthesis in $HepG_2$ cells. Glycogen content was found to be significantly higher in liver tissues of CSE knockout mice compared to that from wild type mice in both fed and 6 hour fasting conditions. NaHS induced reduction of AMPK phosphorylation was shown to mediate NaHS-impaired glucose uptake. Furthermore, H_2S increases glucose production likely through increased phosphoenolpyruvate carboxykinase (PEPCK) activity. In addition, insulin at the physiological range inhibited CSE expression and H_2S production, meanwhile H_2S diminished insulin-stimulated phosphorylation of Akt (a marker of insulin resistance) in $HepG_2$ cells. However, CSE expression was induced in insulin resistant $HepG_2$ cells. In a nutshell, the interaction of H_2S and insulin in liver is suggested to play an important role in regulating insulin sensitivity and glucose metabolism.

1. H_2S and insulin secretion

Insulin release is regulated by physiological substances including various hormones, autacoids, neurotransmitters, and nutrients. Most of these substances regulate

insulin release by modulating their intracellular signalling molecules. Until now, an endogenous signalling molecule directly inhibiting insulin release has not been explored in detail (Kaneko, et al., 2006; Sharp, et al., 1996). From this perspective, H_2S is a novel signalling molecule which plays a suppressive role in insulin release from pancreatic β cells. Therefore, it becomes imperative to investigate the involvement of H_2S in the mechanism of insulin release in the physiological or diabetic status.

Our lab and other groups have reported that H_2S and L-cysteine exert a physiological inhibitory role on insulin secretion from insulin-secreting β cell line (INS-1E, MIN6 and HIT-T15 cells) and isolated rat islets (Yang, et al., 2005; Kaneko, et al., 2006; Ali, et al., 2007). However, this inhibition was shown to occur via varied mechanisms, such as opening of K_{ATP} channels (Yang, et al., 2005), a K_{ATP} independent mechanism (Kaneko, et al., 2006), regulation of intracellular Ca^{2+} concentration (Ali, et al., 2007) and direct inhibition of VDCC in pancreatic β cells (our unpublished data). Therefore, the relative portion of each specific mechanism remains to be investigated in great detail. The above evidence clearly shows that H_2S suppresses insulin secretion from pancreatic islets. It is reasoned that that elevation of the H_2S levels derived from L-cysteine metabolism is possibly involved in a vicious cycle of impaired insulin secretion under diabetic status, since the activities of H_2S-producing enzymes (CBS and CSE) were reported to become higher in diabetic animals and patients (Veldman, et al., 2005; Wijekoon, et al., 2005; Ratnam, et al., 2002; Hargrove, et al., 1989).

Our data suggested that high glucose suppressed CSE gene transcription, CSE protein expression and activity in the physiological condition, further inhibited H_2S production (mainly from CSE) in pancreatic β cells. Considering the inhibitory role of

H_2S to insulin secretion, glucose modulated down-regulation of CSE may mediate and contribute to glucose-stimulated insulin secretion (GSIS). During this process, SP1 phosphorylation by p38 MAPK acts as one molecular link between glucose level and CSE production (Zhang L, et al., 2011). The network mechanism has been summarized in Figure 5-1.

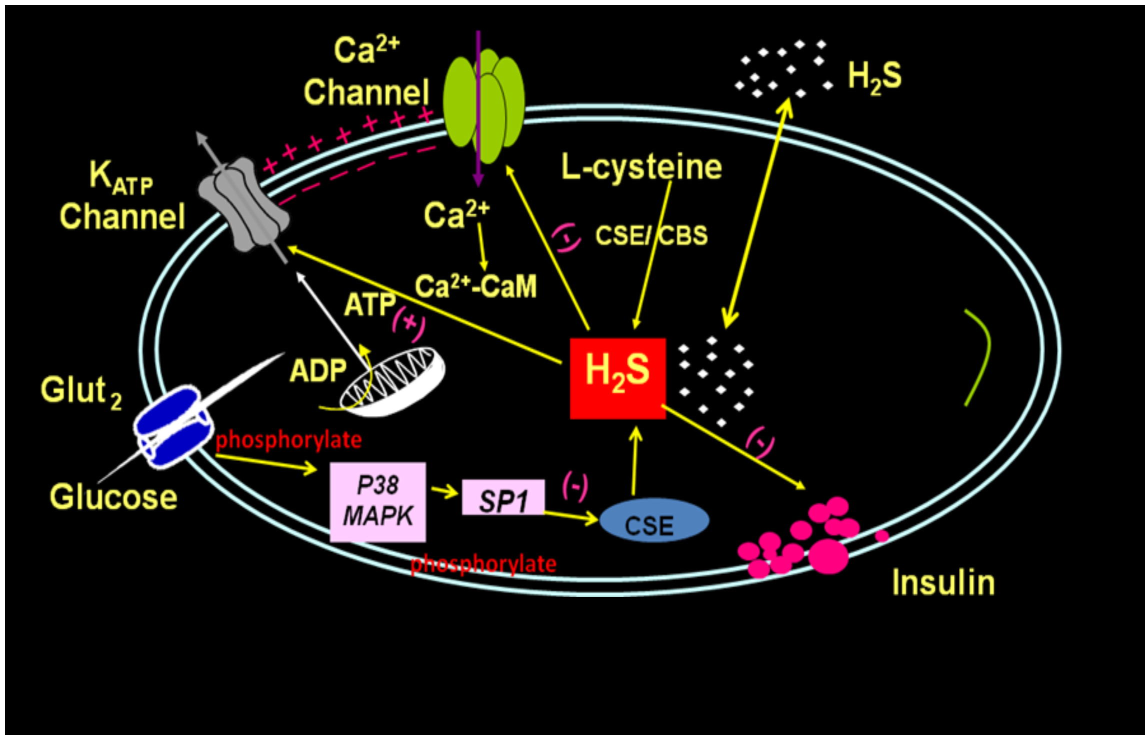


Figure 5-1. Regulation of H_2S on glucose stimulated insulin secretion in pancreatic β-cells

2. CSE/H_2S system and Type 1 diabetes

Type 1 diabetic patients were observed to have decreased homocysteine level (Veldman, et al., 2005). STZ is a diabetogenic agent that caused hyperglycemia due to partial destruction of β cells, within 4–5 days following its injection. In streptozotocin

(STZ)-induced Type 1 diabetic models, hepatic or pancreatic activities of CBS and CSE were significantly up-regulated and were associated with decreased plasma homocysteine levels (Wijekoon, et al., 2005; Ratnam, et al., 2002). The above studies relevant to CBS or CSE mainly focus on altered homocysteine metabolism with altered H_2S metabolism being largely neglected.

Moore's lab (Yusuf, et al., 2005) reported that giving insulin to STZ-injected rats reversed the increase in H_2S synthesizing activity by a direct effect of insulin on CSE expression or activity (Ratnam, et al., 2002). H_2S can reduce β-cell mass (Yang, et al., 2007) and increase K_{ATP} channels activities (Yang, et al., 2005) in pancreatic β cells, both of which are associated with the development of diabetes. In our studies (Yang, et al., 2011), we examined the effects of genetic and pharmacologic ablation of CSE (a major H_2S-producing enzyme in pancreatic β cells) on β-cell functions and their correlation with STZ-induced diabetes. Mice deficient of CSE gene exhibited a delayed onset of diabetic status including less apoptotic death in β cells and improved glucose tolerance after injections of STZ. Administration of PPG to inhibit CSE activity similarly protected WT mice from STZ-induced hyperglycemia and hypoinsulinemia. Injection of NaHS as a H_2S donor instantly increased blood glucose level, decreased plasma insulin, and deteriorated glucose tolerance in mice.

To summarize, our results provide evidence that STZ induces intra-islet H_2S production, and then contributes to the β cell death in type 1 diabetes. Previously, the translation of basic science findings into the clinical settings (bench to bed studies) has demonstrated the therapeutic potential of gaseous signalling molecules like NO (Griffiths, et al., 2005). Our data suggested a potential therapeutic possiblity to protect β cell

function in type 1 diabetics by specifically inhibiting CSE in pancreatic β cells. The simplified relationship between CSE/H_2S system and type 1 diabetes has been outlined in Figure 5-2.

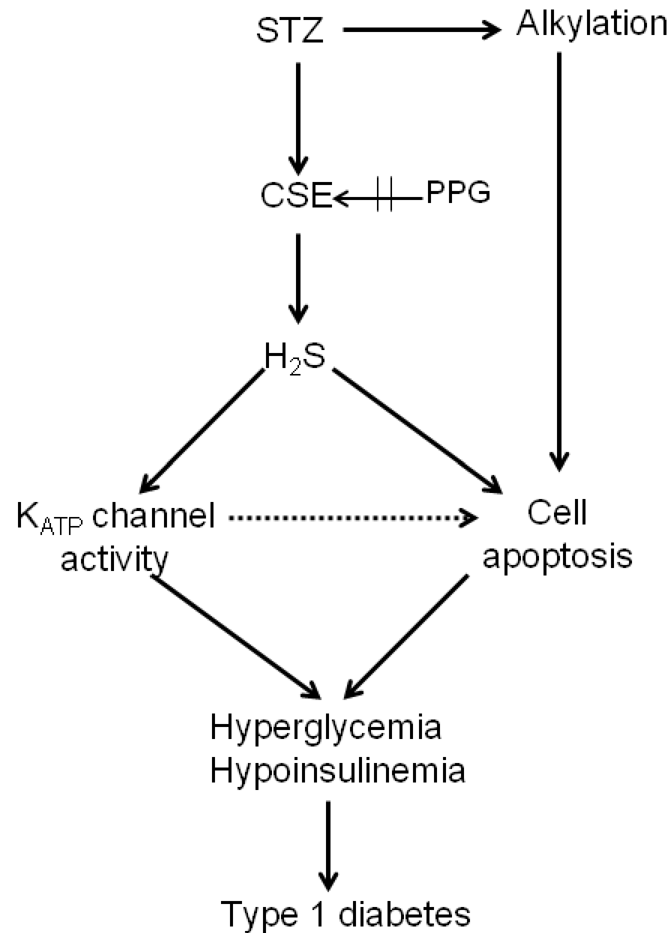


Figure 5-2. Schematic diagram of the pathogenic role of CSE/H_2S system in type 1 diabetes. STZ-induced diabetes is largely mediated by the effect of pancreatic produced H_2S on β cell apoptosis and K_{ATP} channel activity, which lead to hyperglycemia and hypoinsulinemia, both of them are typical symptoms of type 1 diabetes.

3. Role of H_2S in insulin resistance and metabolic syndrome

Insulin resistance (IR) is a condition when the regular amount of hormone insulin becomes less effective at lowering blood glucose levels. Insulin resistance in muscle and adipose cells impairs glucose uptake (and also local storage of glucose as glycogen and triglycerides, respectively), whereas insulin resistance in hepatocytes results in reduced glycogen synthesis and storage and a failure to suppress glucose production and release into the circulation (Le Roith, et al., 2001). Previously, CSE/H_2S system was identified in both rat adipocytes and pre-adipocytes and it is indicated that adipose could secret H_2S and act as an autocoid to inhibit basal and insulin-stimulated glucose uptake and affect the pathogenesis of insulin resistance (Feng, et al., 2009). H_2S induced hepatic insulin resistance was demonstrated in our present study (Chapter 4). As a marker of insulin resistance, impaired phosphorylation of AKT was seen after exposure to H_2S. Impaired glucose uptake, glycogen storage, and increased gluconeogenesis in hepatocytes were also observed after treatment of H_2S, which are symptoms of hepatic insulin resistance. Taken together, H_2S is considered as “a novel insulin resistance regulator”.

The metabolic syndrome is a group of disorders that cause the high risk of developing cardiovascular events and type 2 diabetes and is featured by the symptoms including central obesity, increased blood triglycerides, reduced plasma HDL, elevated blood glucose and hypertension (Aschner, et al., 2010; Mottillo, et al., 2010; Weiss, et al., 2010). The pathogenesis of the metabolic syndrome is controversial, but it is normally considered that insulin resistance is the central reason. However, up to now there is barely any investigation on the role of H_2S in the development of lipogenesis induced by

high lipid and carbohydrate diet. Sulfide poisoning has been indicated to induce metabolic syndrome (Wang, 2012). The role of H_2S in the pathogenesis of hypertension and diabetes has been elaborated in the literature review part, which are two abnormalities of metabolic syndrome. Obesity is another part of metabolic syndrome, and the development of obesity is possibly linked to H_2S metabolism. Indeed, lower plasma H_2S level in obese people has been previously shown (Whiteman, et al., 2010). Recently, a great review has covered many aspects of H_2S effect in various targets of metabolic syndrome, including the relationship of H_2S with pancreatic function and insulin secretion, interplays between H_2S and methylglyoxal, plasma glucose, obesity and hyperlipidemia, as well as hypertension (Desai, et al., 2011). However, all these reports deal with the specific conditions of hypertension, obesity and diabetes solely and they are only limited to the effects of H_2S administrations in cultured cells and animal models. Obviously, more efforts need to be spent before a big picture of the role of H_2S in the metabolic syndrome emerges. Whether the altered H_2S metabolism is the cause or the consequence of obesity and insulin resistance is not yet understood.

4. Limitations of study

For the limitations of H_2S study generally, several laboratories are investigating the role of H_2S in the pathogenesis of diabetes mellitus besides our group. Researchers around the globe have thrown themselves enthusiastically into the task of probing the roles of this novel biologically active gas in both physiological and pathological conditions. However, several fundamental issues remained obscure, such as these: What is the effective and physiological concentration range of endogenous H_2S? To what

extent can H_2S effect be mimicked by NaHS and other donors? How does this gas work exactly at the cellular level?

It's still a controversy if circulating H_2S level is decreased or increased in diabetic patients. Until now, most of the studies were done in rodents. Moore et al. demonstrated the induction of H_2S-producing enzyme CBS in the pancreas of animals treated with β cell toxin streptozotocin (Yusuf, et al., 2005). In addition, CSE protein expression in pancreatic islets and serum H_2S level of ZDF rats (a type 2 diabetes model) was significantly higher than that of age-matched ZL and ZF rats (Wu, et al., 2009). However, it seems like H_2S in the plasma was lower in diabetic conditions in various diabetes model used (Brancaleone, et al., 2008), as well as a small amount of human data. Considering the biggest controversy exist in the methodology of measuring H_2S level in extracellular fluids or in plasma, additional work should be done to determine the absolute value of free forms of H_2S in the circulation of diabetics compared to that of normal control. Ideally, measurement of exhaled H_2S can be a feasible approach to differentiate diabetics and non-diabetics. Future studies need to be conducted to examine the specific role of obesity in the circulating H_2S level of diabetics. A recent investigation suggests adiposity (Whiteman, et al., 2010) is an independent factor contributing to the low level of H_2S in diabetes.

There are also clear contradictory results as to whether H_2S exerts cytoprotective or cytotoxic effect. There is a trend that low concentrations of H_2S is cytoprotective, whereas high level of H_2S starts to induce toxic effect. Cytoprotective mechanisms of H_2S include direct and indirect antioxidant/redox based mechanism (Jha, et al., 2008), upregulation of antioxidant pathway proteins such as thioredoxin (Jha, et al., 2008) and

Hsp90 (Yang Z, et al., 2011). Cytotoxic mechanisms include the depletion of antioxidants (Truong, et al., 2007), pro-oxidative response (Eghbal, et al., 2004), modulation of ERK1/2 phosphorylation and p21Cip/WAK-1(Yang, et al., 2004a), induction of DNA injury and alterations in apoptotic gene expression (Baskar, et al., 2007). However, concerning the varied effects of H_2S in pancreatic β cells, only the concentration differences is not enough to explain the contrasting results. As our lab reported before, endogenous H_2S inhibits insulin secretion (Yang, et al., 2005) and induces endoplasmic-reticulum stress response (Yang, et al., 2007), which results in apoptosis of insulin-secreting β cells. Meanwhile, another group observed that L-cysteine and NaHS increased glutathione content and exerted cytoprotective roles in MIN6 cells. Therefore, cell type differences, cell culture conditions, length of exposure, different H_2S donor, and future studies have to be all considered to answer the bigger question: does H_2S *in vivo*, even in human pancreatic β cells, exert cytoprotective or cytotoxic role?

For the limitation of our pancreas study (Chapter 3), we adopted insulin secreting cell line INS-1E (from rat) and rat pancreatic islets cultured *in vitro* to do our study. It is important to state that in vivo intact islet structures (comprising α, β and δ-cells, which secrete glucagon, insulin and somatostatin respectively) are required to maintain appropriate and pulsatile hormone secretion in response to nutrient stimuli. Therefore, we are not clear if H_2S can modulate the whole hormone system *in vivo* including the proportion of glucagon, insulin or other hormone (like leptin), which may indirectly suppress insulin secretion.

For the limitation of our liver study (Chapter 4), we measured absolute glycogen levels in $HepG_2$ cells by a colorimetric method with glucose as sole substrate; however,

many studies have determined the effects of chemicals on glycogen as incorporation of radioactive glucose, which is more direct. Due to our purpose and limitation of our lab condition, the radioisotope method was not adopted for our study, as it does not consider effects of agents on endogenous glycogen stores. In our study, the reduction of glycogen content by H_2S might be explained by both decreased glucose consumption and increased glycogenolysis. Similarly for the assay of gluconeogenesis, pyruvate and lactate were added as substrates in our studies to produce glucose and we measured glucose production from cell culture medium. The increased glucose production by H_2S is probably associated with both elevated gluconeogenesis from substrates and increased glycogenolysis from glycogen breakdown.

Considering all the literatures reported so far and our own studies, it seems that the inhibition of pancreatic and hepatic H_2S biosynthesis is beneficial for diabetes, whereas supplementation of H_2S emerges as a therapeutic approach for diabetic complications in cardiovascular systems (Suzuki, et al., 2011). Clearly, more investigations are needed to develop a valid therapeutic approach to diabetes related to H_2S biosynthesis.

CONCLUSIONS

Our studies demonstrated the important role of CSE/H_2S system in glucose homeostasis. Therefore, high glucose would offer two different effects: down-regulating CSE expression and inhibiting CSE activity. The latter may function as a physiological response responsible for glucose induced insulin secretion. The reduced H_2S production would lead to increased insulin release in pancreatic β-cells since H_2S itself can open K_{ATP} channels and hyperpolarize the membrane to inhibit insulin release.

In the hormone level of whole body, insulin was observed to decrease CSE expression and H_2S production in liver cells. Considering that H_2S itself increased glycogenolysis and gluconeogenesis, but inhibited glucose utilization in liver cells, decreased H_2S affected by insulin will improve glucose utilization, decrease glucose production from liver, thereby glucose level will go back to normal. It is a circle of physiological regulation. This theory has been summarized in Figure 5-3. However, imbalanced overproduction of H_2S in the liver or pancreatic β cells would exert the opposite effect and might constitute one of pathogenic mechanisms for diabetes related to H_2S.

In this context, a hypothetic model of 'sweetened rotten egg (H_2S)' for physiological regulation of pancreatic and liver function is proposed. This model integrates the role of pancreas and liver in regulation of 'sweet' glucose metabolism and inhibitory role of the 'rotten egg' gas H_2S in insulin release and glucose utilization. As abnormal insulin release from pancreas and impaired insulin action in liver are ubiquitous

for many disorders of insulin resistance syndrome, including obesity and hypertension, a critical evaluation of the role of H_2S as a 'sweetened rotten egg' in pancreas and liver will shed light on understanding of pathogenesis and management of these disorders.

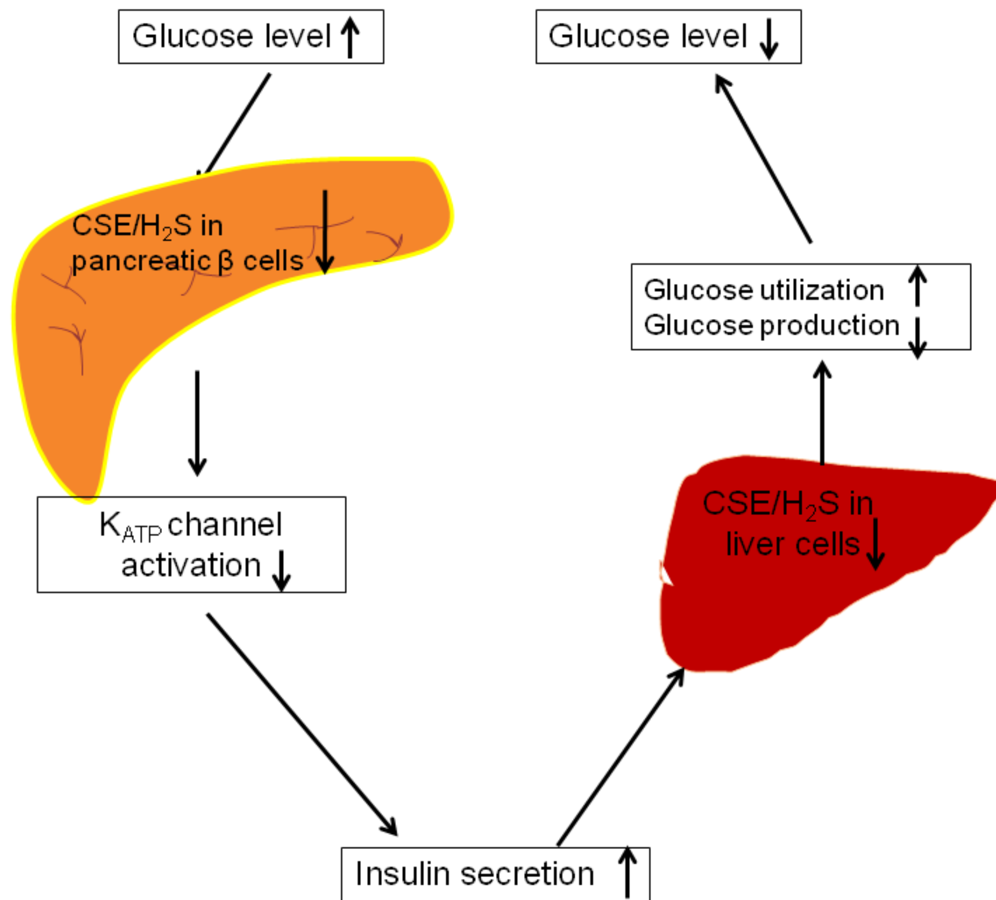


Figure 5-3. Summarized mechanism of the role of CSE/H_2S system in glucose homeostasis

SIGNIFICANCE OF THE STUDY

Diabetes is one of the fastest growing and most costly metabolic disorders worldwide, it's more common in developed countries, but now its incidence is also increasing rapidly in developing countries due to urbanization and lifestyle changes. Until now, the mechanisms of its pathogenesis have not been fully understood (Mathis, et al., 2001).

Approximately, 285 million people worldwide are affected by diabetes. With 7 million more people developing diabetes annually, this number is expected to reach 438 million by 2030. Among them, more than 9 million are Canadians suffering from diabetes or prediabetes. An estimated 90% of diabetic patients are T2DM due to a number of factors including sedentary life style, aging and obesity, et al. The personal costs of diabetes are largely due to the increased likelihood of complications such as diabetic neuropathy, nephropathy, cardiovascular disease, retinopathy, stroke, amputation and erectile dysfunction. Almost 80% of people with diabetes will die as a result of cardiovascular emergency. Overall, diabetes is a contributing factor of deaths of over 41, 500 Canadians each year. Furthermore, the financial burden of diabetes and its complications is huge. The direct cost for each diabetic patient for medication and equipments ranges from $1,000 to $15,000 per year. It is estimated that the Canadian healthcare system will spend $16.9 billion a year on diabetes by 2020 (Canadian Diabetes Association, http://www.diabetes.ca/diabetes-and-you/what/prevalence/).

Clinical therapeutic targets based on the current mechanisms have not achieved as much as expected. Diabetes still mystifies and fascinates both practitioners and investigators due to its elusive causes and multitudes of symptoms. As a novel gasotransmitter, H_2S has been shown to contribute to or be involved in the development and progression of diabetes (Wu, et al., 2009; Yang, et al., 2011). However, very little is known about the molecular regulatory mechanisms of CSE gene, a major H_2S-producing enzyme in β cells as well as some other insulin sensitive tissues (such as liver), during the development of diabetes. The novelty of our experiments is that for the first time, we have displayed the interaction between glucose metabolism and CSE gene expression and H_2S production in both pancreatic β cells and liver cells, and unveiled the underlying molecular mechanisms. Clarifying the role of CSE/H_2S system in glucose homeostasis and the development of diabetes is meaningful and may lead to discoveries of new protective interventions or therapeutic treatment for diabetes and associated complications. The derived novel discoveries can either be transformed to pharmaceutical anti-diabetic drugs or to new diagnostic methods for early diagnosis and follow-up the progression of diabetes.

FUTURE DIRECTION

To extend and expand our findings reported in this thesis, we are planning to carry out the following experiments in the future:

1. To further investigate the mechanism of H_2S affected glucose catabolism. The expressions and activities of glucose metabolism-related genes glucose-6-phosphatease (G-6-P), fructose-1,6-bisphosphatease (FBP), hexokinase (HK), glucokinase (GK), pyruvate kinase (PK) and pyruvate carboxylase (PC) in islets, liver, adipose tissue and/or skeletal muscle will be determined. Inflammation (TNF-α, IL-1β, IL-6, monocyte chemotactic protein-1) and apoptosis (caspase-3, CHOP, and SREBP-1c)-related genes in islets and hepatocytes will be detected by real-time PCR, Western blots, and/or ELISA. The activities and expression levels (mRNA and protein) of different enzymes involved in glucose metabolism will be determined and quantified using Real-Time PCR and Western blot analysis.

2. To elucidate the correlation between CSE deficiency, H_2S production and high fat diet (HFD)-induced metabolic syndrome, which lead to central obesity, fasting hyperglycemia, hypercholesteremia and insulin resistance. Briefly, age-matched male CSE WT mice and CSE KO mice will be fed ad libitum a low- or high-fat diet (LFD or HFD), with 10% or 45% kcal from fat, respectively (Research Diets, New Brunswick, NJ). The possible mechanisms for the role of CSE deficiency will be explored.

3. To investigate the possibility of H_2S modulated enzyme activity important for glucose catabolism through sulfhydration. Hexokinase (HK), glucokinase (GK), pyruvate

kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate carboxylase (PC) enzyme activity after H_2S treatment will be measured and sulfhydration of these enzyme protein will be assayed by biotin switch method.

4. To develop new assays to detect free H_2S in real time. The capability to measure free H_2S generated within cells at low concentrations in real time would be a significant advance to H_2S research.

5. To get a clearer idea of the plasma/serum/tissue concentration of H_2S and its physiological significance in terms of predicting the biological relevance of endogenous and exogenous H_2S provided by slow- and fast-releasing donor drugs.

6. To develop H_2S releasing drugs at different rates in a wide range. A better understanding of their precise cellular and intracellular sites of action/breakdown will be of benefit in establishing whether this mediator can be targeted with drugs for therapeutic benefit.

7. To get greater insights into the mechanism of the cell signalling activity of H_2S. It is critical to determine the relative contributions of redox mechanisms and intracellular protein S-sulfhydration and their interconnection with the known effects of this gaseous signalling molecule on ion channels, transcription factors, and kinases, which seem to be the major molecular targets of H_2S.

8. To clarify the role of H_2S in diabetes caused inflammation and explore potential treatment strategy

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APPENDIX

Before the present thesis is going into print, some manuscripts and abstracts produced from thesis data have been published or submitted for publication in the peer reviewed journals or communicated in the national and international conferences. These publications are listed below.

Articles published or submitted in peer-reviewed journals

1). Zhang L, Yang G, Tang G, Wu L, and Wang R*. (2011) Rat pancreatic level of cystathionine γ-lyase is regulated by glucose level via specificity protein 1 (SP1) phosphorylation. *Diabetologia*, 54(10):2615-2625. (Impact Factor: 6.973).

2). 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. *American Journal of Pathology,* 179(2):869-879. (Impact Factor: 5.971).

3). Zhang L, Yang G, Wu L, Wang R*. (2011) H_2S impairs glucose utilization but increases gluconeogenesis in $HepG_2$ hepatoma cells (revised by *Endocrinology*).

4). Tang G, Zhang L, Yang G, Wu L, Wang R*.(2011) H_2S-induced inhibition of L-type Ca^{2+} channels and insulin secretion in mouse pancreatic beta cells (submitted to *Endocrinology*).

Abstracts communicated in conferences

1). Zhang L, Yang G, Tang G, Wu L, Wang R. Glucose represses cystathionine gamma-lyase expression in pancreatic beta cells. **The First International Conference of**

Hydrogen Sulfide in Biology and Medicine Conference held at Shanghai, China, June 26-28, 2009

2). Zhang L, Yang G, Wu L, Wang R. H_2S impairs insulin-stimulated glucose utilization and increases gluconeogenesis in $HepG_2$ hepatoma cells. **CPS (Canadian Physiology Society)/CAP Net Winter meeting 2011 on "Physiological mechanisms of perception cognition and action"** held at Sainte Adele, Quebec, February 10-12, 2011