ENHANCED METHYLGLYOXAL FORMATION IN CYSTATHIONINE γ-LYASE KNOCKOUT MICE

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ABSTRACT

Methylglyoxal (MG) is a reactive glucose metabolite and a known causative factor for hypertension and diabetes. Hydrogen sulfide (H_2S), on the other hand, is a gasotransmitter with multifaceted physiological functions, including anti-oxidant and vasodilatory properties. The present study demonstrates that MG and H_2S can interact with and modulate each other's functions. Upon *in vitro* incubations, we found that MG and H_2S can directly interact to form three possible MG-H_2S adducts. Furthermore, the endogenous production level of MG or H_2S was significantly reduced in a concentration-dependent manner in rat vascular smooth muscle cells (A-10 cells) treated with NaHS, a H_2S donor, or MG, respectively. Indeed, MG-treated A-10 cells exhibited a concentration-dependent down-regulation of the protein and activity level of cystathionine γ-lyase (CSE), the main H_2S-generating enzyme in the vasculature. Moreover, H_2S can induce the inhibition of MG-generated ROS production in a concentration-dependent manner in A-10 cells. In 6-22 week-old CSE knockout male mice ($CSE^{-/-}$), mice with lower levels of vascular H_2S, we observed a significant elevation in MG levels in both plasma and renal extracts. Renal triosephosphates were also significantly increased in the 6-22 week-old $CSE^{-/-}$ mice. To identify the source of the elevated renal MG levels, we found that the activity of fructose-1,6-bisphosphatase (FBPase), the rate-limiting enzyme in gluconeogenesis, was significantly down-regulated, along with lower levels of its product (fructose-6-phosphate) and higher levels of its substrate (fructose-1,6-bisphosphate) in the kidney of 6-22 week-old $CSE^{-/-}$ mice. We have also observed lower levels of the gluconeogenic regulator, peroxisome

proliferator-activated receptor-γ coactivator (PGC)-1α, and its down-stream targets, FBPase-1 and -2, phosphoenolpyruvate carboxykinase (PEPCK), and estrogen-related receptor (ERR)α mRNA expression levels in renal extracts from 6-22 week-old $CSE^{-/-}$ mice. Likewise, FBPase-1 and -2 mRNA levels were also significantly down-regulated in aorta tissues from 14-16 week-old $CSE^{-/-}$ mice. Administration of 30 and 50 μM NaHS induced a significant increase in FBPase-1 and PGC-1α in rat A-10 cells. We have also observed a significant up-regulation of PEPCK and ERRα mRNA expression levels in 50 μM NaHS-treated A-10 cells, further confirming the involvement of H_2S in regulating the rate of gluconeogenesis and MG formation. Overall, this unique study demonstrates the existence of a negative correlation between MG and H_2S in the vasculature. Further elucidation of this cross-talk phenomenon between MG and H_2S could lead to more elaborate and effective therapeutic regimens to combat metabolic syndrome and its related health complications.

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To my family,

for their unconditional

love and support

TABLE OF CONTENTS

CHAPTER 5: DISCUSSION AND CONCLUSION

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

3-MST	3-mercaptopyruvate sulfurtransferase
γ-GC	γ-glutamylcysteine synthetase
AC	adenylate cyclase
ACTH	adrenocorticotropic hormone
AGEs	advanced glycation endproducts
AMO	amino oxidase
AR	adrenergic receptor
BAT	brown adipose tissue
BCA	β-cyanoalanine
BMI	body mass index
BP	blood pressure
CaMK	calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CBS	cystathionine β-synthetase
CEL	*N*ε-carboxyethyl-lysine
cGMP	cyclic guanosine monophosphate
CML	*N*ε-carboxymethyl-lysine
CNS	central nervous system
CO	carbon monoxide

CREB	cAMP response element-binding
CRH	corticotropin-releasing hormone
CSE	cystathionine γ-lyase
$CSE^{-/-}$	cystathionine γ-lyase-knockout
DHAP	dihydroxyacetone phosphate
DPI	diphenyliodonium
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
eNOS	endothelial NO synthase
ER	estrogen receptor
ERK	extracellular signal-regulated kinases
ERRα/β/γ	estrogen-related receptor-α/β/γ
F-1,6-P	fructose-1,6-phosphate
F-6-P	fructose-6-phosphate
FBPase	fructose-1,6-bisphosphatase
FOXO1	forkhead box O1
G6Pase	glucose-6-phosphatase
GA3P	glyceraldehyde 3-phosphate
GK	glucokinase
Gly-I	glyoxalase-I

Gly-II	glyoxalase-II
GSH	reduced glutathione
GSH-Px	glutathione peroxidase
GSSG	oxidized glutathione
GSSG-Red	glutathione reductase
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulfide
HDL	high-density lipoprotein
HNF4α	hepatocyte nuclear factor
HRT	hormone replacement therapy
ICAM-1	inter-cell adhesion molecule-1
IL	interleukin
JNK	c-Jun N-terminal kinases
K_{ATP}	K^+-dependent-ATP channels
LDL	low-density lipoprotein
MAPK	mitogen-activated protein kinases
MCAO	middle cerebral artery occlusion
MG	methylglyoxal
NAC	N-acetyl cysteine
NaHS	sodium hydrosulfide

NF-κB	nuclear factor-kappaB
NMDA	*N*-methyl-D-aspartate
NO	nitric oxide
$O_2^{\cdot -}$	superoxide anion
$ONOO^-$	peroxynitrite
PBS	phosphate-buffered saline
PEPCK	phosphoenolpyruvate carboxykinase
PFK	phosphofructokinase
PGC-1α/β	peroxisome proliferator-activated receptor-γ coactivator-1α/β
PK	pyruvate kinase
PNS	peripheral nervous system
PPG	$_{DL}$-propargylglycine
RAGE	receptor for advanced glycated endproducts
RCS	reactive carbonyl species
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAM	*S*-adenosyl-$_L$-methionine
SD	sprague-dawley
SHRs	spontaneous hypertensive rats
SOD	superoxide dismutase

SSAO	semicarbazide-sensitive amine oxidase
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TNF-α/β	tumor necrosis factor-α/β
WKY	Wistar Kyoto
VCAM-1	vascular cell adhesion molecule-1
VSMCs	vascular smooth muscle cells
ZDF	Zucker diabetic fatty
ZF	Zucker fatty
ZL	Zucker lean

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 Metabolic syndrome

Metabolic syndrome, also known as syndrome X, represents a cluster of risk factors that can increase an individual's chance of developing type 2 diabetes mellitus (T2DM) and other cardiovascular diseases (Ford et al. 2002; Alberti et al. 2005). This collection of risk factors includes abdominal obesity (a waist circumference of greater than 102 cm in men and 88 cm in women), high blood pressure (BP; 130/85 mm Hg or higher), elevated fasting blood glucose levels ($\geq$ 5.6 mM or 100 mg/dl), low high-density lipoprotein (HDL) cholesterol ($\leq$ 1.03 mM or 40 mg/dl in men and 1.29 mM or 50 mg/dl in women), and high triglycerides ($\geq$ 1.7 mM or 150 mg/dl) (Alberti et al. 2005). If an individual exhibits three or more of the above described conditions, then he/she is considered to have metabolic syndrome. A study done by Ford et al (2002) demonstrated that the prevalence of metabolic syndrome increases with age.

There is still a great debate on what is the exact cause of metabolic syndrome. Most studies have focused on insulin resistance such as the effects of insulin on glucose metabolism, lipid metabolism, protein synthesis, as well as cell-cycle control and proliferation (Bernal-Mizrachi and Semenkovich 2006). Genetics, older age and lifestyle, including a high-fat diet and inactivity, also appears to play a role. Currently, there is a rise in metabolic syndrome, particularly due to growing rates of abdominal obesity and high BP (Ford et al. 2002). The rise in the diagnosis of metabolic syndrome is of great concern, because metabolic syndrome is a substantial risk factor for cardiovascular diseases, including hypertension and T2DM (Bernal-Mizrachi and Semenkovich 2006).

1.1 Hypertension

Hypertension, or high BP, is a medical condition defined as individuals with chronically elevated arterial BP. The optimal BP reading from a healthy individual should be < 120 mm Hg, systolic, and < 80 mm Hg, diastolic (Carretero and Oparil 2000). However, individuals with BP at or above 140/90 mm Hg are strongly recommended for immediate drug treatment (Chobanian et al. 2003).

There is a strong correlation between high BP and the risk of developing cardiovascular diseases, such as stroke, myocardial infarction, and heart failure (Carretero and Oparil 2000). Indeed, hypertension is the leading risk factor for stroke (O'Donnell 2010). Cardiovascular diseases are responsible for approximately 30% of all deaths worldwide (Murray and Lopez 1997; WHO 2002). In 2000, it was estimated that approximately 1 billion people were hypertensive (Kearney et al. 2005). If there are no serious interventions, that number is predicted to increase by 29%, to nearly 1.56 billion people in the year 2025 (Kearney et al. 2005).

1.1.1 Types of hypertension

To date, hypertension is classified into two distinct categories: primary (essential) hypertension and secondary hypertension. Essential hypertension, for which the medical cause is not clear, represents about 90–95% of cases. The remaining 5–10% of cases, referred to as secondary hypertension pathology, are caused by conditions that affect the kidneys, arteries, heart, or endocrine system.

1.1.2 Pathogenesis of essential hypertension

Despite the fact that no clear cause is known in the pathophysiological development of essential hypertension, numerous functional abnormalities have been identified (Figure 1-1). These pathophysiologic factors include, but are not limited to, enhanced sympathetic nervous system activity, overproduction of sodium-retaining hormones and vasoconstrictors, inappropriate renin secretion, increased production of angiotension II and aldosterone, abnormalities of resistance vessels, as well as increased oxidative stress, endothelial dysfunction, and vascular remodelling (Oparil et al. 2003). The diagnosis of cardiovascular complications such as diabetes mellitus, insulin resistance, and obesity are also linked to the pathogenesis of essential hypertension (Oparil et al. 2003).

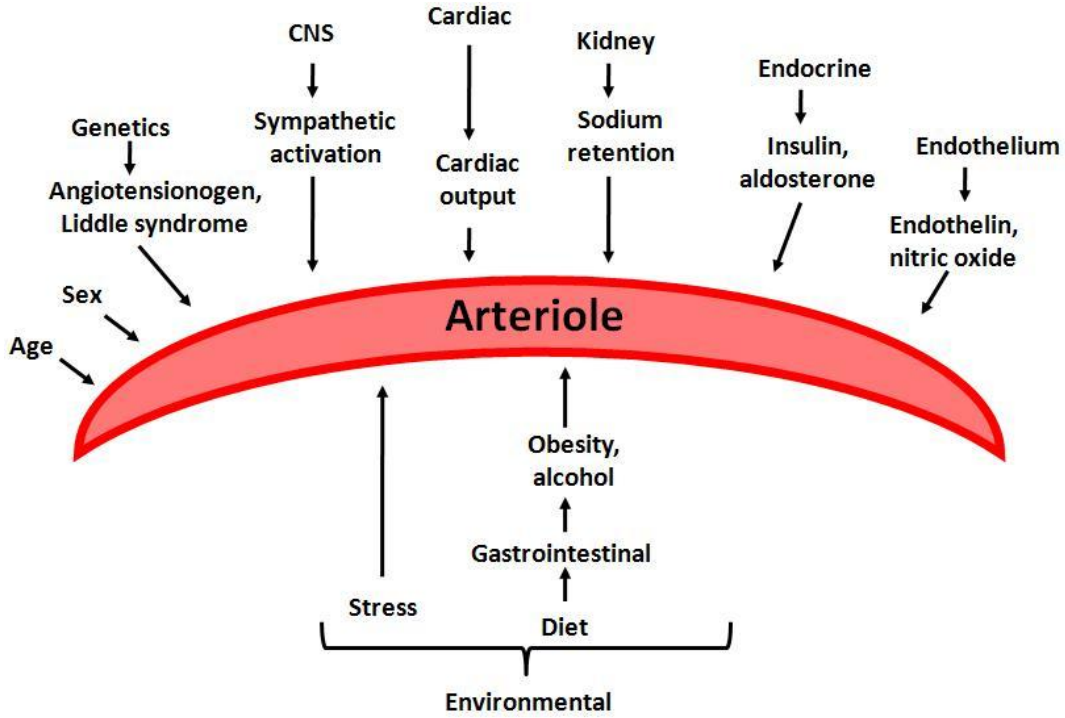

Figure 1-1: Proposed schemes for the pathophysiological development of essential hypertension. CNS: central nervous system. (Modified from *Ann. Intern. Med.* 139:761-776, 2003)

1.2 Diabetes

Diabetes mellitus is a chronic, heterogeneous metabolic illness in which a person has high blood sugar either because the body does not produce enough insulin (type 1 diabetes mellitus; T1DM), or because cells do not respond to the insulin that is produced (T2DM) (Canadian Diabetes Association 2008). In 2000, 171 million people around the world had been diagnosed with diabetes, which is projected to increase to 366 million by 2030 (Wild et al. 2004). The impact of diabetes is also felt in Canada, where approximately 5.5% of the population had been diagnosed with diabetes in 2005 (Landolt et al. 2005).

All forms of diabetes are characterized by chronic hyperglycemia, where the fasting plasma glucose levels are $\geq$ 7.0 mM (126 mg/dl) and $\geq$ 11.1 mM (200 mg/dl) from the glucose tolerance test (WHO 2006). Obesity is strongly associated with an increased risk for the development of T2DM (Mokdad et al. 2001) by contributing to the increased endogenous glucose production (Roden et al. 2000; Staehr et al. 2003). Prolonged exposure to hyperglycemia could increase the risk of developing diabetes-specific microvascular complications, such as microvascular damage to the eyes (retinopathy), kidney (nephropathy), and nerves (neuropathy) (Brownlee 2001), as well as macrovascular complications, including ischaemic heart disease, stroke, and peripheral vascular disease (WHO 2006).

1.2.1 Hyperglycemia

Despite wide variations in daily food intake and physical activity, plasma glucose levels are tightly maintained in the range of approximately 3.9 to 8.9 mM (70 to 160 mg/dl) (Gerich 2000). This complex homeostatic-regulating system involves the precise balancing of glucose production, its reabsorption, as well as its use in the peripheral tissues, which is achieved through a network of hormones, neural pathways, and glucose transport proteins (Marsenic 2009). However, if these mechanisms fail in a way that allows glucose to rise to abnormal levels, hyperglycemia is the result. Hyperglycemia, or high blood sugar, is a chronic elevation in the plasma glucose levels that are higher than 11.1 mM (200 mg/dl), which could lead to organ damage (Brownlee 2001). Hyperglycemia is commonly associated with T2DM, due to the defects of T2DM in both insulin secretion and tissue sensitivity to insulin (Brownlee 2001), as well as obesity (Mokdad et al. 2001; Roden et al. 2000; Staehr et al. 2003).

1.2.2. Obesity

Obesity is a medical condition that has the potential to reduce life expectancy and/or increase cardiovascular diseases, such as T2DM and hypertension (Haslam and James 2005). Obesity is the 6th most important risk factor contributing to the overall burden of disease worldwide (Ezzati et al. 2002), and alarmingly, it is estimated that 1.1 billion adults are overweight, including 312 million of whom are obese, and 10% of children are classified as overweight or obese (Haslam and James 2005). However, with the new Asian body mass index (BMI) of 23.0 kg/m^2 classified as overweight, this number is projected to be about 1.7 billion (James et al. 2004). The

WHO recognizes an individual as overweight if the BMI, a measurement which compares weight and height of an individual, is 25.0 kg/m^2 and is obese if at 30.0 kg/m^2 or higher (WHO 2000). However, the risks of developing hypertension, diabetes, and dyslipidaemia increase from a BMI of approximately 21.0 kg/m^2 (James et al. 2004).

Increased in fat deposition can lead to an increase in the secretion of products such as cytokines and tumour necrosis factor-α (TNF-α), which could potentially lead to insulin resistance (Haslam and James 2005). TNF-α has a paracrine suppressive effect on adiponectin secretion, a powerful insulin sensitizer. Thus, with an expanded adipocyte mass, less adiponectin will be secreted (Haslam and James 2005). Additionally, increased fat deposit in the pancreatic islet cells can decrease the islets' capacity to maintain the increased insulin output in the insulin resistant state (Haslam and James 2005). Dieting and physical exercise are the mainstays of treatment for obesity, but better management and prevention is needed to combat the increasingly growing epidemic of obesity.

2.0 Hydrogen sulfide (H_2S)

In the past, hydrogen sulfide (H_2S) was considered mainly as a toxic gas and an environmental hazard. However, this all changed upon the discovery that this "gas of rotten eggs" was actually being produced in mammals, including humans (Wang 2002). In fact, H_2S is the most recent addition to the endogenous gasotransmitter family that includes nitric oxide (NO) and carbon monoxide (CO). This endogenous physiological regulator/modulator has emerged as a major

player in the immune system, as well as the peripheral and central nervous system (PNS, CNS, respectively) (Szabó 2007).

2.1 Formation of H_2S

2.1.1 Enzymatic synthesis of H_2S

2.1.1.1 The pyridoxal-5´-phosphate-dependent enzymes

In mammalian cells, H_2S is synthesized endogenously by the pyridoxal-5´-phosphate-dependent enzymes including cystathionine γ-lyase (CSE; EC 4.4.1.1) and cystathionine β-synthase (CBS; EC 4.2.1.22) (Wang 2002). The substrate of CSE and CBS, $_L$-cysteine, can be made available from alimentary sources, or endogenous proteins (Szabó 2007). This important amino acid can also be synthesized from $_L$-methionine through the trans-sulfuration pathway, which uses homocysteine as an intermediate (Szabó 2007) (Figure 1-2). Indeed, these enzymes are expressed in a tissue-specific manner. For example, CSE is mainly expressed in the liver and kidney (Ishii et al. 2004; Tripatara et al. 2009), pancreas (Wu et al. 2009), as well as in vascular smooth muscle cells (VSMCs) (Chang et al. 2010; d'Edmmanuele et al. 2009). CBS, on the other hand, is the predominant H_2S-producing enzyme in the CNS (Tan et al. 2010).

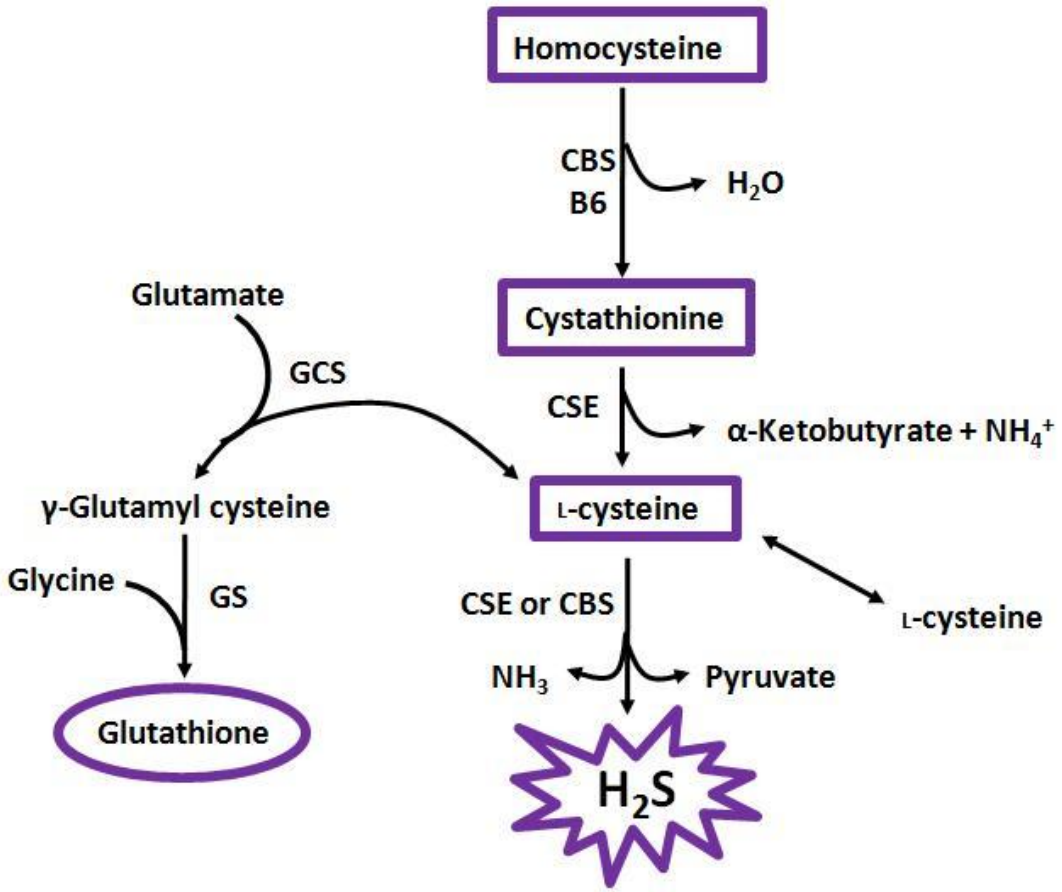

Figure 1-2: Endogenous synthesis of H_2S in mammalian cells. B6: Vitamin B6; CBS: cystathionine β-synthase; CSE: cystathionine γ-lyase; GCS: γ-glutamyl cysteine synthase; GS: glutathionine synthase. (Modified from *Kid. Int.* 76:700-704, 2002)

2.1.1.2 3-mercaptopyruvate sulfurtransferase

Recently, Kimura and colleagues have identified a new H_2S-generating enzyme, 3-mercaptopyruvate sulfurtransferase (3-MST; EC 2.8.1.2), which can produce H_2S in the brain (Shibuya et al. 2009a) and in the endothelium (Shibuya et al. 2009b). This unique mechanism requires 3-mercaptopyruvate, which is produced by cysteine aminotransferase from cysteine and α-ketoglutarate, as a precursor for 3-MST-induced H_2S generation. This discovery introduces the possibility that production and release of H_2S from the endothelium could act as a smooth

muscle relaxant. More research is required in order to identify endogenous regulators, modulators, and other locations where 3-MST may be found in mammalian cells.

2.1.2 Non-enzymatic synthesis of H_2S

The non-enzymatic generation of H_2S is a less significant source of this endogenous physiologic regulator (Searcy and Lee 1998). It is thought that this unique pathway generates H_2S from the reduction of elemental sulfur produced from the reducing equivalents of oxidized glucose, which occurs during glycolysis (Searcy and Lee 1998). In fact, this is the major source of non-enzymatic production of H_2S (Searcy and Lee 1998). To a lesser extent of H_2S production, the phosphogluconate pathway was also described as a non-enzymatic mechanism in erythrocytes (Searcy and Lee 1998). However, more research is needed in order to better understand the production of H_2S through these non-enzymatic pathways.

2.2 Metabolism of H_2S

In order to maintain a proper physiological balance of H_2S, the mammalian cell must be able to metabolize excess amounts of H_2S. This endogenous gasotransmitter can either be oxidized to sulfate in the mitochondria, with GSH acting as an intermediate, or methylated to CH_3SCH_3 in the cytosol (Wang 2002). Furthermore, H_2S can be scavenged by methemoglobin or oxidized glutathione (GSSG) (Wang 2002). Indeed, haemoglobin acts as a "sink" for H_2S in the blood stream, and may compete with other gasotransmitters, such as NO and CO, for binding (Wang 2002). It is thought that H_2S binds to haemoglobin through the attractive forces of the iron

molecule (Szabó 2007). Moreover, the binding of one gasotransmitter could affect the binding probability of other gases, thus altering their bioavailability (Wang 2002).

2.3 H_2S concentration and its various effects

Throughout the years, many groups have reported on the paradoxical effects of H_2S on mammalian cells. In fact, it can be speculated that this contradiction could reside in the amount of H_2S, which could lead to this endogenous gasotransmitter producing cytoprotective or cytotoxic effects.

2.3.1 Low concentrations: cytoprotective effects

2.3.1.1 Anti-oxidant properties

Many reports have shown that low concentrations of exogenously applied H_2S (approximately 10-100 μM) can have anti-oxidant capabilities (Chang et al. 2010; Yan et al. 2006; Kimura and Kimura 2004; Kimura et al. 2006; Whitman et al. 2004; Ali et al. 2006). Indeed, H_2S can significantly increase intracellular levels of the potent anti-oxidant GSH in rat aortic vascular smooth muscle cells (A-10 cells) (Chang et al. 2010), in rat primary cortical neurons (Kimura and Kimura 2004), and in HT22 immortalized hippocampal cells (Kimura et al. 2006). Increased GSH upon H_2S administration could be due to H_2S-induced enhancement of the activity of γ-glutamylcysteine synthetase (γ-GC), resulting in increased γ-GC expression levels, which is the precursor for $_L$-cysteine production and thus GSH production (Kimura et al. 2004, 2006). Furthermore, Kimura et al (2004) also demonstrated that H_2S can enhance the cysteine/glutamate

antiporter (x_c^- system), thus increasing available cysteine for glutamate production. Lastly, due to its reducing abilities, H_2S can also act as an oxidant scavenger. H_2S has been shown to scavenge peroxynitrite ($ONOO^-$) in SH-SY5Y human neuroblastoma cells (Whitman et al. 2004) and in A-10 rat VSMCs (Yan et al. 2006), NO in rat aortic rings (Ali et al. 2006), as well as homocysteine- (Yan et al. 2006) and methylglyoxal (MG)-induced hydrogen peroxide (H_2O_2) and $ONOO^-$ production in A-10 cells (Chang et al. 2010) (the latter will be discussed in more detail in section 3.3.1).

2.3.1.2 Apoptotic effects

The reducing ability of H_2S allows it to regulate cellular signal transduction pathways, leading to the alteration of various genes and gene products expression (Szabó 2007). For instance, H_2S has been shown to induce DNA fragmentation (apoptotic phenotype) due to caspase activation, specifically caspase-3, in human aortic SMCs (Yang et al. 2006). The H_2S-induced DNA fragmentation mechanism most likely occurs *via* the activation of extracellular signal-regulated kinases (ERK) and p38 mitogen-activated protein kinases (MAPK) pathways (Yang et al. 2006). MAPK represents important signal transduction machinery and can influence cell growth, differentiation, and apoptosis (Yang et al. 2004). Additionally, H_2S was also shown to inhibit cell proliferation through the increased activation of ERK and $p21^{Cip/WAK-1}$ in human embryonic kidney (HEK)-293 cells (Yang et al. 2004) and to increase other apoptotic signaling proteins, such as p53, as well as causing the translocation of Bax and cytochrome c (in human pulmonary fibroblasts) (Baskar et al. 2007). Furthermore, H_2S can indirectly inhibit the activation of nuclear

factor-κB (NF-κB) pathway (Oh et al. 2006). Activation of NF-κB has been reported to be essential for proliferation of VSMCs (Bellas et al. 1995). In fact, due to its ability to induce cell apoptosis or inhibit proliferation, H_2S can be used to improve the hypertrophy/hyperplasia state of VSMCs, as well as decrease aortic ring thickening seen in spontaneous hypertensive rats (SHRs) (Shi et al. 2007; Zhao et al. 2001). Overall, this suggests that H_2S has an important role in maintaining vascular integrity.

2.3.1.3 Physiologic vasodilator

Interestingly, unlike CO and NO, H_2S can function as a vasorelaxant through the activation of K^+-dependent-ATP (K_{ATP}) channels in VSMCs (Yang et al. 2005; Zhao et al. 2001, 2003) and in pancreatic β-cells (Cook et al. 1988; Yang et al. 2005; Ali et al. 2007) (the latter will be discussed in more detail in section 2.3.3.3.1). Generally, upon the opening of the K_{ATP} channels in VSMCs, the membrane hyperpolarizes, which causes the voltage-dependent Ca^{2+} channels to close and reduces intracellular Ca^{2+} levels (Szabó 2007). Ca^{2+} plays an important role in the contractile responses of VSMCs, where a low level of intracellular Ca^{2+} results in vasodilation (Szabó 2007).

Zhao et al (2001) were the first group to demonstrate that intravenous injection of H_2S (2.8 and 14 mM/kg) significantly decreased BP in Sprague Dawley (SD) rats, which was successfully attenuated by the K_{ATP} channel inhibitor glibenclamide. These authors also showed that H_2S induced relaxation in isolated rat aortic rings by opening the K_{ATP} channels (Zhao et al. 2001).

The hypotensive responses of H_2S had no effect on heart rate in the SD rats (Zhao et al. 2001). However, the exact mechanism by which H_2S activates K_{ATP} channels in VSMCs is unclear.

2.3.1.3.1 Hypertension in mice with the genetic knockout of the CSE gene

Additionally, the administration of $_{DL}$-propargylglycine (PPG), a CSE inhibitor, significantly increased the BP in SD rats (Zhao et al. 2003). The genetic knockout of CSE in mice caused impaired endothelium-dependent vasorelaxation and an age-related increase in BP, among other findings (Yang et al. 2008). At 7 weeks of age, both male and female $CSE^{-/-}$ mice exhibited higher BP than their wild-type counterparts, which increased in an age-related fashion until at 12 weeks of age the male $CSE^{-/-}$ had a BP reading 18 mm Hg higher than the control mice (Yang et al. 2008). Indeed, upon intravenous bolus injections of NaHS, a H_2S donor, the systolic BP significantly decreased in both $CSE^{-/-}$ and $CSE^{+/+}$ mice, but the magnitude of decrease was greater in the $CSE^{-/-}$ mice, suggesting an enhanced sensitivity of H_2S stimulation in the $CSE^{-/-}$ mice (Yang et al. 2008). Therefore, H_2S is a vital physiologic vasodilator and regulator of BP and, quite possibly, it could be an endothelium-derived relaxing factor (EDRF), or an endothelium-derived hyperpolarizing factor (EDHF), as was first proposed by Rui Wang (Wang 2009).

2.3.1.4 Anti-inflammatory effects

Apart from the previously mentioned H_2S mechanisms, H_2S can also play an important role in inflammation. H_2S was found to be generated at sites of inflammation and can modulate the

ability of neutrophils to cause tissue damage (Zanardo et al. 2006). Because of this, H_2S was shown to exert protective effects in animal models of inflammation and inflammation-related pain (Szabó 2007; Zanardo et al. 2006). Additionally, Moore and colleagues (2007) demonstrated that a sulfide-releasing diclofenac derivative reduced tissue neutrophil infiltration and interleukin (IL)-1β levels, up-regulated IL-10 levels, and attenuated the activation of NK-κB in an endotoxin-induced lung and liver inflammation model. Also, a H_2S-releasing modified anti-inflammatory compound exerted therapeutic effects in rodent models of inflammation (Distrutti et al. 2006; Baskar et al. 2008). Furthermore, H_2S-induced anti-inflammatory actions decreased leukocyte rolling velocity and also suppressed expression of some leukocyte and endothelial adhesion molecules (Zanardo et al. 2006). This mechanism likely occurs through the activation of K_{ATP} channels, since pre-treatment with glibenclamide (a K_{ATP} channel antagonist) reversed the effects of H_2S donors, and the effect was mimicked by pinacidil (a K_{ATP} channel agonist) (Zanardo et al 2006).

It must be noted that there are some contradicting reports regarding the role of H_2S and inflammation. Zhang et al (2007) demonstrated that H_2S injection up-regulated leukocyte attachment and rolling in blood vessels and also increased the intercellular adhesion molecule-1 levels in sepsis mice, and showed that this was attenuated by PPG (Zhang et al. 2007). Additionally, Zhi et al (2007) showed that H_2S administration increased the generation of the pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, through activation of the ERK-NF-κB

signaling pathway in human monocytes. More research is needed in order to clearly identify H_2S as an anti- or pro-inflammatory agent.

2.3.1.5 Suspended animation

Due to its affinity for cytochrome c oxidase, H_2S has been linked to the phenomenon of H_2S-induced suspended animation (Blackstone et al. 2005). To explain this, mice were exposed to 80 ppm H_2S in the air, which resulted in a decrease in their breathing rate from 120 to 10 breaths per minute and body temperature from 37 °C to an ambient temperature of 13°C, along with decreased carbon dioxide production and oxygen consumption (Blackstone et al. 2005). Interestingly, these mice survived this exposure to H_2S for 6 hours and afterwards showed no negative health consequences (Blackstone et al. 2005).

So what could this mean for us? Due to the fact that H_2S can slow metabolic rate and induce a hibernation-like state, this could be used as a life saving tool in emergency-related situations (Szabó 2007). For example, H_2S could provide emergency personnel more precious time to transport victims suffering from trauma to the hospital (Szabó 2007). By slowing the metabolic rate of the victim, such as reducing the respiration rate and temperature, etc, could slow the biological chain reaction of events that occur in trauma-related situations. If researchers are able to safely manipulate the side effects of H_2S poisoning, it is possible that this hazardous gas could be used as a life saving tool.

2.3.2 H_2S-releasing drugs

Currently, research regarding the role of H_2S in various aspects of the metabolic syndrome, including the conditions of hypertension, obesity, and diabetes, is still in its infancy. However, there is promising potential in some novel H_2S-releasing drugs that could be used as preventive agents/treatments for the metabolic syndrome (Figure 1-3). These include a new H_2S-releasing compound that has anti-hypertensive and vasodilator properties known as morpholin-4-ium 4 methoxyphenyl (morpholino) phosphinodithioate (GYY4137) (Li et al. 2008), as well as an H_2S-releasing phosphodiesterase inhibitor compound, which is a selective inhibitor of cyclic guanosine monophosphate (cGMP) phosphodiesterase type-V, and is used in conditions of endothelial dysfunction (Sparatore and Wallace 2006). Also, a new H_2S-conjugated moiety of statin (simvastatin), which can reduce platelet aggregation and increase platelet cyclic adenosine monophosphate (cAMP), shows potential (Wallace et al. 2009). Additionally, a series of H_2S-releasing compounds are being tested to determine their effectiveness as anti-inflammatory agents (Scherrer and Sparatore 2006; Sparatore et al. 2009; Li et al. 2007; Distrutti et al. 2006; Baskar et al. 2008). Inflammation is associated with the metabolic syndrome, including diabetes, obesity, and hyperlipidemia (Desai et al. 2011). Unfortunately, there is much uncertainty regarding the therapeutic usefulness of H_2S in inflammation, since the idea of H_2S having anti-inflammatory properties is controversial. More research is needed in order to clarify whether this physiological regulator has pro- or anti-inflammatory properties, or if the effects are related to concentration.

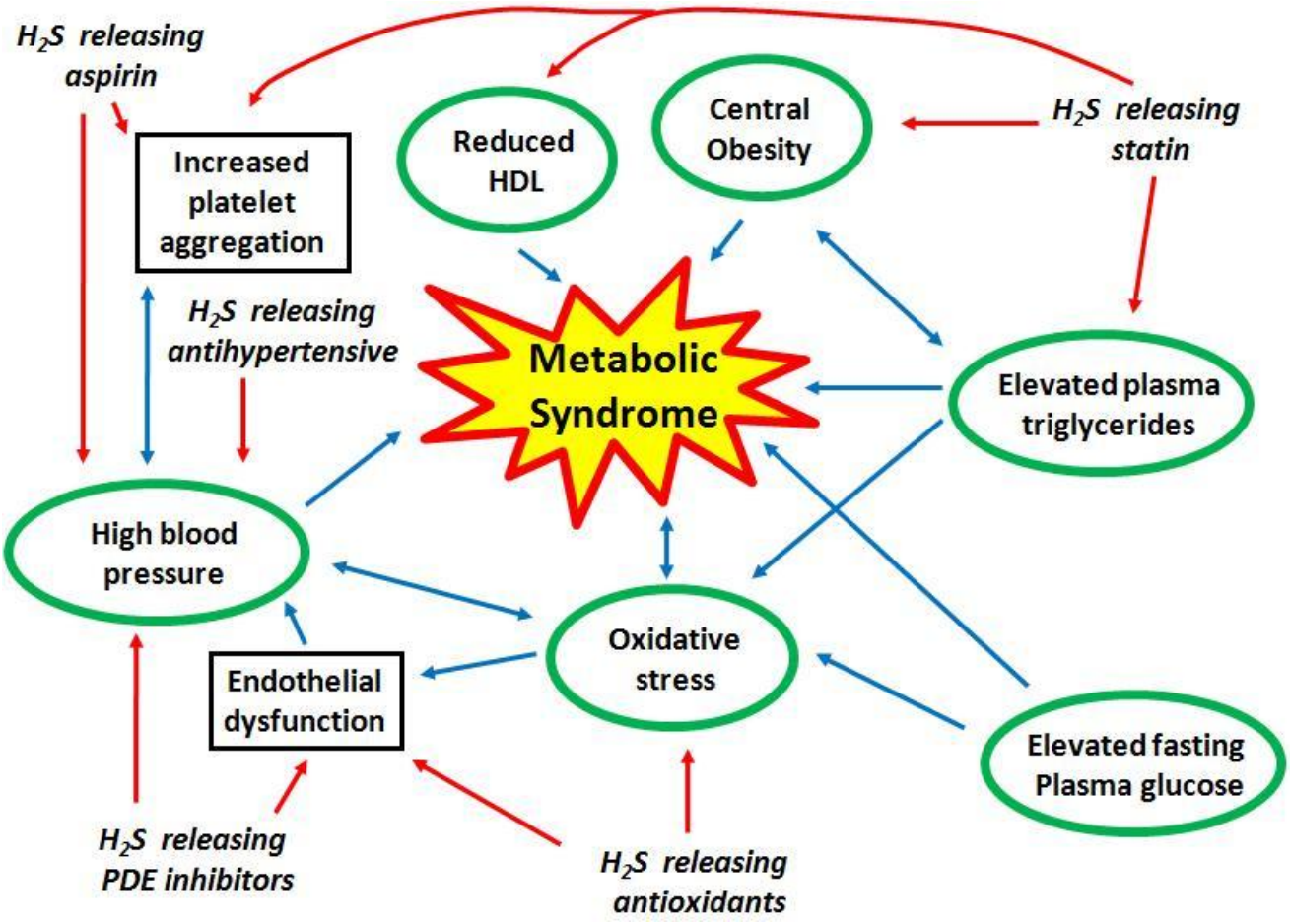

Figure 1-3: The components of the metabolic syndrome and the therapeutic potential of H_2S-releasing drugs. The blue arrows illustrate the major pathological factors that contribute to the development of the metabolic disorder and the red arrows represent the antagonizing actions of H_2S releasing drugs against these factors. (Modified from *Expert Rev. Clin. Pharmacol.* 4:63-73, 2011)

2.3.3 High concentrations: cytotoxic effects

For about 300 years, H_2S has been chemically known to humans, and throughout most of its known existence, it has been identified as an extremely hazardous gas (Reiffenstein et al. 1992). H_2S poisoning usually occurs by inhalation (Reiffenstein et al. 1992). This section will outline the main mechanisms of action regarding fatal H_2S poisoning.

2.3.3.1 Inhibition of cellular respiration

In respect to its high chemical reactivity, H_2S has a strong affinity for cytochrome c oxidase (Szabó 2007). It can be argued that H_2S is far more potent than cyanide (Reiffenstein et al. 1992). The main mechanism of H_2S toxicity is its high affinity for cytochrome c oxidase, and is therefore similar to that of cyanide toxicity (Lowicka and Beltowski 2007). Briefly, cytochrome c oxidase is a key factor in the electron transport chain within the mitochondrion, which regulates cellular respiration (Lowicka and Beltowski 2007). If the activity of cytochrome c oxidase is inhibited, it would arrest aerobic metabolism (Lowicka and Beltowski 2007). H_2S can inhibit cellular respiration by binding to the copper centre of cytochrome c oxidase, thus blocking the regulator of cellular oxygen consumption (Hill et al. 1984).

Because H_2S can inhibit cytochrome c oxidase, and cytochrome c oxidase is found in virtually all cell types, H_2S is classified as a broad-spectrum poison, meaning that it can poison several different systems in the body (Reiffenstein et al. 1992). For example, when a fatally high level of H_2S is inhaled (500-1000 ppm), it will inhibit the cytochrome c oxidase in the brain, reduce oxygen uptake into cells, and inhibit the reuptake of $_L$-glutamate, an excitatory neurotransmitter, thus quickly leading to death (Nicholson et al. 1998). In fact, approximately 500 ppm of H_2S will cause loss of consciousness and between 500-1000 ppm will result in respiratory paralysis, neural paralysis, and cardiac arrhythmias, eventually leading to death (Reiffenstein et al. 1992) (Table 1-1).

Table 1-1: Various concentrations of H_2S and their effects on human physiologic responses. (Modified from *Ann. Rev. Pharmacol. Toxicol.* 109-134, 1992)

Concentration of H_2S (ppm)	Physiological Effects
0.003-0.02	Odour detection
3-10	Obvious unpleasant odour
20-30	Strong offensive odour ("rotten eggs")
50	Conjunctival (eye) irritation
50-100	Irritation of respiratory tract
100-200	Loss of smell (olfactory fatigue)
250-500	Pulmonary edema
500	Anxiety, headache, ataxia, dizziness, stimulation of respiration, amnesia, unconsciousness
500-1000	Respiratory paralysis leading to death, neural paralysis, cardiac arrhythmias, death

2.3.3.2 Oxidative stress

Although low levels of H_2S (approximately 10-100 µM) may have anti-oxidant capabilities, concentrations of H_2S in the millimolar range tend to generate free radicals and oxidants. In the presence of peroxidase and H_2O_2, H_2S yields the free radicals $SH^{\cdot}$ and $S^{\cdot}$ (Wang 2002). These free radicals enable H_2S to be a highly reactive molecule (Wang 2002). In fact, it was demonstrated that high amounts of exogenous H_2S depleted GSH levels in rat primary

hepatocytes (Truong et al. 2006). Additionally, the authors found that H_2S also increased the formation of reactive oxygen species (ROS), presumably by the inhibition of the electron transport chain through cytochrome c oxidase, as well as significantly decreasing available GSH levels (Truong et al. 2006).

2.3.3.3 Inhibition of insulin secretion

2.3.3.3.1 Activation of K_{ATP} channels

Mounting evidence in recent years has indicated that H_2S can act as an endogenous modulator of insulin secretion from β-cells (Yang et al. 2005; Ali et al. 2007; Kaneko et al. 2006, 2009; Yang et al. 2007; Wu et al. 2009). This is largely, if not only, due to H_2S-induced activation of the K_{ATP} channels in insulin-secreting cell lines, such as INS-1E at 100 μM NaHS (Yang et al. 2005) and HIT-T15 at 100 μM H_2S (Ali et al. 2007). When glucose enters pancreatic β-cells, the intracellular levels of ATP increase, which will then inhibit K_{ATP} channels, depolarize the plasma membrane, encourage the opening of Ca^{2+} channels, and cause insulin secretion (Desai et al. 2011). However, upon H_2S-induced K_{ATP} channel activation, the plasma membrane will not depolarize, the Ca^{2+}-channels will not open, and insulin will not be released from pancreatic β-cells. Ali et al (2007) demonstrated that the administration of 10 μM glibenclamide, an anti-diabetic drug that inhibits the opening of K_{ATP} channels, blocked 100 μM NaHS-induced inhibition of insulin secretion from pancreatic β-cells. Another group showed that $_L$-cysteine (the precursor for H_2S formation) and NaHS reduced intracellular Ca^{2+} levels and ATP

production, which also prevented insulin release in isolated mouse islets and in MIN6 cells (mouse β-cell line) (Kaneko et al. 2006).

2.3.3.3.2 Induced apoptosis of pancreatic β-cells

Another piece of evidence that H_2S could be involved in the maladaptive role of insulin secretion is the fact that this K_{ATP} channel activator can also induce apoptosis in β-cells. In fact, by activating the p38 MAPK pathway and up-regulating BiP and CHOP (indicators of endoplasmic reticulum stress), overexpression of CSE induced apoptosis of INS-IE cells (Yang et al. 2007). In agreement, it was shown that NaHS treatment can induce apoptosis in isolated pancreatic acinar cells (exocrine cells that assist in digestion), by causing phosphatidylserine externalization, which is an indicator of early stages of apoptosis (Cao et al. 2006). In fact, Cao et al (2006) also demonstrated that H_2S can induce apoptosis in these cells by activating both mitochondrial and death receptor pathways.

However, H_2S-induced apoptosis in pancreatic β-cells is controversial. On the contrary, Kaneko et al (2006) showed that exogenous 3 mM $_L$-cysteine and 100 μM NaHS prevented 20 mM glucose-induced apoptosis in β-cells, and in fact, increased total glutathione levels. Of note, β-cells are highly susceptible to glucotoxicity because of their low anti-oxidant defense mechanisms (Desai et al. 2011). Furthermore, this same group also demonstrated that 2 mM PPG (a CSE inhibitor) blocked the protective effects of 3 mM $_L$-cysteine against glucose-induced apoptosis in β-cells (Kaneko et al. 2006). Overall, the discrepancy between H_2S-induced

apoptosis in INS-IE cells and H_2S anti-apoptotic affects in isolated mouse islets could be due to different methods used to induce apoptosis. As well, the INS-IE cells were derived from a tumour cell line, which could introduce variable biological differences, such as the cell survival and apoptotic pathways. Therefore, further study is needed in order to determine the exact role H_2S plays in pancreatic β-cell survival mechanisms.

2.4 H_2S and hormones

2.4.1 Insulin

Please see section 2.3.3.3.

2.4.2 Corticotropin-releasing hormone

In addition to the regulation of insulin secretion, H_2S may regulate other hormones. Interestingly, H_2S was shown to decrease the release of corticotropin-releasing hormone (CRH) from the hypothalamus in a concentration-dependent manner (Russo et al. 2000). CRH is secreted in response to biological stress, which then stimulates the synthesis and release of corticotropin, also known as adrenocorticotropic hormone (ACTH), from the anterior pituitary gland (Kimura 2002). ACTH then stimulates the production and release of glucocorticoids, which can affect carbohydrate metabolism, by enhancing gluconeogenesis and lipolysis, as well as immune function, due to its potent anti-inflammatory and immunosuppressive properties (Kimura 2002). Hideo Kimura (2002) also showed that *S*-adenosyl-$_L$-methionine (SAM; a CBS activator) administration mimicked H_2S effects by inhibiting KCl-induced CRH release (Russo

et al. 2000). These results suggest that H_2S plays an important role in regulating the response of the hypothalamo-pituitary axis.

2.4.3 Testosterone

H_2S production has also been linked to testosterone levels. Eto and Kimura (2002) showed that testosterone can regulate the brain H_2S level by enhancing the activity level of SAM. Endogenous H_2S levels are significantly less in the female brain than in the male brain (Eto and Kimura 2002). These authors showed that testosterone administration increased the production of H_2S levels in the female brain to the levels that were comparable to those in the male brain. Additionally, significantly lower testosterone, SAM, and H_2S levels were observed in brain matter from castrated male mice (Eto and Kimura 2002).

It has been suggested by Cirino and associates (2009) that H_2S could be the key player in testosterone-induced vasorelaxation in the vascular system (Bucci et al. 2009). These authors showed that testosterone induced a concentration-dependent vasorelaxation in rat aortic rings, which was attenuated by PPG and β-cyanoalanine (BCA), both specific CSE inhibitors. Bucci et al (2009) also demonstrated that testosterone can increase the conversion of $_L$-cysteine to H_2S, which was significantly abrogated by PPG and BCA (Bucci et al. 2009). Overall, these results open a new window regarding the interconnected mechanisms between sexual reproduction hormones and H_2S. However, it has yet to be determined if estrogen or progesterone, the main female-dominant hormones, may also influence the production rate of H_2S.

2.5 The association between H_2S and diseases

The production of H_2S is a tightly regulated mechanism, where any minuscule change could have severe outcomes. The pathologic implications of H_2S have been linked to many diseases (Table 1-2). Due to its diverse and potent physiological actions, overproduction of H_2S has been connected to the pathogenesis of septic shock, diabetes mellitus, and Down syndrome, just to name a few. On the other hand, abnormally low levels of H_2S have been blamed for hypertension and may also be connected to Alzheimer's disease.

Table 1-2: Diseases associated with abnormal production levels of H_2S. (Modified from *Pharmacol Reports*, 59:4-24, 2007).

Increased H_2S formation:
Down syndrome
septic shock
colitis
diabetes mellitus
febrile seizures
Decreased H_2S formation:
hypertensive $CSE^{-/-}$ mice
spontaneously hypertensive rats
arterial hypertension induced by NOS blockade
myocardial ischemia/reperfusion injury
liver cirrhosis
Alzheimer's disease

2.5.1 Overproduction of H_2S

2.5.1.1 Septic shock

Due to its potent vasodilator properties, H_2S, along with NO and CO, has been implicated in septic shock. Septic shock is characterized by severe vasodilatation and hypotension that is commonly caused by an overwhelming infection with gram-negative bacteria (Lowicka and Beltowski 2007). Upon lipopolysaccharide administration, CSE expression and activity is significantly up-regulated in the kidney and liver (Li et al. 2005), which likely contributes to the excessive H_2S levels (Hui et al. 2003; Lyons et al. 2001). Furthermore, H_2S has negative inotropic effects, which could also contribute to this disease (Geng et al. 2004; Lowicka and Beltowski 2007).

2.5.1.2 Diabetes mellitus

There is a strong correlation between high H_2S levels and the deterioration of insulin release in pancreatic β-cells (Yang et al. 2005; Ali et al. 2007; Kaneko et al. 2006, 2009; Yang et al. 2007; Wu et al. 2009). This is mainly due to the association of H_2S and the increased opening probability of K_{ATP} channels (Lowicka and Beltowski 2007). Indeed, streptozotocin-induced diabetic rats, rats that have no insulin-producing β-cells, exhibited increased mRNA and activities of CSE and CBS in the liver (Yusuf et al. 2005; Hargrove et al. 1989; Nieman et al. 2004; Jacobs et al. 1998) and pancreas (Yusuf et al. 2005). Yet, interestingly enough, insulin treatment (8 U/kg, s.c., for 5 d) attenuated the up-regulation of both CSE and CBS mRNA and activity levels in these type 1 diabetic rats (Yusuf et al. 2005). Additionally, Wu et al (2009)

showed that Zucker diabetic fatty (ZDF) rats exhibited higher levels of pancreatic CSE and H_2S production than their counterparts, Zucker fatty (ZF) and the Zucker lean (ZL) rats. These data suggest that inhibition of H_2S in the pancreas could be therapeutic in diabetic conditions. However, more research is needed in order to better understand the relationship between H_2S and hormones in diabetes mellitus.

2.5.1.3 Down syndrome

In addition to its involvement in diabetes mellitus, H_2S is also associated with Down syndrome. Down syndrome is a chromosomal disease caused by the presence of an extra chromosome, the 21st chromosome. Patients with Down syndrome have a significant overexpression of CBS by 166% in fibroblasts (Chadefaux et al. 1985) and by 1,200% in myeloblasts (Taub et al. 1999). In agreement, low concentrations of plasma homocysteine, the substrate of CBS, were detected in Down syndrome patients (Chadefaux et al. 1988). Likewise, Chadefaux-Vekemans and associates (2003) observed a significant increase in the level of thiosulfate, the main catabolite of H_2S, in the urinary excretion from Down syndrome patients, thus supporting the notion that H_2S is linked to Down syndrome.

Interestingly, it was pointed out by Chadefaux-Vekemans and associates (2003) that the biological and clinical signs of Down syndrome mimic chronic H_2S poisoning. This includes reduced sensory nerve conduction velocity and impaired color vision and contrast sensitivity in workers exposed to carbon disulfide (Takebayashi et al. 1988; Raitta et al. 1981; Vanhoorne et

al. 1996) and in Down syndrome patients (Christensen et al. 1988; Rocco et al. 1977; Perez-Carpinell et al. 1994). These authors suggested that low doses of sodium nitrite (or nitrate, which is a nitrite precursor) could be used to combat the overproduction of H_2S in Down syndrome patients, since nitrite is used to treat acute H_2S poisoning.

2.5.2 Underproduction of H_2S

2.5.2.1 Hypertension

Approximately 90-95% of hypertension is caused by unknown factors, which is known as essential hypertension. Throughout the decade, there has been indirect evidence suggesting H_2S as a regulator of BP (Wang 2002; Zhao et al. 2001; Kimura 2002; Mok et al. 2004; Fiorucci et al. 2005). The first physiological evidence that suggests H_2S has vasorelaxant properties was demonstrated by Wang and associates (2001). These authors showed that injection of H_2S at 2.8 and 14 mM/kg bodyweight induced a concentration-dependent decrease in the mean arterial BP in anaesthetized SD rats by 12.5 ± 2.1 and 29.8 ± 7.6 Hg mm, respectively (Zhao et al. 2001). Moreover, this depressive effect of H_2S was mimicked by pinacidil (a K_{ATP} channel opener) and attenuated by glibenclamide (a K_{ATP} channel blocker) in SD rats (Zhao et al. 2001), which is consistent with findings from other groups (Cheng et al. 2004; Ali et al. 2006).

Given this, Rui Wang and associates (2008) generated mice with the knockout of the gene encoding CSE, which resulted in these mice becoming hypertensive. The $CSE^{-/-}$ mice also developed impaired endothelium-dependent vasorelaxation upon methacholine (a vasorelaxant)

administration in mesenteric arteries that were preconstricted with phenylephrine (Yang et al. 2008). Interestingly, both male and female $CSE^{-/-}$ mice, as young as 7 weeks old, exhibited significantly higher BP readings than the age-matched wild-type mice (Yang et al. 2008). At 12 weeks of age, this age-dependent increase in BP in $CSE^{-/-}$ mice increased further to 135 Hg mm, which was about 18 mm Hg higher than the wild-type mice (Yang et al. 2008). The H_2S/CSE system was likely responsible for the elevated BP readings in the $CSE^{-/-}$ mice, because no difference was observed in the H_2S level in the brain, the endothelial NO synthase (eNOS) protein was unchanged, the kidney architecture was preserved, and the administration of $_L$-methionine, a homocysteine precursor, did not increase BP (Yang et al. 2008). Thus, alterations of the CNS, impaired eNOS function, renal damage, or excess homocysteine levels were not causative factors for the observed high BP in the $CSE^{-/-}$ mice (Yang et al. 2008). This exciting discovery by Rui Wang and associates points to the possibility of H_2S being the next EDRF in the cardiovasculature (Wang 2009). Thus, pharmacological approaches that employ H_2S-releasing drugs could be an excellent approach for the treatment of hypertension.

2.5.2.2 Alzheimer's disease

Alzheimer's disease is an age-related, degenerative disease that is the most common cause for dementia. It was demonstrated that SAM, a CBS activator, is significantly decreased in the serum of patients with Alzheimer's disease (Morrison et al. 1996), whereas homocysteine levels are elevated (Clarke et al. 1998). These findings suggest decreased CBS activity and thus H_2S in patients with Alzheimer's disease. Likewise, Kimura and associates (2002) demonstrated that

endogenous levels of H_2S, along with the enzymatic activity of CBS, are significantly lowered in the brains of patients with Alzheimer's disease. However, the relationship between H_2S and Alzheimer's disease is unclear and it is also not certain if lack of H_2S may be involved in the etiology of Alzheimer's. More research is needed in this field.

3.0 Methylglyoxal

MG was first chemically characterized in 1885 by the German researcher Dr Baumann (Baumann 1885). Since then, MG is known to be produced as a byproduct of sugar, fat, and protein metabolism, and can be found in virtually all mammalian cells (Kalapos 2008). Indeed, MG and its adducts, advanced glycation endproducts (AGEs) and ROS, are involved in normal physiological functions, such as cellular transduction systems, including ERK 1/2 (Du et al. 2003; Blanc et al. 2003), c-Jun N-terminal kinases (JNK) (Kyriakis and Avruch, 1996; Du et al. 2000), and p38 MAPK pathways (Kyriakis and Avruch 1996), tissue remodeling maintenance and normal functions of the primary immune response (Di Loreto et al. 2004). However, overproduction of MG could result in endothelial dysfunction (Wu and Juurlink 2002), wall inflammation, and vasoconstriction (Pedchendo et al. 2005); thus, leading to several insulin resistance diseases, such as hypertension (Wang et al. 2004, 2005, 2008; Vasdev et al. 1998a, b; Wu and Juurlink 2002; Wu 2006; Tomaschitz et al. 2010) and diabetes mellitus (Wang et al. 2007; Riboulet-Chavey et al. 2006; McLellan et al. 1994).

3.1 Formation of MG

Methylglyoxal is mainly generated during glucose metabolism. Less significant sources of MG are fatty acid and amino acid metabolism (Figure 1-4). This section will outline the various metabolic pathways of MG formation.

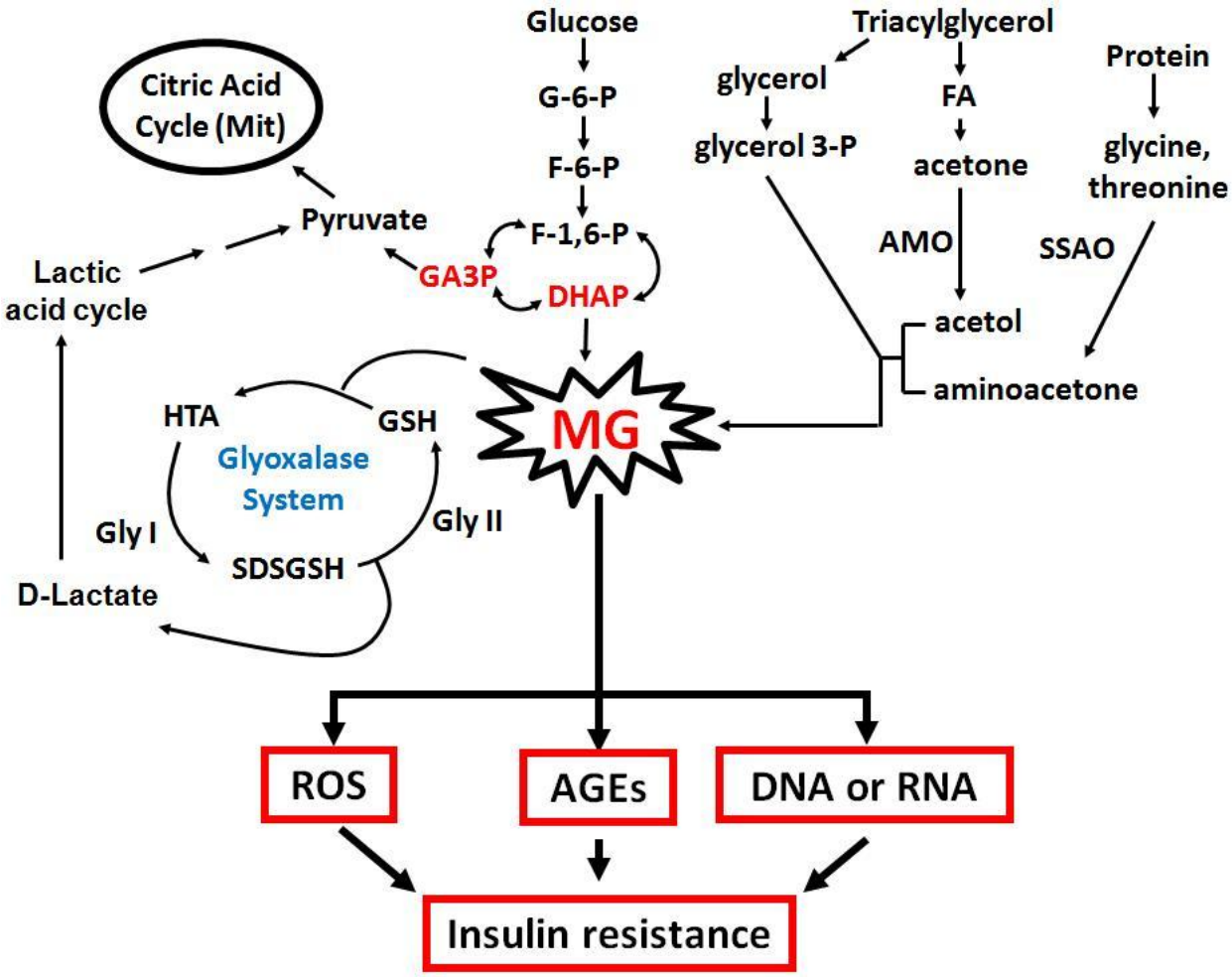

Figure 1-4: Endogenous formation of MG in mammalian cells. AGEs: advanced glycated endproducts; AMO: amino oxidase; DHAP: dihydroxyacetone phosphate; G-6-P: glucose-6-phosphate; GA3P: glyceraldehyde 3-phosphate; glycerol 3-P: glycerol 3-phosphate; F-1,6-P: fructose-1,6-bisphospahte; F-6-P: fructose-6-phosphate; FA: fatty acids; Gly-I & II: glyoxalase I & II; GSH: reduced glutathionine; HTA: hemithioacetyl; MG: methylglyoxal; Mit: mitochondria; ROS: reactive oxygen species; SDSGSH: S-*D*-lactoylglutathione; SSAO: semicarbazide-sensitive amine oxidase. (Modified from *Can. J. Physiol. Pharmacol.* 84:1229-1238, 2006)

3.1.1 Non-enzymatic MG formation

As a member of the reactive carbonyl species (RCS), MG is formed mainly through the fragmentation and elimination of phosphate from the triosephosphates dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GA3P) (Wu 2006; Figure 3.1-1). The triosephosphate pool, in turn, is regulated by both glycolytic and gluconeogenic pathways. For example, elevated levels of plasma glucose levels (e.g. 100 mg/dl or 5.6 mM) would enhance cellular glycolysis and thus MG generation (Chang and Wu 2006; Wu 2006), whereas, upon enhanced gluconeogenesis, that occurs during starvation periods, MG production would likely be lessened. Although it is currently unknown how MG enters the cell, by increasing available glucose in cultured cells, such as human red blood cells, bovine endothelial cells, or aortic smooth muscle cells, MG levels are significantly elevated (Thornalley 1988; Thornalley 1996; Wang et al. 2006). By increasing glucose, MG-induced ROS (Dhar et al. 2010; Wang et al. 2006), as well as MG-induced AGE formation are also increased (Thornalley 2003).

3.1.2 Enzymatic MG formation

This dicarbonyl molecule can also be generated by lipolysis, which uses acetone as a precursor, (Casazza et al. 1984; Koop and Casazza 1985), or by the metabolism of threonine (Ma et al. 1989) or aminoacetone (Lyles and Chalmers 1992) from protein catabolism (Figure 3.1-1). MG derived from acetone is synthesized by acetal monooxygenase by amino oxidase (AMO) (Koop and Casazza 1985). In protein catabolism, semicarbazide-sensitive amine oxidase (SSAO) catalyzes the conversion of aminoacetone to MG (Casazza et al. 1984; Lyles and Chalmers

1992), and aminoacetone was shown to be a significant source of MG formation in A-10 cells (Dhar et al. 2010). Currently, two forms of SSAO are known, a soluble form, typically found in the blood stream, and a membrane-bound form, located on VSMCs (Ekblom 1998; Tressel et al. 1986). Significantly elevated serum SSAO activity levels were found in patients with diabetic complications, including retinopathy or nephropathy (Yu et al. 2003), although, the underlying mechanisms are unclear (Wu and Juurlink 2002).

3.2 Metabolism of MG

3.2.1 Glyoxalase system

MG is a reactive intermediate that is inevitably produced under normal conditions and, therefore, there is a MG detoxification pathway. The degradation of MG occurs mainly by the ubiquitous glyoxalase system, which is present in the cytosol of all mammalian cells (Chang and Wu 2006). This MG detoxification system consists of two enzymes, glyoxalase-I (Gly-I) and glyoxalase-II (Gly-II), as well as the cofactor, GSH (Chang and Wu 2006) (Figure 3.1-1). The process involves the irreversible conversion of MG to S-$_{D}$-lactoylglutathione, *via* Gly-I activity with GSH as a cofactor, then to $_{D}$-lactate by Gly-II activity. At this stage, $_{D}$-lactate can be further converted to pyruvate, which can either be metabolized through gluconeogenesis or enter the citric acid cycle (Chang and Wu 2006).

3.3 Cellular toxicity of MG

The net output of MG in a cell is the summation of its generation and degeneration. However, as a consequence of overconsumption of foods high in carbohydrates, or fat, or beverages containing high amounts of ethanol, or coffee, can upset this delicate balance, leading to an accumulation of MG. Therefore, this can produce deleterious effects.

3.3.1 Production of reactive oxygen species (ROS)

The generation of free radicals and ROS is needed for normal physiological functions, such as cellular redox signaling, tissue remodeling maintenance, and normal functions of the primary immune response (Yan et al. 2006; Chang and Wu 2006; Wu 2006). However, overproduction of free radicals and ROS contributes to the development of endothelial dysfunctions, wall inflammation, and vasoconstriction, thus leading to several insulin resistance diseases, such as hypertension, atherosclerosis, and diabetes mellitus (Chang and Wu 2006; Wu 2006: Kalapos 2008). Oxidative stress occurs when there is an imbalance between oxidants and anti-oxidants, which could be from increased production and/or decreased degradation of ROS (Chang and Wu 2006).

MG is also known to generate $ONOO^-$, H_2O_2, and superoxide anions ($O_2^{\cdot-}$) *via* non-enzymatic reactions (Chang et al. 2005). $ONOO^-$ is formed when $O_2^{\cdot-}$ interacts with NO, which also decreases the bioavailability of NO (Chang and Wu 2006). Indeed, MG induced a concentration-dependent increase in ROS/reactive nitrogen species (RNS) in A-10 cells, which was attenuated

by GSH and N-acetyl cysteine (NAC), a MG scavenger, superoxide dismutase (SOD; a $O_2^{\cdot-}$ scavenger), and diphenyliodonium (DPI; a NADPH oxidase inhibitor) (Chang et al. 2005). Furthermore, it was also demonstrated that MG can enhance NADPH oxidase-mediated production of H_2O_2 in rat kidney mesangial cells, which was abrogated by SOD (Ho et al. 2007), as well as enhance H_2O_2 levels in rat hepatocytes (Kalapos et al. 1994). Moreover, Wu and Juurlink (2002) showed that MG administration induced oxidative stress in isolated VSMCs from SHRs, due to higher levels of GSSG, lower levels of GSH, and the impairment of the enzymatic activities of glutathione reductase (GSSG-Red) and glutathione peroxidase (GSH-Px) with comparison to the wild-type VSMCs. Additionally, it was demonstrated that MG can enhance H_2O_2 levels in neutrophils (Ward and McLeish 2004) and induce platelet H_2O_2 accumulation and aggregation (Leoncini and Poggi 1996).

3.3.2 Interaction with anti-oxidant enzymes

Because MG is a reactive carbonyl, it can directly interact with anti-oxidant enzymes, such as GSH-Px (Paget et al. 1998) and GSSG-Red (Blakytny et al. 1992) *via* glycation. Normally, GSH-Px scavenges H_2O_2, by using GSH as a cofactor, which is then oxidized to GSSG (Desai et al. 2010). GSSG-Red then reduces 1 mole of GSSG to form 2 moles of GSH, thus replenishing cellular GSH levels (Desai et al. 2010). However, this MG-induced impairment of the GSH recycling system, involving the main enzymes GSSG-Red and GSH-Px, could shift the balance of oxidants and anti-oxidants to higher levels of oxidants, thus leading to oxidative stress (Desai

et al. 2010). This deleterious phenomenon would impair the detoxification of MG, thereby enhancing its half-life and pro-oxidant potential.

3.3.3 Modification of proteins

Due to its electrophilic nature, MG can readily react with specific arginine, lysine, or sulfhydryl residues in enzymes, lipids, DNA, and receptors (Chang and Wu 2006; Wu 2008). As a result, this will lead to alterations in biological functions in VSMCs and ECs located within the blood vessel walls (Wu 2008). As the most reactive AGEs precursor, MG can undergo an irreversible glycation reaction on the targeted protein to yield AGEs (Chang and Wu 2006; Wu 2008). These highly reactive species will either directly interact with other cellular proteins, nucleic acids, or with their receptors (RAGE). RAGE signal transduction mechanisms are known to induce oxidative stress (Wu 2008). Higher levels of MG precursors, such as glucose and fructose (Wu and Juurlink 2002), can lead to higher levels of MG and MG-generated AGEs.

MG can readily react with arginine or lysine residues in proteins, leading to glycation, and this can also irreversibly inhibit enzyme activity. Arginine and lysine are common occurring amino acids in the catalytic active sites of enzymes (Wu and Juurlink 2002). As mentioned previously, MG can increase oxidative stress by inactivating GSH-Px and GSSG-Red *via* glycation (please see section 3.3.2). Likewise, Wu and Juurlink (2001) showed that GSH expression and the activities of GSSG-Red and GSH-Px were lower in VSMCs from hypertensive rats. This suggests a link between MG-induced AGE formation and decreased GSH expression.

Interestingly, these glycation reactions with selected amino acid residue on the targeted protein are highly selective (Chang and Wu 2006). For example, the reaction of MG with arginine produces hydroimidazolone Nε-(-5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (Ahmed et al. 2003) and argpyrimidine (Ahmed et al. 2002), whereas the irreversible reaction of MG with lysine leads to the formation of *N*ε-carboxymethyl-lysine (CML) and *N*ε-carboxyethyl-lysine (CEL). Moreover, MG-induced formation of AGEs, including CEL, are labeled as indicators of carbonyl overload *in vivo* (Singh et al. 2001) and they are also connected to age (Ando et al. 1999; Li et al. 1996). At the cellular level, the deleterious effects of MG-induced AGE formation would lead to the inactivation of enzymes, receptors, protein carriers, and structural proteins (Chang and Wu 2006), whereas clinically, AGE production has been implicated in the development of neuropathy, retinopathy, and nephropathy in diabetic patients (Sugiyama et al. 1996).

3.3.4 Modification of nucleic acids

MG reacts readily with not only arginine and lysine amino acid residues on proteins, but also with guanyl residues in DNA and RNA strands. This can lead to translational, as well as transcriptional abnormalities within a cell (Thornalley 1996). However, more research is needed in this field.

3.3.5 Pro-inflammatory effects

In cardiovascular diseases, one of the major effects of oxidative stress is the induction of pro-inflammatory molecules (Ogata et al. 2000). NF-κB plays key roles in regulating cell survival and in the immune response to infection. Upon activation of NF-κB by pro-inflammatory cytokines (TNF-α, or IL-1), pro-inflammatory responses are induced by promoting the expression of genes that mediate inflammatory reactions such as adhesion molecules (inter-cell adhesion molecule-1; ICAM-1 and vascular cell adhesion molecule-1; VCAM-1), and other cytokines (IL-8, TNF-β) (Ogata et al. 2000; Marumo et al. 1997). Thus, it is not a surprise to know that NF-κB has been linked to diseases in which inflammation is an issue, including insulin resistance diseases and atherosclerosis (Marumo et al. 1997).

In light of this, MG-induced ROS and AGE generation can play an important role in activation of NF-κB, by for example, degrading IκBα (an inhibitor of κB). It was shown by Wu and Juurlink (2002) that MG-induced H_2O_2 can activate NF-κB p65 in VSMC in SHRs. It was also observed that NF-κB can be activated by $O_2^{\cdot -}$ and H_2O_2 (Canty et al. 1999; Ogata et al. 2000), as well as $ONOO^-$ (Cooke and Davidge 2002) in endothelial cells. It was further demonstrated by Wu et al (2004) that significantly higher expression levels of NF-κB occur in the kidney of SHRs. Likewise, MG administration elicited the activation of NF-κB p65 in cultured rat VSMCs and from the aorta (Wu and Juurlink 2002) and mesenteric artery (Wu 2005). In both studies, NAC significantly decreased the MG-induced inflammatory responses. Lastly, it was shown that MG administration significantly increased both the transcriptional and translational expression of

IL-1β and nerve growth factor in hippocampal neural cells (Di Loreto et al. 2004). Thus, overproduction of MG can elicit pro-inflammatory responses in the vasculature.

3.4 The association between MG and diseases

At normal physiological levels, MG regulates signal transduction systems and various homeostatic mechanisms of cellular functions, balances redox reactions, and influences cell survival. However, abnormally high levels of MG and MG-induced production of ROS and AGEs are implicated in the alteration of vascular reactivity, wall inflammation, and endothelial dysfunction. The Western diet (foods high in carbohydrates and/or fats, as well as beverages with high amounts of sugar), as well as lack of exercise, does not help the situation. Numerous studies have linked high levels of MG and MG adducts to the impairment of the cardiovascular system, resulting in diabetes, hypertension, heart disease, and stroke, which are the number one killers in North America (WHO 2007).

3.4.1 Hypertension

High MG levels are associated with the development of high BP in SHRs and may be a causative factor for the development of hypertension in this model (Wang et al. 2004, 2005, 2008; Vasdev et al. 1998a, b; Kamencic et al. 2000). Wistar Kyoto (WKY) rats treated with either MG (0.2% to 0.8%) (Vasdev et al., 1998a) or fructose (4%) (Vasdev et al. 1998b), a precursor for MG formation (in drinking water), showed a progressive increase in systolic BP during treatment. The WKY rats that were treated with both MG and NAC, a MG scavenger, did not develop high

BP (Vasdev et al. 1998a). These authors also found that the MG treated rats showed smooth muscle cell hyperplasia in the small arteries and arterioles of the kidney, whereas the MG+NAC treated rats showed no such change (Vasdev et al. 1998a). VSMC proliferation is one of the characteristics of hypertension (Irani 2000). The hyperplasia in the resistance arteries may be due to the cell proliferative effects of MG, since MG can activate both ERKs (Du et al. 2003; Blanc et al. 2003) and NF-κB p65 pathways in VSMCs (Wu and Juurlink 2002). These pathways promote cellular proliferation and survival (Chang and Wu 2006). Proliferation and apoptosis of VSMCs are important cellular events of vascular remodeling (Yang et al. 2004), but overstimulation of these pathways severely effects vascular integrity (Figure 3.4.1-1).

Additionally, Wang et al (2004, 2005) demonstrated that plasma MG levels in the SHRs progressively increased with age and were associated with increased BP in SHRs compared with the age-matched WKY rats. In fact, MG-induced CEL and CML formation was significantly elevated in the vasculature of SHRs when compared to age-matched WKY rats (Wang et al. 2004, 2005). In this study, no observed difference in BP was seen between the SHRs and WKY rats at 5 weeks, but this changed at 8 weeks. At this age, SHRs exhibited a significant increase in systolic BP along with a progressive increase in MG and the MG-induced AGE products, CEL and CML, in both the aorta and kidney of SHRs (Wang et al. 2004, 2005). Thus, the increase of MG and MG-induced AGE formation may be a causative factor for hypertension development (Figure 1-5).

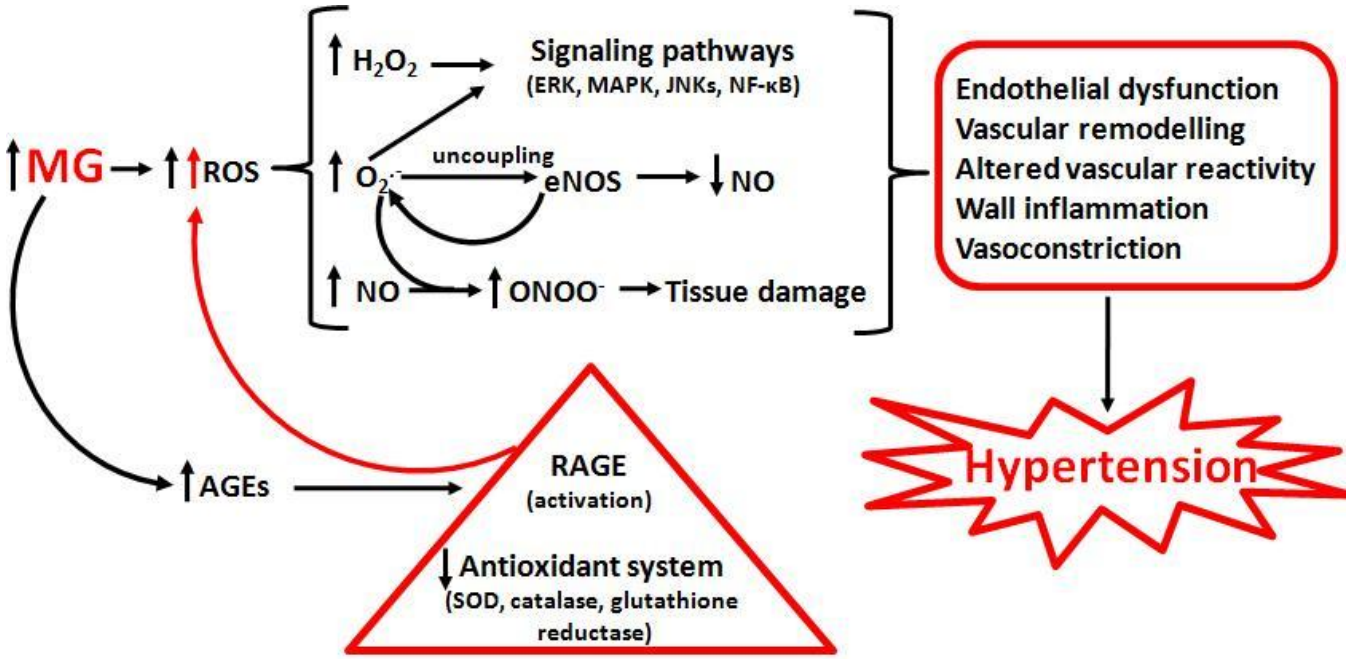

Figure 1-5: The association of MG with the pathophysiological development of hypertension. AGEs: advanced glycation endproducts; eNOS: endothelial nitric oxide synthase; ERKs: extracellular signal-regulated kinases; H_2O_2: hydrogen peroxide; JNKs: c-Jun N-terminal kinases; MAPK: mitogen-activated protein kinase; MG: methylglyoxal; NO: nitric oxide; NF-κB: nuclear factor kappa B; $O_2^{\cdot-}$: superoxide anion; $ONOO^-$: peroxynitrite; RAGE: receptor for AGEs; ROS: reactive oxygen species; and SOD: superoxide dismutase. (Modified from *Can. J. Physiol. Pharmacol.* 84:1229-1238, 2006).

3.4.2 Diabetic complications

Not only is this reactive aldehyde connected to hypertension, MG is also linked to diabetes. In a clinical study conducted by McLellan et al (1994), MG serum levels increased by 5-6-fold in type 1 diabetic patients, and by 2-3-fold in type 2 diabetic patients. In fact, the circulating levels of MG could be due to abnormal elevations in the activities of plasma SSAO (a MG-producing enzyme in protein catabolism) and in plasma AMO (a MG-producing enzyme in lipolysis) (Ekbom 1998). Likewise, Yu et al (2003) also reported increased serum SSAO activity in

patients suffering from diabetic complications, such as retinopathy and nephropathy. Since MG is an important precursor for AGE formation, plasma concentrations of imidazolone (Kilhovd et al. 2003) and argpyrimidine (Wilker et al. 2001), both MG-induced AGEs, are significantly increased in diabetics. In agreement, two inhibitors of AGE formation (aminoguanidine and pyridoxamine) were demonstrated to decrease or abolish the development of retinopathy, nephropathy, and neuropathy in diabetic rats (Hammes et al. 2003; Alderson et al. 2003). Schmidt et al (1999) reported significant increases in RAGE expression in both endothelium and VSMCs of diabetic patients. Moreover, these authors also reported that activation of RAGE, a cascade of pro-inflammatory processes were induced, such as the activation of NF-κB and enhanced expression of cell adhesion molecules. Thus, it was suggested by Bourajjaj et al (2003) that chronic inflammation may play a role in microvascular complications in patients with diabetes mellitus. Overall, there is strong evidence suggesting that the maladaptive overproduction of MG in diabetic patients may be linked to micro- and macrovascular complications, likely due to its AGEs producing and pro-inflammatory properties.

3.4.3 Aging

Aging is a multifactorial process that affects the cell, organ, and whole body level (Desai et al. 2009). Oxidative stress is thought to play an important role in age and age-related diseases (Desai et al. 2010). The free radical theory of aging, first proposed by Denham Harman in 1956, states that throughout an organism's lifespan, the endogenous production of free radicals, inevitably produced as by-products of cellular metabolism, would ultimately react with and

cause irreversible damage to cellular function and tissue constituents, eventually leading to disease and death. In fact, the main source for free radical production is from the mitochondria, specifically the electron transport chain (Harman 2001). Aged organisms show increased free radicals and oxidatively-damaged mitochondrial DNA (Beckman and Ames 1998). Damaged mitochondrial DNA can result in the production of dysfunctional enzymes and structural abnormalities in the electron transport chain, which can further increase the production of ROS (Beckman and Ames 1998). Wang et al (2009) showed that MG can react with complex III, which would disrupt the flow of electrons in the electron transport chain, thus uncoupling electron flow, resulting in increased production of $O_2^{\cdot -}$ and $ONOO^-$ in A-10 cells. Wang et al (2009) also showed that MG inhibited the activity of MnSOD (mitochondrial SOD), which is the first line of defence for overproduction of $O_2^{\cdot -}$ in the mitochondria, thus further enhancing oxidative stress in A-10 cells. Overall, being a major source of free radical and ROS production, increased MG levels could be a characteristic feature of aging.

Additionally, many studies have shown that excessively high levels of glucose and caloric intake increase oxidative stress, which can shorten life span (McCay et al. 1935; Weindruch and Walford 1988; Szatrowski and Nathan 1991; Simic and Bertgold 1991). As mentioned before, MG-induced AGE formation has been linked to aging (section 3.3.3) (Ando et al. 1999; Li et al. 1996). Once MG reacts with a protein, it becomes a stable complex. Because of this, the measurements of AGEs, such as CEL, are representative markers of the status of oxidative stress and cumulative markers of oxidative damage to proteins in aging (Degenhardt et al. 1998;

Kilhovd et al. 2003). In diabetes, the destructive effects of MG-induced AGE formation are seen later on in age, due to the development of macrovascular damage to the eyes, kidneys, and nerves (Sugiyama et al. 1996). Overall, the accumulation of MG and AGEs can be seen as one of the causative factors in the phenomenon known as aging.

4.0 Gluconeogenesis

Glucose is a major energy source for all mammalian cells and therefore proper measures must be in place to ensure against hypoglycaemia (Pilkis and Granner 1992). Gluconeogenesis is the *de novo* synthesis of glucose and it occurs during periods of fasting, starvation, low-carbohydrate diets, or intense physical activity (Pilkis and Granner 1992). The rate of gluconeogenesis is determined by the unidirectional enzymes phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase), and glucose-6-phosphatase (G6Pase) (Marsenic 2009). These gluconeogenic enzymes are controlled at the transcriptional level by key hormones, particularly insulin, glucagon, glucocorticoids, and catecholamines (Pilkis and Granner 1992). During times of starvation or intense physical activity, plasma levels of glucagon, glucocorticoids, and catecholamines will increase, leading to increased activities of G6Pase, FBPase, and PEPCK, and to a coordinated decrease of glycolytic enzymatic activity (Hers and Hue 1983; Pilkis et al. 1988; Pilkis and Granner 1992) *via* increased intracellular levels of cAMP (Solomon et al. 1988), as well as the phosphorylation of several enzymes, and/or by changes in allosteric effectors (Pilkis et al. 1988; Hers and Van Schaftingen 1982). The opposite occurs during food consumption. By a series of mechanisms, β-cells in the pancreas will increase

insulin secretion and decrease the secretion of counter-regulatory hormones, resulting in suppressed glycogenolysis and gluconeogenesis and increased activities of glycolytic enzymes, including pyruvate kinase (PK), phosphofructokinase (PFK), and glucokinase (GK) (Pilkis and Granner 1992; Pilkis et al. 1988).

4.1 Gluconeogenesis and its association with T2DM

It is widely accepted that endogenous glucose production in T2DM is inappropriately increased during times of fasting and the postprandial period as a result of elevated gluconeogenesis (Boden et al. 2001; Magnusson et al. 1992; Gastaldelli et al. 2000; DeFronzo 1999). In fact, the rate of glucose production is increased by approximately 25-100% in patients with T2DM compared to non-diabetic patients (Hundal et al. 2000). Endogenous glucose production occurs through two processes: glycogenolysis and gluconeogenesis. Glycogenolysis involves the breakdown of glycogen to glucose-6-phosphate and its subsequent hydrolysis by glucose-6-phosphatase to free glucose (Gerich et al. 2001), whereas gluconeogenesis involves the *de novo* synthesis of glucose-6-phosphate from noncarbohydrate precursors, such as lactate, glycerol, and amino acids, where glucose-6-phosphate is subsequently hydrolyzed to glucose (Gerich et al. 2001). The only organs capable of generating sufficient glucose to be released into the circulation are the liver and kidney (Gerich et al. 2001).

4.1.1 Drug therapy that targets gluconeogenesis in T2DM

4.1.1.1 Metformin

Because gluconeogenesis is abnormally elevated in T2DM, it is a target of therapy for patients with diabetes. One anti-diabetic drug that targets gluconeogenesis in T2DM is metformin. Metformin has been available for treatment of T2DM for nearly 8 years, and it is the most widely prescribed anti-hyperglycemic agent (Hundal and Inzucchi 2003). It is widely held that metformin significantly decreases plasma glucose levels in T2DM by inhibiting gluconeogenesis and by increasing glucose uptake into the cell (Hundal et al. 2000; Natali and Ferrannini, 2006; Inzucchi et al. 1998; Perriello et al. 1994). Although its precise mechanism is controversial, metformin can decrease hepatic gluconeogenesis by inhibiting hepatic lactate uptake (Radziuk et al. 1997), reducing the intracellular hepatic ATP concentration (Argaud et al. 1993), and by inhibiting PEPCK activity (Large and Beylot, 1999). In addition, metformin can lower plasma free fatty acids by 10-30% in diabetic subjects (Hundal et al. 2000). Since fatty acids are known to increase the rate of gluconeogenesis (Sindelar et al. 1997), the metformin-induced decrease in free fatty acids may also contribute to the reduced rates of gluconeogenesis (Hundal and Inzucchi, 2003).

4.1.1.2 Inhibitors of key gluconeogenic enzymes

Since the rate of gluconeogenesis is determined by G6Pase, FBPase, and PEPCK, these three enzymes form the major control points in gluconeogenesis and thus have all been targets of drug discovery efforts (Figure 1-6). However, inhibition of PEPCK and G6Pase has proven to be

problematic. For example, due to PEPCK involvement in the early mitochondrial steps of gluconeogenesis, there are multiple potential side effects, such as increased mitochondrial red-ox state, inhibition of the tricarboxylic acid cycle, and a reduction in beta-oxidation of fats leading to hepatic steatosis (Burgess et al. 2004). Additionally, PEPCK inhibition does not inhibit endogenous glucose production when the substrate glycerol is in abundance (Burgess et al. 2004; Poelje et al. 2007). Inhibition of G6Pase, on the other hand, has also significant mechanistic concerns. Because G6Pase catalyzes the final step in gluconeogenesis, as well as glycogenolysis, inhibition of this enzyme represents substantial risk for the development of hypoglycaemia, which could be fatal (Poelje et al. 2007).

The development of a FBPase inhibitor, however, is thought to be a more logical target for pharmacological intervention. FBPase is the second-to-last enzyme in gluconeogenesis, meaning that it is not involved in the first step of glucose production, by the mitochondria, and not in the last step, such as the breakdown of glycogen. This theoretically reduces the risk of hypoglycaemia, lacticemia, and hyperlipidemia (Poelje et al. 2007). In fact, individuals who are genetically deficient in FBPase have near normal biochemical and clinical parameters (Gitzelmann et al. 1995). Furthermore, FBPase expression is significantly up-regulated in diabetic animal models (Kodama et al. 1994; Lamont et al. 2006). Recently, preliminary trails using a FBPase-specific inhibitor, CS-917, were completed suggesting clinically relevant glucose lowering was achieved in patients with T2DM without eliciting the risk of hypoglycaemia,

lacticemia, and hyperlipidemia (Triscari et al. 2006; Bruce et al. 2006; Walker et al. 2006). However, larger scale and longer term clinical trials are still needed.

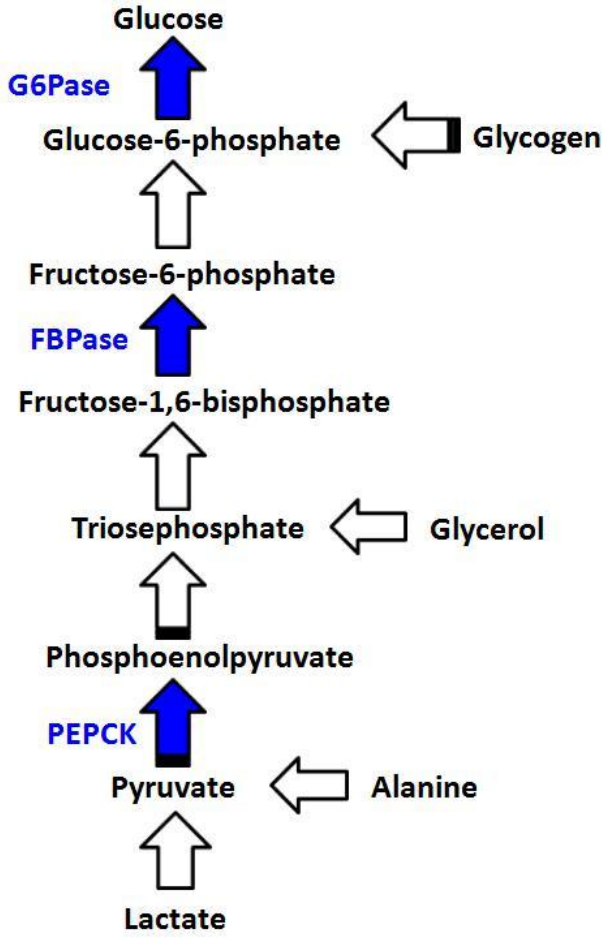

Figure 1-6: Regulatory steps in gluconeogenesis and glycogenolysis. The blue arrows represent the rate-limiting steps in gluconeogenesis and/or glycogenolysis. FBPase: fructose-1,6-bisphosphatase; G6Pase: glucose-6-phosphatase; and PEPCK: phosphoenolpyruvate carboxykinase. (Modified from *Curr. Opin. Drug. Discov. Devel*. 10:430-437, 2007)

4.1.2 PGC-1α and its association with gluconeogenesis

4.1.2.1 PGC-1α and its stimulating effects

The peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α is a transcriptional coactivator that induces many physiological stimuli. PGC-1α is a potent stimulator of

mitochondrial biogenesis and respiration (Kelly and Scrapulla 2004; Lehman et al. 2000; Lin et al. 2002; Puigserver et al. 1998). This gives PGC-1α the ability to regulate adaptive thermogenesis in brown adipose tissue (BAT) (Puigserver et al. 1998), and fiber-type switching in skeletal muscle (Lin et al. 2002), as well as to stimulate β-oxidation of fatty acids and gluconeogenesis in the liver (Herzig et al. 2001; Puigserver et al. 2003; Rhee et al. 2003; Yoon et al. 2001).

4.1.2.1.1 Up-regulation of G6Pase, FBPase, and PEPCK

During the fasting state, glucagon secretion will increase, which will then increase the intracellular cAMP levels in the liver. As a result, the transcription factor cAMP response element-binding (CREB) will become activated, thus leading to the induction of the gene expression of PGC-1α (Puigserver, 2005). Once PGC-1α is activated, it will bind to co-activators such as hepatocyte nuclear factor (HNF4α) and forkhead box O1 (FOXO1), which will then lead to the induction of G6Pase, FBPase, and PEPCK gene expression (Puigserver 2005). Yoon et al (2001) showed that subjecting mice to a 24-hr fast induces a 3.7-fold increase in PGC-1α mRNA, which was reversed by refeeding. These authors also showed that by performing a time course of fasting, an increase in PGC-1α mRNA was observed after 2 hr and peaked after 5 hr of food deprivation, and PEPCK mRNA also exhibited a similar response pattern to PGC-1α. Additionally, PGC-1α markedly increased the mRNA expression of G6Pase, FBPase, and PEPCK in rat hepatocytes with an adenovirus-based vector for PGC-1α.

Furthermore, when rats were injected with adenoviruses expressing PGC-1α, glucose output and insulin secretion was significantly elevated after 5 days (Yoon et al. 2001).

During food consumption, on the other hand, gluconeogenesis will be "turned off" and glycolysis will be "turned on." Through the actions of insulin, the Akt pathway will be activated, leading to the phosphorylation of FOXO1, marking it for degradation (Puigserver 2005). Without FOXO1, PGC-1α would be unable to bind and localize to the promoter chromatin region of the gluconeogenic genes, thus significantly decreasing the transcriptional activity of gluconeogenic enzymes (Puigserver 2005). Meanwhile, the activities of glycolytic enzymes will increase, whereas the gluconeogenic enzymatic activities will decrease, leading to enhanced glucose metabolism (Puigserver 2005). Overall, insulin is the dominant regulator of gluconeogenesis.

4.1.2.1.2 Up-regulation of ERRα

Of course, all potent regulators come with their own unique suppressors. The estrogen-related receptor-α (ERRα) is an orphan nuclear receptor that shares a significant sequence similarity to the estrogen receptor (ER) (Schreiber et al. 2003). ERRα, along with ERRβ and ERRγ, recognize and bind to similar DNA sequences recognized by ERs (Schreiber et al. 2003). However, the *in vivo* function of ERRα is still unclear (Schreiber et al. 2003). PGC-1α and ERRα are both predominantly expressed in organs with high metabolic needs such as the skeletal muscle and kidneys (Ichida et al. 2002). Indeed, Ichida et al (2002) showed that after starving

mice overnight, both PGC-1α and ERRα were significantly up-regulated at the transcriptional level. In fact, when PGC-1α is induced, it regulates the expression of ERRα mRNA, as well as its transcriptional activity (Schreiber et al. 2003), where in turn, ERRα can significantly repress PGC-1α transcriptional activity (Ichida et al. 2002; Herzog et al. 2006). Thus, ERRα has opposing effects on genes important for gluconeogenesis.

4.1.2.2 PGC-1α and its induction in ROS-detoxifying enzymes

PGC-1α is also a broad and powerful regulator of ROS metabolism (St-Pierre et al. 2006). This is due to the ability of PGC-1α to be a potent stimulator of mitochondrial biogenesis, which would consequently cause the production of ROS. Mitochondrial metabolism is responsible for the majority of ROS production in cells (Balaban et al. 2005). This occurs when unpaired electrons escape from the electron transport chain and react with molecular oxygen, generating $O_2^{\cdot -}$, and thus $ONOO^-$ (Brown and Borutaite, 2001).

ROS-detoxifying enzymes are the first line of defense to combat the deleterious effects of excess ROS production. These anti-oxidant enzymes include, but are not limited to, SOD1 and manganese SOD2, catalase, and GSH-Px (St-Pierre et al. 2006). In fact, Bruce Spiegelman and associates (2006) showed that PGC-1α can stimulate mitochondrial electron transport while suppressing ROS levels, which is accomplished by increasing the expression and activity of SOD1 and 2, catalase, and GSH-Px (Figure 1-7). This mechanism thus allows tissues, such as brown fat and skeletal muscle, to increase mitochondrial metabolism without causing self-

inflicted oxidative damage (St-Pierre et al. 2006). The authors also reported that *PGC-1α* null mice displayed a blunted induction of the ROS defense system and were more sensitive to oxidative stress than their wild-type counterparts. For example, Spiegelman and associates (2006) showed that approximately half of fibroblasts isolated from *PGC-1α* null mice died after exposure to 1.5 mM H_2O_2 while only 25% of the wild-type fibroblasts died. Furthermore, exposure to 1.5 mM H_2O_2 caused death to more than 80% of cells lacking both PGC-1α and –β *via* iRNA (St-Pierre et al. 2006). Thus, PGC-1α may serve as an adaptive regulator of ROS production, ensuring balance between the metabolic requirements of the cell and its cytotoxic protection.

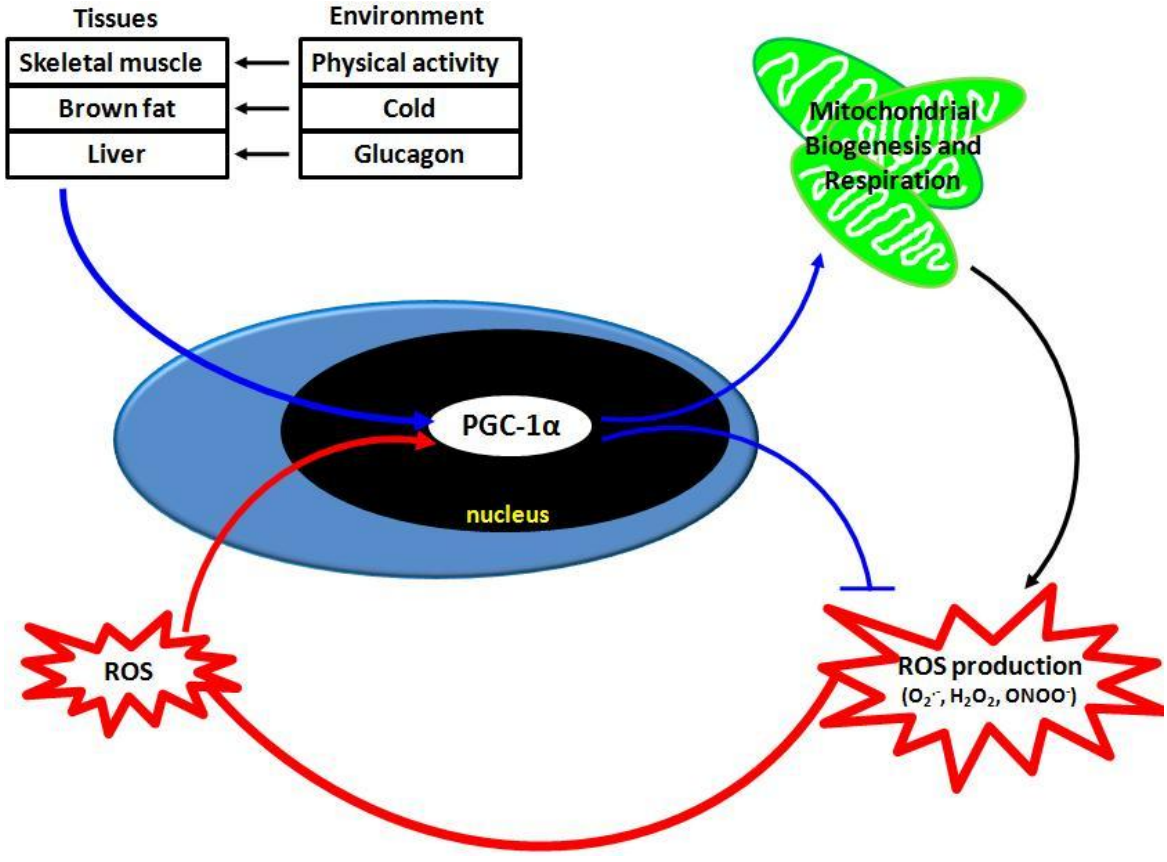

Figure 1-7: PGC-1α plays a key role in the ROS homeostatic cycle. Physiological stimuli by the environment, such as cold, starvation, or physical activity, can increase the gene expression

level of PGC-1α in brown fat, liver, and in skeletal muscle, respectively, in order to increase energy and/or heat production *via* mitochondrial biogenesis. However, enhanced mitochondrial biogenesis can lead to increased ROS levels, thus PGC-1α also induces anti-oxidant responses to prevent overproduction of ROS levels in the mammalian cell. PGC-1α: peroxisome proliferator-activated receptor-γ coactivator-1α; and ROS: reactive oxygen species. (Modified from *Cell.* 127:397-408, 2006)

4.1.2.3 PGC-1α and its interaction with NO and CO

There are many upstream effectors that can induce PGC-1α expression and activity. For instance, in BAT and liver tissues, the β-adrenergic/cAMP pathway activates *PGC1A* gene transcription, whereas and in striated muscles, calcineurin A and calcium/calmodulin-dependent protein kinase (CaMK) activates PGC-1α expression (Finck and Kelly 2006). Recently, NO, a gasotransmitter originally identified as a vasodilator, was shown to activate mitochondrial biogenesis through the induction of PGC-1α (Figure 1-8) (Nisoli et al. 2003). Nisoli et al (2003) found that overexpression of NO, cGMP, or eNOS significantly increased mitochondrial numbers in cells as diverse as brown adipocytes and 3T3-L1 (a mouse white fat cell line), U937 (a human monocytic cell line), and HeLa (a human cervical cancer cell line) cells. Furthermore, both male and female *eNOS* null mice exhibited decreased numbers of mitochondria in brain, liver, and heart tissues, which resulted in decreased energy metabolism and weight gain (Nisoli et al. 2003).

Another gasotransmitter, CO, was also shown to be involved in adaptive oxidative metabolism by optimizing mitochondrial biogenesis (Suliman et al. 2007). Suliman and associates showed that in the mouse heart and in isolated cardiomyocytes, activation of both guanylate cyclase and

the pro-survival kinase Akt/PKB, CO also induced the expression and activity of PGC-1α. It has yet to be determined whether H_2S could also induce the expression of PGC-1α.

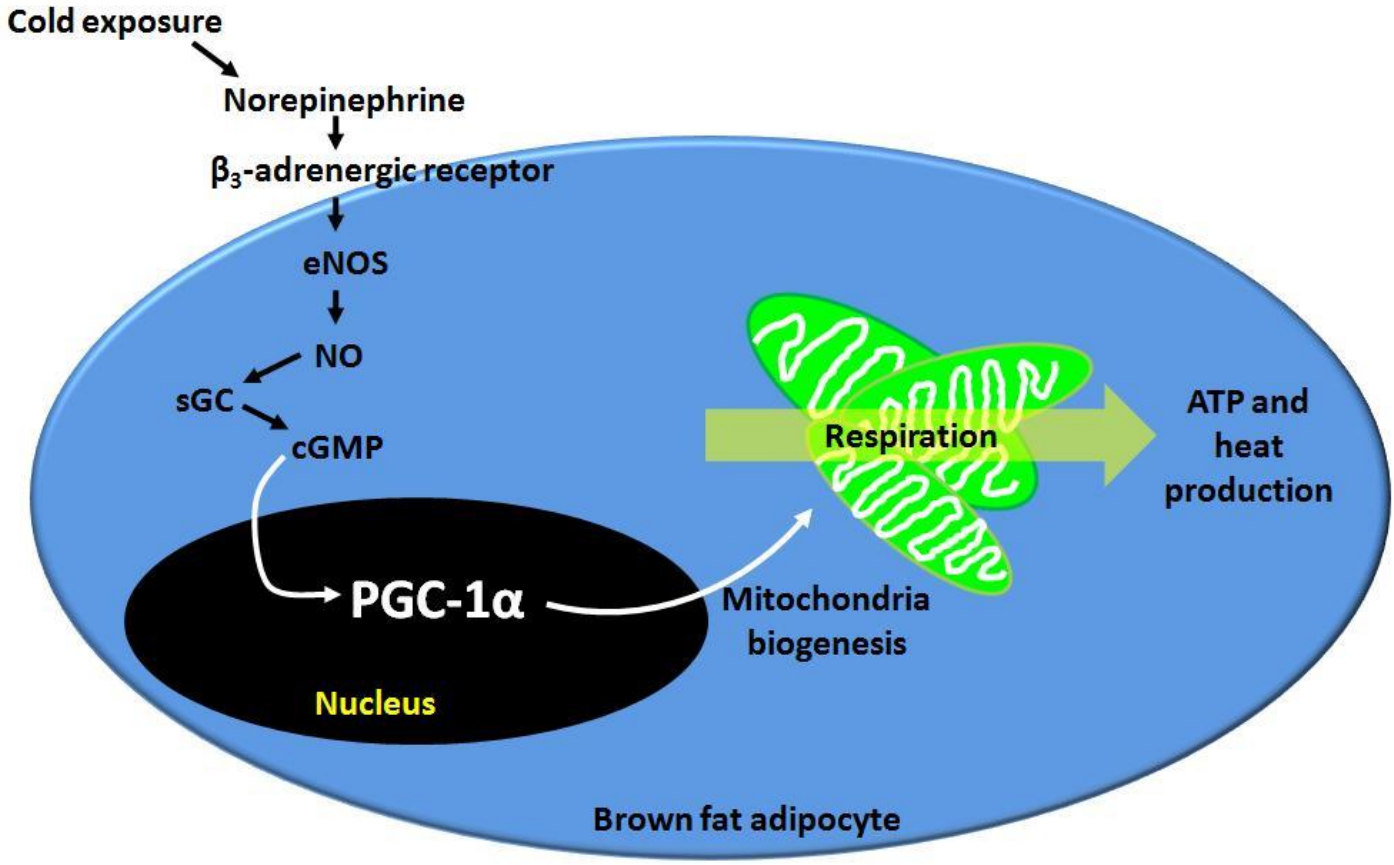

Figure 1-8: NO mediated PGC-1α-induction and mitochondria biogenesis in brown adipocytes. Upon cold exposure, norepinephrine is released into the blood stream, which interacts with and activates β_3-adrenergic receptors in brown fat adipocytes, leading to elevated levels of intracellular calcium ions and cAMP. This activates eNOS and induces NO production, where NO activates sGC and increases cGMP formation. Expression of PGC-1α is enhanced by cGMP, which stimulates mitochondrial biogenesis, leading to enhanced mitochondrial respiration, and finally energy and heat production. eNOS: endothelial nitric oxide synthase; NO: nitric oxide; PGC-1α: peroxisome proliferator-activated receptor-γ coactivator-1α; and sGC: soluble guanylate cyclase. (Modified from *Science.* 299:838-839, 2003)

4.1.2.4 PGC-1α and its association with diabetes mellitus

Both animal and human studies have shown that altered PGC-1α signaling could lead to glucose intolerance, insulin resistance, and diabetes (Yoon et al. 2001; Koo et al. 2004; Andrulionyte et al. 2004; Ek et al. 2001; Hara et al. 2002; Vimaleswaran et al. 2005; Oberkofler et al. 2004). PGC-1α activity is abnormally elevated in the diabetic liver in the fasted state (Finck and Kelly 2006), which could be a main contributing factor for hyperglycemia. Additionally, PGC-1α may promote insulin resistance by inducing TRB-3, an inhibitor of Akt signaling, thus interfering with insulin signaling (Koo et al. 2004). In fact, FBPase, a downstream target of PGC-1α, was up-regulated 5-fold in pancreatic islets from diabetes-susceptible obese BTBR mice compared with the diabetes-resistant C57BL/6 mice (Lan et al. 2003). Additionally, Kebede et al (2008) showed that up-regulation of FBPase can have detrimental effects to β-cells, because it can decrease the cell proliferation rate, as well as impair insulin secretion by depressing glucose-induced insulin secretion. Thus, FBPase overexpression in β-cells could result in reduced glycolytic flux and energy production (Kebede et al. 2008). However, the precise mechanism by which cross-talk occurs between insulin signaling and PGC-1α activity in the diabetic state is currently unknown and is an active field in diabetic research (Finck and Kelly 2006).

4.3 Renal gluconeogenesis

The kidney plays a vital role in BP regulation (Tomaschitz et al. 2010), but its role in glucose metabolism is often ignored (Gerich and Meyer 2001). Until recently, it was believed that the liver was solely responsible for gluconeogenesis, and that renal gluconeogenesis became

significant only during prolonged fasting or acidosis (Gerich 2000; Gerich et al. 2001; Roden and Bernroider 2003). However, it is now recognized that the kidney has a significant role in glucose homeostasis *via* gluconeogenesis and reabsorption of filtered glucose (Meyer et al. 2002a; Gerich et al. 2001; Meyer et al. 2004). Renal gluconeogenesis has been estimated to account for 20 ± 2% of total glucose release (Gerich and Meyer 2001). However, in relation to T2DM, renal release of glucose is significantly elevated in the fasting state. Meyer et al (1998) showed that the absolute increase in renal glucose release is comparable to that of the liver in magnitude [2.60 and 2.21 μmol/(kg min) for liver and kidneys, respectively]. In fact, the relative increase in renal gluconeogenesis is substantially greater than the increase in hepatic gluconeogenesis (300 vs. 30%) (Meyer et al. 1998).

The proximal tubule, located within the renal cortex, is the only segment of the nephron capable of gluconeogenesis, because this is the precise location of key gluconeogenic enzymes such as G6Pase, FBPase, and PEPCK (Gerich 2000; Guder and Ross 1984; Schoolwerth et al. 1988). Indeed, these three gluconeogenic enzymes are active along the entire length of the proximal tubule (Conjard et al. 2001).

5.0 Rationale and hypothesis

Recent studies have concluded that MG regulates signal transduction systems, balances redox reactions, and influences cell survival. However, abnormally high levels of MG and MG-induced production of ROS and AGEs are implicated in alterations of vascular reactivity, wall inflammation, oxidative stress, and endothelial dysfunction. H_2S, on other hand, can induce reconditioning and cardiac protective effects. For example, H_2S is a scavenger for ROS and RNS and can indirectly increase GSH levels, thus combating oxidative stress, as well as reducing inflammation and promoting cellular apoptosis. However, overproduction of H_2S has been linked to the pathogenesis of septic shock and diabetes.

With this in mind, the cell must have highly sophisticated regulation mechanisms in place in order to tightly control these potent endogenous and influential molecules. Thus, it is logical to propose that a link exists between H_2S and MG. Since MG and H_2S are both involved in opposing pathways (pro-oxidant vs. anti-oxidant, induction of proliferation vs. apoptosis, pro-inflammatory vs. anti-inflammatory), it is possible a negative correlation exists between MG-induced responses and H_2S-induced effects. Elucidation of a possible relationship between MG and H_2S in physiological and pathophysiological conditions could lead to more elaborate and effective therapeutic treatments to combat oxidative stress and its implications.

A crosstalk phenomenon may occur between MG and H_2S, such as the down-regulation of endogenous synthesis of the opposing molecule, as well as the attenuation of its downstream

effect, possibly to maintain balance in the cell. If crosstalk does occur between MG and H_2S, it may play an important role in the overall picture of cellular physiology, and disruption of this balance may lead to different pathophysiological conditions. **Therefore, my hypothesis is that a physiological balance between MG and H_2S plays an important role in the regulation of glucose metabolism and that an imbalance in this relationship may be one contributing factor in the development of some metabolic disorders** (Figure 1-9)

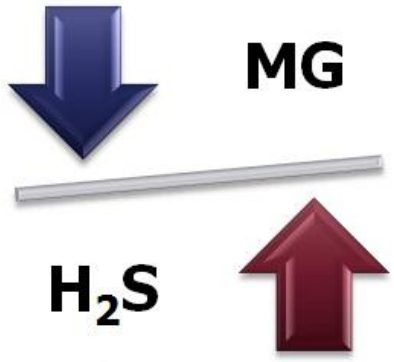

Figure 1-9: Hypothesis: physiological balance between MG and H_2S is needed to maintain normal glucose metabolism and cellular function.

6. 0 Objectives and experimental approaches

This thesis is mainly focusing on the possible connection between MG and H_2S. Not much is known in this field and therefore this project has been divided into two consecutive studies.

6.1 Study 1: Interactions of methylglyoxal and hydrogen sulfide in rat vascular smooth muscle cells

MG and H_2S are both produced in vascular tissues. We first need to determine if an interaction, either direct or indirect, occurs, and if so, what are the physiological outcomes (Figure 1-10). Thus, in order to accurately study the *in vivo* and *in vitro* interaction of MG and H_2S we will:

1. Determine if a chemical-to-chemical interaction can occur between MG and H_2S in a cell-free medium.
2. Investigate if administration of MG and H_2S will decrease the endogenous level of one another in A-10 cells.
3. Study the oxidative stress of A-10 cells when exposed to exogenous levels of MG, H_2S, or both by using the DCF assay. Furthermore, it is essential to analyze any changes in the endogenous levels of $_L$-cysteine, homocysteine, and GSH.
4. Analyze whether or not MG can affect the expression levels and activity of the dominant H_2S-producing enzyme in the vasculature, CSE, since MG is known to react with and modify proteins.

This study would provide us with novel insight if cross-talk does in fact occur between H_2S and MG in rat aortic VSMCs (A-10 cells), whether this be indirect and/or direct. These discoveries may help unveil complex pathologic mechanisms of various diseases such as hypertension and other forms of insulin resistance syndrome with altered cysteine/homocysteine metabolism.

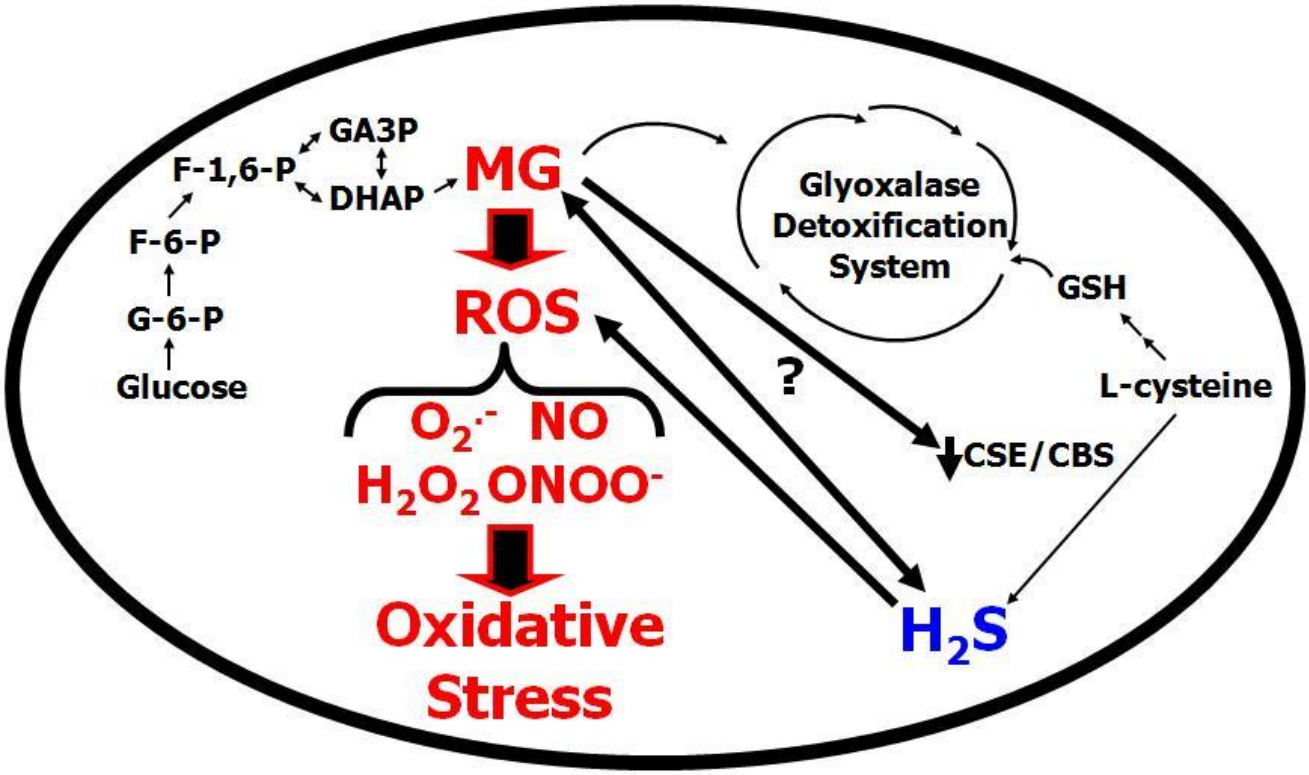

Figure 1-10: Schematic diagram for the layout of Study 1.

6.2 Study 2: Increased renal methylglyoxal formation with down-regulation of PGC-1α-FBPase pathway in cystathionine γ-lyase knockout mice

After observing that H_2S has cytoprotective properties against MG, due to its MG scavenging abilities, abolishing MG-induced ROS production, and up-regulating GSH expression levels, we sought to determine whether MG levels and gluconeogenic enzymes are altered in kidneys of 6-

22 week-old $CSE^{-/-}$ male mice. These knockout mice were generated by our lab and collaborators, and both male and female $CSE^{-/-}$ mice developed hypertension (Yang et al., 2008). The kidney was the tissue of choice in this study, because these organs have high metabolic rates of MG, and thus would provide us with an accurate assessment of any alteration in the MG formation pathway. Therefore, to investigate if a physiological balance occurs between MG and H_2S, we plan to analyze age-related changes in MG levels, gluconeogenic enzymes, and transcription factors in the kidneys of $CSE^{-/-}$ male mice, ages 6-22 weeks old (Figure 1-11), with the intention of determining:

1. Altered plasma glucose and MG levels in 6-22 week-old $CSE^{-/-}$ and $CSE^{+/+}$ mice.
2. Age-influenced changes in the MG levels, along with the MG precursors, DHAP and GA3P, in the renal tissues of $CSE^{-/-}$ and $CSE^{+/+}$ mice.
3. Age-related alterations in the enzymatic activities of FBPase, which catalyzes the conversion of fructose-1,6-bisphosphate (F-1,6-P) to fructose-6-phosphate (F-6-P), and the counter glycolysis enzyme, PFK, which catalyzes the conversion of F-6-P to F-1,6-P, in the kidney of $CSE^{-/-}$ and $CSE^{+/+}$ mice. We also plan to analyze the F-1,6-P and F-6-P levels in the renal tissues of $CSE^{-/-}$ and $CSE^{+/+}$ mice.
4. Altered mRNA levels of the main rate-limiting gluconeogenic enzymes, FBPase-1,-2 and PEPCK, along with the mRNA levels of the gluconeogenic regulators, PGC-1α and ERRα, in the $CSE^{-/-}$ and $CSE^{+/+}$ mice.

5. Finally, in cultured A-10 cells, we intend on determining a H_2S-induced up-regulation of PGC-1α, ERRα, and FBPase-1 and -2 mRNA levels in NaHS-treated VSMCs.

Performing a gluconeogenic study in the CSE knockout mice, and thus lower levels of vascular H_2S, would provide us with the information needed to determine if the endogenous production of MG can be influenced by H_2S, likely by influencing the rate of gluconeogenesis. Additionally, in regards to the drug activity of metformin, possible alterations of FBPase in the presence of decreased H_2S could provide novel insights into the use of FBPase inhibitors to treat hyperglycemia in diabetic patients. Figure 1-11 summarizes our experimental objectives.

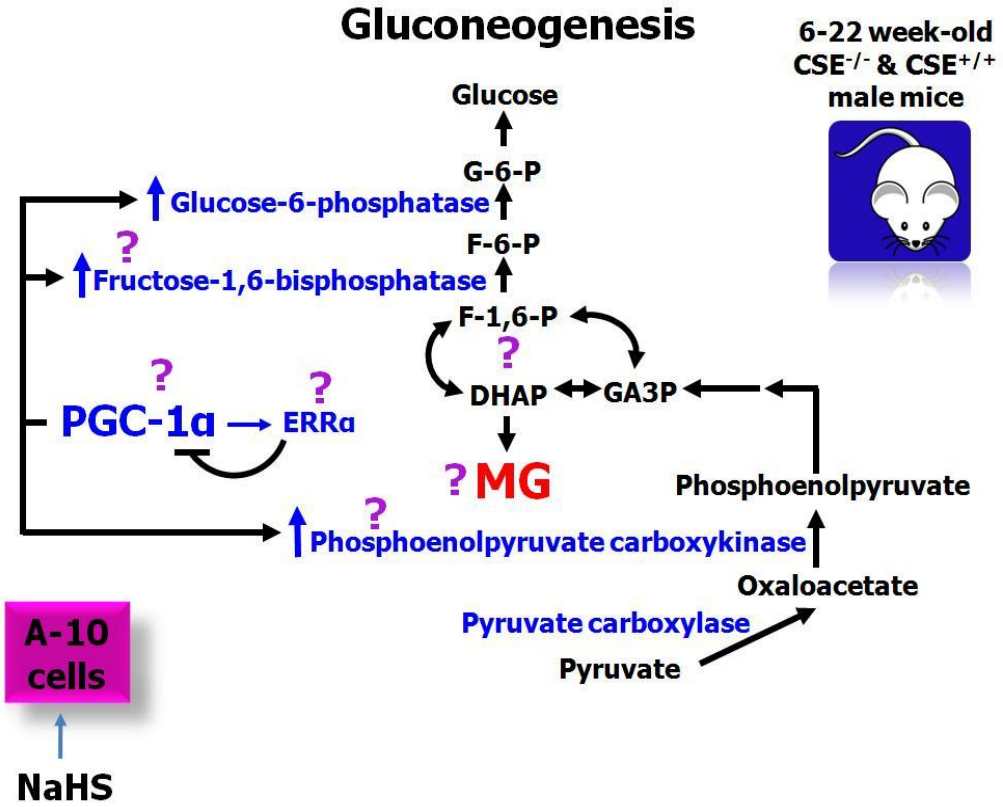

Figure 1-11: Schematic diagram for the layout of Study 2.

CHAPTER 2

GENERAL METHODOLOGY

VSMC PREPARATION

A rat thoracic aortic smooth muscle cell line (A-10 cells) was obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, as described in our previous study (Chang et al. 2010). Cultured cells were grown to 60~80% of confluence before being starved in serum-free DMEM for 24 h and then exposed to MG or NaHS, a H_2S donor, for 24 h. Treated and untreated cells were washed with ice-cold phosphate-buffered saline (PBS), and then harvested by trypsinization. For the determination of oxidized DCF production, cells were seeded in 96-well plates with equal amount of cells in each well (~$4x10^4$ cells) and treated as indicated above.

ANIMALS AND TISSUE PREPARATION

Male 6-22 week-old $CSE^{+/+}$ and $CSE^{-/-}$ mice were housed in a temperature-regulated animal facility and exposed to a 12 h light/dark cycle with free access to food and water. All animal experiments were conducted in accordance with protocols approved by the Animal Health Care Committee of the University of Saskatchewan, in accordance with the guidelines of the Canadian Council on Animal Care. Prior to harvesting tissues, mice were starved for 16 h. Kidneys and aortas were isolated in ice-cold PBS, cleaned, and snap-frozen in liquid nitrogen immediately. Tissues were pulverized with a Mikro-Dismembrator (B. Braun Biotech International, PA, USA) and stored at -80 °C until processing.

MG MEASUREMENT

Quantitation of MG was performed by the widely accepted *o*-phenylenediamine (*o*-PD)-based assay as described (Chang et al. 2010). A-10 cells were collected in a cell lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and a proteinase inhibitor cocktail) and kidney samples were prepared in ice-cold 50 mM sodium phosphate monobasic buffer (pH 4.5). Samples were sonicated twice for 15 s on ice and then centrifuged at 12,000 rpm at 4 °C for 10 min. A portion of the supernatant was used for protein determination *via* the bicinchoninic acid procedure using bicinchoninic acid (BCA) standards. The supernatant from cell or kidney homogenate were incubated with a final concentration of 10 mM *o*-PD (derivatizing agent) and 0.45 N perchloric acid (PCA) with 50 μM EDTA for 24 h at room temperature and protected from light. The quinoxaline formed between dicarbonyl compounds and *o*-PD, as well as the internal standard (5-methylquinoxaline) were measured using a Hitachi D-7000 high-performance liquid chromatography (HPLC) system (Hitachi Ltd., Ontario, Canada). A Nova-Pak C18 column was used (Waters, MA, USA). The mobile phase was composed of 8% (v/v) of 50 mM NaH_2PO_4 (pH 4.5), 17% (v/v) of HPLC grade acetonitrile and 75% of water. Samples were measured in triplicate and calibrated by comparison with a 2-methylquinoxaline standard.

MEASUREMENT OF REDUCED GSH LEVELS

Levels of reduced GSH in the supernatant of lysed cells were determined by derivation with 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) and reverse-phase HPLC using ultraviolet detection, as described in our previous study (Chang et al. 2010). The reaction mixture contained 250 μL 500 mM Tris–HCl buffer (pH 8.9), 65 μL sample or standard, 10 μL internal standard (400 μM D(-)-penicillamine in cold 5% sulfosalicylic acid containing 0.1 mM EDTA), and 175 μL 10 mM DTNB made up in 0.5 mM K_2HPO_4 (pH 7.2). After 5 min of derivatization, the mixture was acidified with 21.5 μL 7 M H_3PO_4, and 50 μL of the mixture was injected into the HPLC system. Chromatography was accomplished using isocratic elution on a Supelcosil LC-18-T column (150 x 4.6 mm, 3 μm) incubated in a 37 °C water bath. The mobile phase consisted of 12.5% methanol (v/v) and 100 mM KH_2PO_4 (pH 3.85) at a flow rate of 0.9 mL/min. GSH–DTNB derivatives were detected by ultraviolet absorbance at 345 nm. After 10 min of isocratic elution, the methanol concentration increased to 40% and pumped for 8 min to elute excess DTNB reagent from the column. The methanol concentration then decreased to 12.5% and was pumped for 7 min before the next sample injection. For analyte quantification, standard curves were constructed by spiking the supernatant with various known amounts of GSH (Sigma, Ontario, Canada). Samples were run in duplicate. Data was collected digitally with D-7000 HPLC System Manager (HSM) software and peak areas were quantified.

MEASUREMENT OF ENZYME ACTIVITIES

FBPase activity

Fructose-1,6-bisphosphatase (FBPase) activity was assayed by the spectrophotometic method, as described (Pontremoli et al. 1965). This assay coupled the production of F-6-P to the reduction of $NADP^+$, which was monitored directly at 340 nm. The assay mixture contained 40 mM glycine buffer (pH 9.1), 1.0 mM EDTA, 2.0 mM $MgCl_2$, 0.6 mM $NADP^+$, and 1.2 U/mL for both glucose-6-phosphate dehydrogenase and phosphoglucose isomerase. The reaction mixture was equilibrated for 10 min at 37 °C. The reaction was initiated by the addition of 70 μM of F-1,6-P and the absorbance was measured at 340 nm in a Multiskan Spectrum (Thermo Labsystems).

PFK activity

Phosphofructokinase (PFK) activity was measured by determining the amount of F-1,6-P, as described (Furuya and Uyeda 1981). The assay mixture contained 50 mM Tris buffer (pH 8.0), 1.0 mM EDTA, 5.0 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.2 mM NADH, 1.0 mM F-6-P, 1.5 U/mL aldolase, and 1.0 U/mL of both triosephosphate isomerase and glycerophosphate dehydrogenase. The reaction was initiated by the addition of 0.5 mM ATP and the decrease in absorbance was measured at 340 nm in a Multiskan Spectrum (Thermo Labsystems).

CSE activity

CSE enzyme activity was determined by measuring the production rate of H_2S as reported (Wu et al. 2009; Chang et al. 2010). Collected A-10 cells were suspended in 400 μL of ice-cold

potassium phosphate buffer (50 mM, pH 6.8) supplemented with a proteinase inhibitor cocktail and lysed by sonication on ice. The supernatant (100 μL) was added to 1 mL of reaction mixture containing (mM): 100 potassium phosphate buffer (pH 7.4), 10 $_L$-cysteine, and 2 pyridoxal-5'-phosphate. Cryovial test tubes (2 mL) were used as the center wells, each containing 0.5 mL 1% zinc acetate as trapping solution. Reaction was performed in a 25 mL Erlenmeyer flask (Pyrex, USA). The flasks containing the reaction mixture and center wells were flushed with N_2 gas before being sealed with a double layer of parafilm. Reaction was initiated by transferring the flasks from ice to a 37 ^oC shaking water bath. After incubating at 37 ^oC for 90 min, reaction was stopped by adding 0.5 mL of 50% trichloroacetic acid. The flasks were sealed again and incubated at 37 ^oC for another 60 min to ensure a complete trapping of released H_2S gas from the mixture. Contents of the center wells were then transferred to test tubes, each containing 0.5 mL of double distilled water. Subsequently, 0.5 mL of 20 mM N,N-dimethyl-p-phenylenediamine sulfate in 7.2 N HCl was added immediately followed by addition of 0.5 mL 30 mM $FeCl_3$ in 1.2 N HCl. The mixture was kept at room temperature, protected from light, for 20 min followed by recording the absorbance at 670 nm in a Multiskan Spectrum (Thermo Labsystems). H_2S concentration was calculated using a calibration curve of standard NaHS solutions.

RNA ISOLATION AND REAL-TIME QUANTITATIVE PCR

Total RNA was isolated using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. First strand cDNA was prepared from total RNA (5 μg) by reverse transcription using M-MLV reverse transcriptase (Invitrogen) and oligo(dT) primer. Real-time quantitative

PCR was performed on the iCycler iQ Real-time PCR Detection System (Bio-Rad, Nazareth). The forward and reverse primers of either mouse or rat CSE, PGC-1α, FBPase-1 and -2, PEPCK, ERRα, and β-actin were used. The PCR conditions were as follows: denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 30 s. Specificity of the amplification was determined by melting curve analysis.

CHAPTER 3

INTERACTIONS OF METHYLGLYOXAL AND HYDROGEN SULFIDE IN RAT VASCULAR SMOOTH MUSCLE CELLS

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ABSTRACT

Background: Hydrogen sulfide (H_2S) is a gasotransmitter with multifaceted physiological functions, including the regulation of glucose metabolism. Methylglyoxal (MG) is an intermediate of glucose metabolism, playing an important role in the pathogenesis of insulin resistance syndrome. In the present study, we investigated the effect of MG on H_2S synthesis and the interaction between these two endogenous substances.

Materials and Methods: In A-10 cells, HPLC was used to determine reduced glutathione (GSH), $_L$-cysteine, homocysteine, and MG levels. DCFH-DA probe was used to measure reactive oxygen species (ROS) generation in cultured vascular smooth muscle cells (VSMCs). Microelectrode specific for H_2S was used to measure H_2S levels. Western blot was used to determine protein expression levels of cystathionine γ-lyase (CSE). Spectrophotometer method was used to measure CSE activity levels.

Results: In cultured VSMCs, MG (10, 30, and 50 μM) significantly decreased cellular H_2S levels in a concentration-dependent manner, while H_2S donor, NaHS (30, 60, and 90 μM), significantly decreased cellular MG levels. The expression level and activity of H_2S -producing enzyme, CSE, were significantly decreased by MG treatment. NaHS (30-90 μM) significantly inhibited MG (10 or 30 μM)-induced ROS production. Cellular levels of GSH, $_L$-cysteine, and homocysteine were also increased by MG or NaHS treatment. Furthermore, a direct reaction of H_2S with MG in both concentration- and time-dependent manners was observed in *in vitro* incubations.

Conclusions: MG regulates H_2S level in VSMCs by down-regulating CSE protein expression and directly reacting with H_2S molecule. Interaction of MG with H_2S may be important for glucose metabolism and the development of insulin resistance syndrome.

Key words: Hydrogen sulfide ■ methylglyoxal ■ vascular smooth muscle cells ■ reactive oxygen species

INTRODUCTION

Hydrogen sulfide (H_2S) is the third gasotransmitter with multifaceted physiological functions [1, 2]. Two pyridoxal-5'-phosphate-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), are responsible for the majority of endogenous H_2S production in mammalian tissues using $_L$-cysteine as the substrate [3, 4]. The expression of CSE and CBS is tissue specific. For instance, CBS is the major H_2S producing enzyme in the nervous system, whereas CSE is mainly expressed in vascular and non-vascular smooth muscle cells [1, 2, 5]. Another less important endogenous source of H_2S is the non-enzymatic reduction of elemental sulfur to H_2S using reducing equivalents obtained from the oxidation of glucose [6].

H_2S exerts a host of biological effects on various types of cells and tissues. At micromolar concentrations, H_2S can have cytoprotective effects [7], while at millimolar concentrations it has been shown to be cytotoxic [8-10]. Previous studies have also proved that H_2S up-regulates the expression of anti-inflammatory and cytoprotective genes including heme oxygenase-1 in pulmonary artery smooth muscle cells [11] and macrophages [12]. The vascular relaxation effect of H_2S was proved largely due to the opening of K_{ATP} channels [13, 14]. In line with its vasorelaxant effect, a H_2S donor was shown to induce a transient hypotensive response in animals [1, 13]. In patients with coronary heart disease, plasma H_2S level was reduced from ~50 to ~25 μM [15]. However, it should be noted that the value of plasma H_2S measured by another group using a different method is substantially different with the values above [16]. We recently

showed that CSE deficiency and reduced endogenous H_2S production in vascular tissues resulted in the development of hypertension in CSE gene knockout mice [5].

Methylglyoxal (MG) is a metabolite of sugar, protein, and fatty acid, formed in virtually all mammalian cells, including vascular smooth muscle cells (VSMCs) [17]. Increased MG production has been reported in human red blood cells, bovine endothelial cells, and VSMCs under hyperglycemic conditions or with increased availability of MG precursors such as fructose [18]. We recently discovered that hypertension in spontaneously hypertensive rats was related to increased MG levels in plasma and vascular tissues in an age-dependent fashion [19, 20]. It has been reported that an elevated MG level is associated with oxidative stress in vascular tissues [21, 22]. MG can induce the production of reactive oxygen species (ROS), including peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2), and superoxide anion ($O_2^{\cdot-}$) in cultured VSMCs [23]. Moreover, as a highly reactive dicarbonyl molecule, MG can interact with the side chains of arginine, lysine, and cysteine residues in proteins to yield different types of advanced glycation endproducts [24]. In the present study, we investigated the interaction of MG and H_2S in VSMCs and the cellular effects of this interaction.

MATERIALS AND METHODS

VSMC preparation

Rat thoracic aortic smooth muscle cell line (A-10 cells) was obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) containing

10% bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, as described in our previous study [23]. Cultured cells were grown to 60~80% of confluence before starved in serum-free DMEM for 24 h and then exposed to MG or H_2S treatments for 24 h. Treated and untreated cells were washed with ice-cold phosphate-buffered saline (PBS), and then harvested by trypsinization. For the determination of oxidized DCF production, cells were seeded in 96-well plates with equal amount of cells in each well (~ 4×10^4 cells) and treated as indicated above.

Measurement of cellular H_2S level

H_2S was measured using a microelectrode as previously described [14, 25]. Briefly, harvested cells were resuspended in 400 μL of cell lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and a proteinase inhibitor cocktail). H_2S in cell lysis (200 μL) was released by adding 5 ml of 80% sulfuric acid in a sealed filtering flask bubbled with N_2 gas for 15 min. Released H_2S was carried by N_2 gas to an absorber container (15 mL test tube), containing 1 mL of 1 M NaOH into which H_2S was absorbed. H_2S level in NaOH was measured with a microelectrode specific for sulfide (Lasar Research Laboratories Inc). H_2S concentration was calculated using a standard curve of NaHS at different concentrations and is expressed in nmol/mg protein.

Measurement of cellular MG level

Collected cells were lysed in a cell lysis buffer containing a proteinase inhibitor cocktail (as stated above). MG in the supernatant of cell lysis was measured as previously reported [20, 25]. Briefly, samples were incubated with 10 mM *o*-phenylenediamine (*o*-PD, derivatizing agent) for 3 h at room temperature and protected from light. The quinoxaline formed between dicarbonyl compounds and *o*-PD, as well as the internal standard (5-methylquinoxaline) were measured using a Hitachi D-7000 high-performance liquid chromatography (HPLC) system (Hitachi Ltd., Mississauga, Ontario, Canada) [20]. A Nova-Pak C18 column was used (Waters, MA, USA). The mobile phase was composed of 8% (v/v) of 50 mM NaH_2PO_4 (pH 4.5), 17% (v/v) of HPLC grade acetonitrile and 75% of water. Samples were measured in triplicates.

Measurement of CSE activity

CSE enzyme activity was determined by measuring the production rate of H_2S as reported [14]. Briefly, collected cell were suspended in 400 μL of ice-cold potassium phosphate buffer (50 mM, pH 6.8) supplemented with proteinase inhibitor cocktail and lysed by sonication on ice. Supernatant (100 μL) was added to 1 mL of reaction mixture containing (mM): 100 potassium phosphate buffer (pH 7.4), 10 $_L$-cysteine, and 2 pyridoxal-5'-phosphate. Cryovial test tubes (2 mL) were used as the center wells, each containing 0.5 mL 1% zinc acetate as trapping solution. Reaction was performed in a 25 mL Erlenmeyer flask (Pyrex, USA). The flasks containing the reaction mixture and center wells were flushed with N_2 gas before being sealed with a double layer of parafilm. Reaction was initiated by transferring the flasks from ice to a 37 ^oC shaking

water bath. After incubating at 37 ^{o}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 ^{o}C for another 60 min to ensure a complete trapping of released H_2S gas from the mixture. Contents of the center wells were then transferred to test tubes, each containing 0.5 mL of water. Subsequently, 0.5 mL of 20 mM N,N-dimethyl-p-phenylenediamine sulfate in 7.2 N HCl was added immediately followed by addition of 0.5 mL 30 mM $FeCl_3$ in 1.2 N HCl. The mixture was kept at room temperature, protected from light, for 20 min followed by recording the absorbance at 670 nm with a spectrophotometer. H_2S concentration was calculated using a calibration curve of standard NaHS solutions.

RNA isolation and Real-time quantitative PCR

Total RNA was isolated using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. First strand cDNA was prepared from total RNA (5 μg) by reverse transcription using M-MLV reverse transcriptase (Invitrogen) and oligo(dT) primer. Real-time quantitative PCR was performed on the iCycler iQ Real-time PCR Detection System (Bio-Rad, Nazareth). The primers of rat CSE were as following: forward 5'-GGACAAGAGCCGGAGCAATGGAGT-3', reverse 5'- CCCCGAGGCGAAGGTCAAACAGT -3'. The primers for rat β-actin was: forward 5'-CGTTGACATCCGTAAAGAC-3' and reverse 5'-TAGGAGCCAGGGCAGTA-3'. The PCR conditions were as follows: denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 30 s.

Specificity of the amplification was determined by melting curve analysis. Data were expressed as a ratio of the quantity of CSE mRNA to the quantity of β-actin mRNA.

Western blot analysis of CSE expression

Total proteins were extracted from harvested cells with 300 μL of cell lysis buffer as described above. Proteins (40 μg) were subject to Western blot analysis according to the procedure reported [26]. The primary antibody dilutions were 1:500 for antibodies against CSE (Abnova, Novus Biologicals, Inc.) and 1:5000 for β-actin. Western blots were digitized with Chemi Genius^2 Bio Imaging System (SynGene), quantified using software of GeneTools from SynGene and normalized against the quantity of loaded β-actin.

Measurement of ROS production

The formation of oxidized DCF was determined by a DCFH assay as described previously with minor modification [23]. Briefly, starved cells were loaded with a membrane-permeable and non-fluorescent probe DCFH-DA for 2 h at 37 °C in phenol red-free DMEM, protected from light. Thereafter, the cells were washed 3 times with phenol red-free DMEM to remove the excess probe, followed by the treatment with or without MG or MG plus NaHS at desired concentrations for different time periods in phenol red-free DMEM. Once inside cells, DCFH-DA becomes the membrane-impermeable $DCFH_2$ in the presence of cytosolic esterases and further oxidized by H_2O_2 or $ONOO^-$ to form oxidized DCF with detectable fluorescence. Oxidized DCF was quantified by monitoring DCF fluorescence intensity with excitation at

485 nm and emission at 527 nm with a Fluoroskan Ascent plate reader (Thermo Labsystem) using Ascent software and expressed in arbitrary units.

Measurement of GSH, $_{L}$-cysteine, and homocysteine levels

Levels of reduced glutathione (GSH), $_{L}$-cysteine, and homocysteine in the supernatant of cell lysis were determined by derivation with 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) and reverse-phase HPLC using ultraviolet detection, as described in our previous study [19]. Briefly, the reaction mixture for the analysis of free reduced sulfhydryl groups contained 250 μL 500 mM Tris–HCl buffer (pH 8.9), 65 μL sample or standard, 10 μL internal standard (400 μM D(-)-penicillamine in cold 5% sulfosalicylic acid containing 0.1 mM EDTA), and 175 μL 10 mM DTNB made up in 0.5 mM K_2HPO_4 (pH 7.2). After 5 min of derivatization, the mixture was acidified with 21.5 μL 7 M H_3PO_4, and 50 μL of the mixture was injected into the HPLC system. Chromatography was accomplished using isocratic elution on a Supelcosil LC-18-T column (150 x 4.6 mm, 3 μm) incubated at 37 °C. The mobile phase consisted of 12.5% methanol (v/v) and 100 mM KH_2PO_4 (pH 3.85) at a flow rate of 0.9 ml/min. Sulfhydryl–DTNB derivatives were detected by ultraviolet absorbance at 345 nm. After 10 min of isocratic elution, the methanol concentration was increased to 40% and pumped for 8 min to elute excess DTNB reagent from the column. The methanol concentration was then decreased to 12.5% and pumped for 7 min before the next sample injection. For analyte quantification, standard curves were constructed by spiking the supernatant with various known amounts of GSH, $_{L}$-cysteine, and homocysteine

(Sigma). Samples were run in duplicate. Data was collected digitally with D-7000 HPLC System Manager (HSM) software and peak areas were quantified.

Direct reaction of H_2S with MG

MG (10 μM) was mixed with H_2S at different concentrations (10, 50, and 100 μM) in cell-free PBS buffer and incubated at 37 °C for 1~24 h. H_2S stock solution was prepared by bubbling H_2S in distilled H_2O for 30 mins [13]. After incubation, free MG was measured with HPLC as described above.

Chemicals and data analysis

MG and NaHS were obtained from Sigma-Aldrich (Oakville, Canada). The data are expressed as mean ± SEM from at least three independent experiments. Statistical analyses were performed using Student's *t* test or ANOVA. Statistical significance was considered at $P < 0.05$.

RESULTS

MG treatment decreased H_2S level in VSMCs and vice versa

Cultured VSMCs were treated with MG at different concentrations for 24 h. After MG treatment, H_2S level in cell lysates was significantly decreased in a MG concentration-dependent fashion (Figure 3-1). In another group of experiments, the effect of H_2S treatment on MG level was studied. Cultured VSMCs were treated with H_2S donor, NaHS, at different concentrations for 24

h. After NaHS treatment, MG level in cell lysates was significantly decreased in a NaHS concentration-dependent manner (Figure 3-2).

MG-induced down-regulation of CSE protein expression

In VSMCs, CSE is the major enzyme responsible for H_2S production. Thus, the CSE expression level in MG-treated cells was investigated. Quantitative-PCR results indicated that MG treatment did not significantly affect CSE mRNA levels (Figure 3-3A). Western blot results showed that CSE protein level was significantly lower in 30 and 50 μM MG-treated cells, but not with 10 μM MG treatment (Figures 3-3B & C, respectively). As shown in Figure 3-3D, CSE activity in 30 and 50 μM MG-treated cells was significantly decreased by 14 and 29% in comparison to the untreated control. CSE activity in 10 μM MG-treated group was lower than the untreated control although the difference was not significant.

Effect of H_2S on MG-induced ROS production

ROS formation in VSMC was significantly increased by MG (10, 30, and 50 μM) in both time- and concentration-dependent manners (Figures 3-4A & B). Interestingly, 30 μM NaHS significantly decreased 10 μM MG-induced ROS production, but not 30 or 50 μM MG (Figure 3-4A). The anti-oxidant effect of H_2S was observed within 8 h of application and continued thereafter (Figure 3-4B). NaHS at 60 and 90 μM decreased 10 μM MG-induced ROS production (Figure 3-4C). This effect of NaHS was more potent when the cells were treated with 30 μM MG

(Figure 3-4D). However, NaHS no longer offered the anti-oxidant effect against MG-induced ROS production once its concentration reached 120 μM (Figures 3-4C & D).

GSH, L-cysteine, and homocysteine levels in MG- or NaHS-treated VSMCs

GSH is an important anti-oxidant agent that protects the cell from oxidative stress [27]. After VSMCs were treated with MG for 24 h, cellular GSH level was significantly increased correspondingly to the concentrations of MG (10-50 μM) (Figure 3-5A). Homocysteine is a precursor of L-cysteine synthesis, where L-cysteine is a precursor of GSH**.** MG treatment at 10 μM, but not 30 or 50 μM, significantly increased L-cysteine and homocysteine levels in cultured VSMCs (Figures 3-5B & C, respectively).

The effects of H_2S on GSH, L-cysteine, and homocysteine levels were also investigated. Cellular GSH levels were significantly increased when cells were exposed to NaHS treatment as compared with control cells (Figure 3-6A). However, GSH levels in 60 and 90 μM NaHS-treated cells were significantly lower than that in 30 μM NaHS-treated cells. L-cysteine levels were also significantly increased by NaHS treatments (30, 60, and 90 μM) (Figure 3-6B).

Direct reaction of MG with H_2S

To understand the mechanism of H_2S and MG interaction, we tested whether MG directly reacts with H_2S molecule. For this purpose, MG (10 μM) was mixed with H_2S at different concentrations in cell-free PBS buffer and incubated at 37 °C for 24 h. After incubation, free MG

was measured with HPLC. H_2S at 50 and 100 μM, but not at 10 μM, significantly decreased MG levels (Figure 3-7A). When the H_2S incubation time was less than 4 h, no change in MG level was observed. However, significant decreases in MG levels were detected after 8 h of incubation with the lowest level detected after 18 h incubation with H_2S (Figure 3-7B).

DISCUSSION

Under physiological conditions, MG level is generally higher in vascular tissues than in other types of tissues [19]. CSE is responsible for H_2S production in vascular tissues, endothelium, pancreas, and liver while CBS produces H_2S mainly in brain and kidney [1, 5, 14]. Obviously, MG and H_2S are co-produced in VSMCs. For instance, we found that the interaction of MG and H_2S lowers their respective cellular levels. We also found that CSE protein level was down-regulated by MG (30 and 50 μM) although no change of CSE mRNA level in MG-treated cells was observed. The above results indicate that MG treatment may decrease the translation of CSE mRNA or the stability of CSE proteins. However, the underlying mechanisms of MG treatment on CSE mRNA translation and the protein stability are not yet clear and will need further investigation. Along with decreased CSE protein levels after MG treatments, CSE enzyme activity was also decreased as indicated by a lower production rate of H_2S. Therefore, MG-induced decrease in CSE protein level could at least in part explain the decrease in H_2S level.

Whereas endogenous cellular H_2S level was significantly decreased by 10 μM MG treatment, CSE protein level or CSE activity was not significantly different from the untreated control. This

phenomenon could be explained by a direct reaction between MG and H_2S, considering H_2S as a reducing agent and MG as a reactive dicarbonyl molecule. We found that MG level in the cell-free MG/H_2S mixture was decreased 8 h after the incubation was started. This chemical reaction between MG with H_2S occurred in both time- and concentration-dependent manners. These data suggest that a direct reaction of MG with H_2S may be responsible for lower MG (10 μM)-induced decrease in H_2S level, while MG at 30-50 μM caused both a direct molecule-to-molecule reaction, as well as the down-regulation of CSE protein expression. Consistently, the direct reaction of MG with H_2S may have caused the decreased MG level in H_2S donor-treated VSMCs.

In our previous study, we showed that MG increased ROS production in VSMCs by increasing $ONOO^-$, H_2O_2, and $O_2^{\cdot-}$ levels [23, 24]. We also showed that H_2S protected VSMCs against homocysteine-induced oxidative stress [7]. It was of interest to study the effect of H_2S on MG-induced ROS production. Our results support the notion that H_2S acts as an anti-oxidant [7]. At the concentrations lower than 90 μM, H_2S decreased 10 and 30 μM MG-induced ROS production in a concentration-dependent manner, but had no effect on 50 μM MG-induced ROS generation. This could be due to the fact that MG-induced ROS formation at high concentrations overwhelms the anti-oxidant ability of H_2S. It is also important to note that H_2S at concentration higher than physiological related concentrations, for example 120 μM, fails to inhibit low concentrations of MG (10 and 30 μM)-induced ROS production. This may be related to the toxicity and pro-apoptosis effect of H_2S at high concentrations [8-10].

One of the most important and abundant endogenous anti-oxidant is GSH, which is found at millmolar range in most cells [27]. GSH levels are significantly elevated when the cells are treated with MG (10, 30, and 50 μM) compared to that of the control group. $_{L}$-cysteine availability is the rate-limiting step in GSH synthesis, and homocysteine is the precursor to $_{L}$-cysteine. We observed a corresponding increase of $_{L}$-cysteine and homocysteine levels in cells treated with MG at the concentration of 10 μM, but not 30 or 50 μM. This may be due to the huge consumption of $_{L}$-cysteine and homocysteine in order to maintain GSH at a certain level to compensate the higher ROS levels in cells induced by MG (30 and 50 μM). On the other hand, H_2S treatment of VSMCs also increased GSH level, which may be attributed to H_2S-enhanced activity of γ-glutamylcysteine synthetase [28]. Furthermore, H_2S may cause a feedback inhibition of CSE [29], which could lead to a decreased breakage of $_{L}$-cysteine to produce H_2S. Consistently, the increased level of $_{L}$-cysteine was observed after H_2S treatment. The consequent increased level of $_{L}$-cysteine may inhibit the demethylation of methionine to produce homocysteine [30].

The physiological relevance for the interaction between MG and H_2S has not been previously investigated. Elevated MG level is linked with the development of hypertension and insulin resistance [24, 31, 32]. In vascular tissues, elevated MG level is expected to lead to a decreased H_2S level based on the direct reaction of MG with H_2S and the down-regulation of CSE expression by MG. One of the consequences of abnormally low H_2S level would decrease the opening of K_{ATP} channels and impair vascular relaxation, causing an increased peripheral

circulation resistance and hypertension development or vascular complications of diabetes. In conclusion, MG can react with H_2S and cause a down-regulation of the expression of CSE. MG may reduce H_2S production, whereas H_2S may limit the availability of free MG. As mentioned before, CSE is mainly expressed in vascular tissues, endothelium, pancreas, and liver, while MG is produced virtually all mammalian cells. Therefore, the interactions of MG with H_2S are expected to occur in VSMCs and possibly other types of tissues, which may provide one of future directions for the studies on glucose metabolism and the development of insulin resistance syndromes.

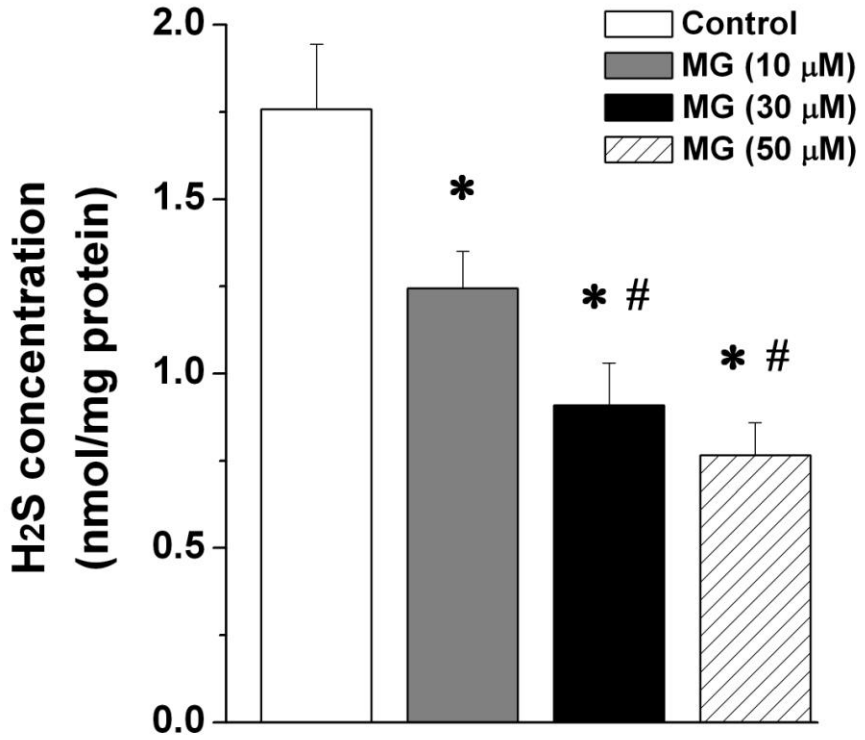


Figure 3-1: **H_2S level in MG-treated VSMCs.** VSMCs were treated with MG at 10, 30, and 50 μM for 24 h, respectively. H_2S level in cell lysis was expressed in nmoL/mg protein. n = 4-6. *$P < 0.05$ *versus* control; $^{\#}P < 0.05$ *versus* MG (10 μM).

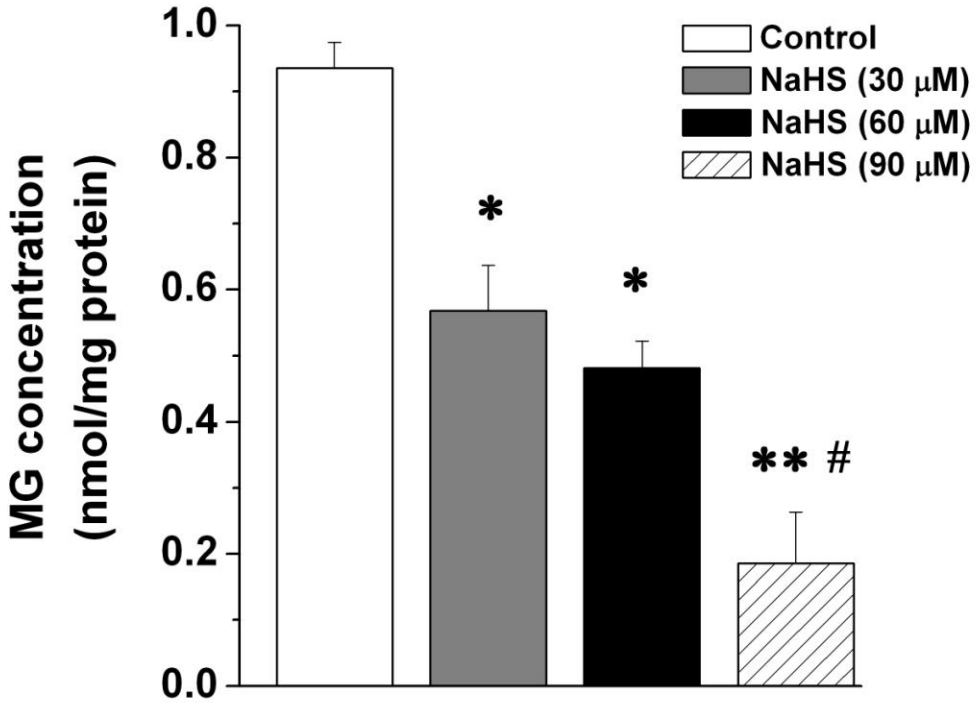


Figure 3-2: MG level in H_2S-treated VSMCs. VSMCs were treated with H_2S donor NaHS at 30, 60, and 90 μM for 24 h, respectively. MG level in cell lysis was measured using HPLC as described in Materials and Methods. $n = 3$. $^{*}P < 0.05$ or $^{**}P < 0.01$ *versus* control; $^{\#}P < 0.05$ *versus* NaHS (30 or 60 μM).

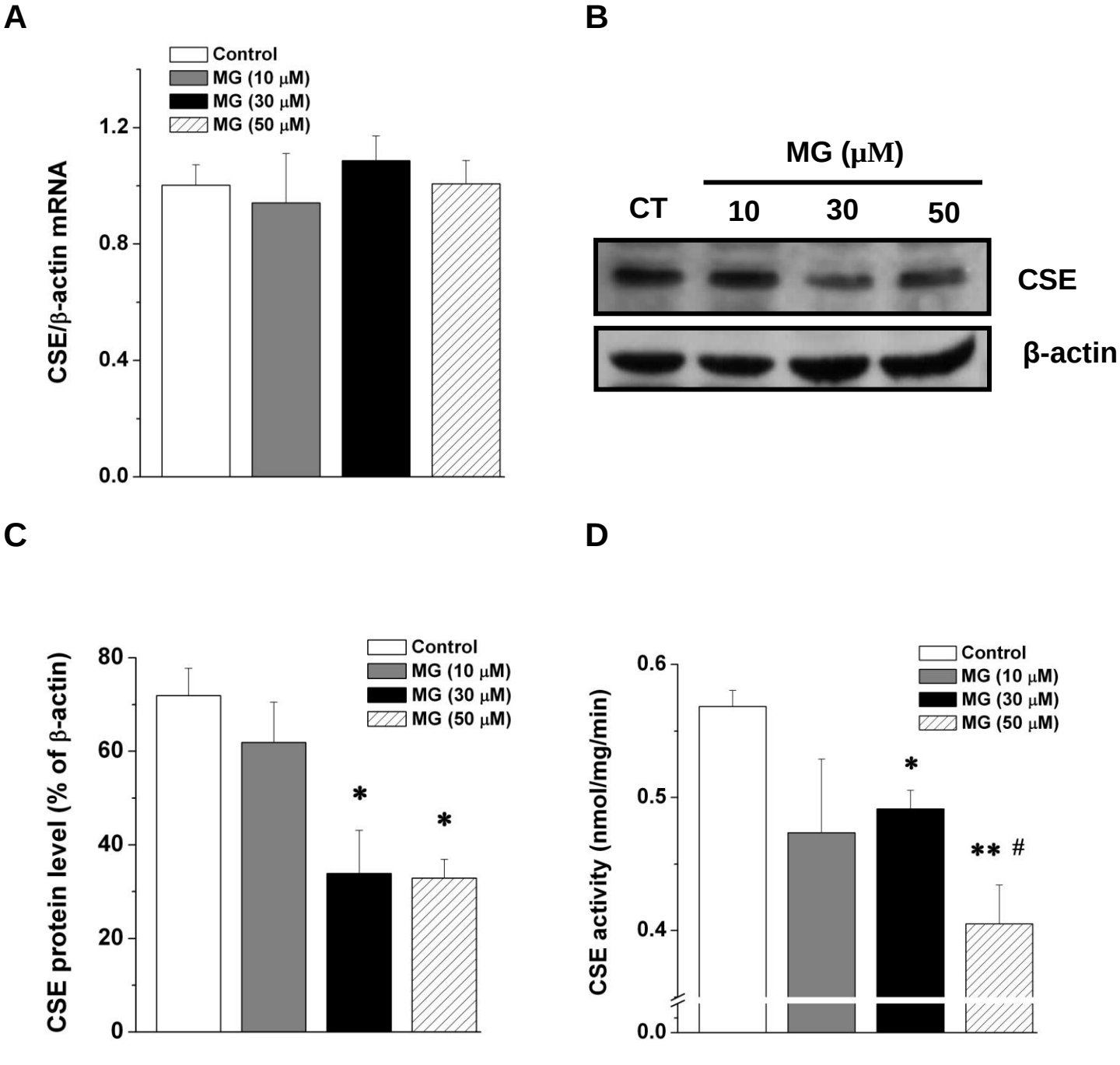
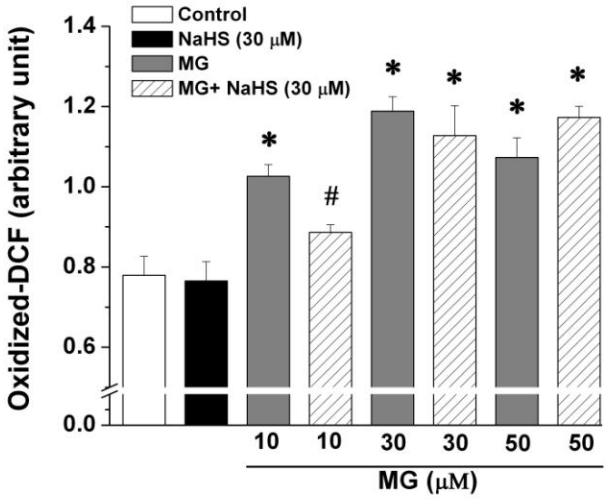
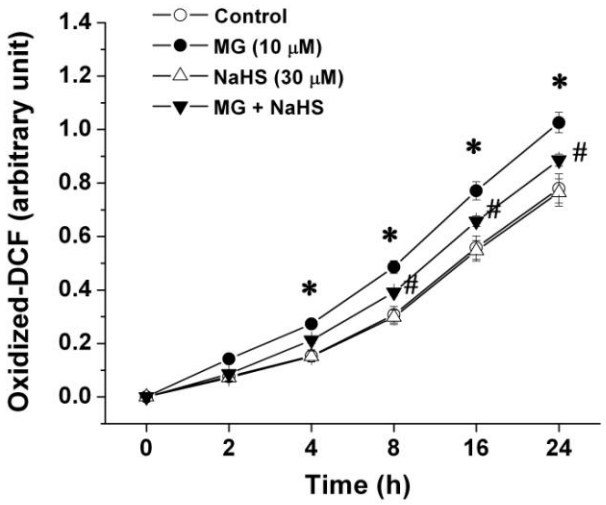

A
Control
MG (10 μM)
MG (30 μM)
MG (50 μM)
CSE/β-actin mRNA
1.2
0.8
0.4
0.0
B
MG (μM)
CT
10
30
50
CSE
β-actin
C
Control
MG (10 μM)
MG (30 μM)
MG (50 μM)
CSE protein level (% of β-actin)
80
60
40
20
0
*
*
D
Control
MG (10 μM)
MG (30 μM)
MG (50 μM)
CSE activity (nmol/mg/min)
0.6
0.5
0.4
0.0
*
** #

Figure 3-3: Effects of MG treatment on CSE expression and activity. VSMCs were treated with MG at 10, 30, and 50 μM for 24 h, respectively. Treated cells were collected for RNA or protein extraction. A. Real-time PCR results of CSE mRNA level in MG-treated cells. $n = 6$ for each group. B & C. CSE protein level in MG-treated cells. $n = 4$ for each group. $^{*}P < 0.05$ *versus* control. D. CSE activity in MG-treated cells. $n = 6$-9. $^{*}P < 0.05$ or $^{**}P < 0.01$ *versus* control; $^{\#}P < 0.05$ *versus* MG (30 μM).

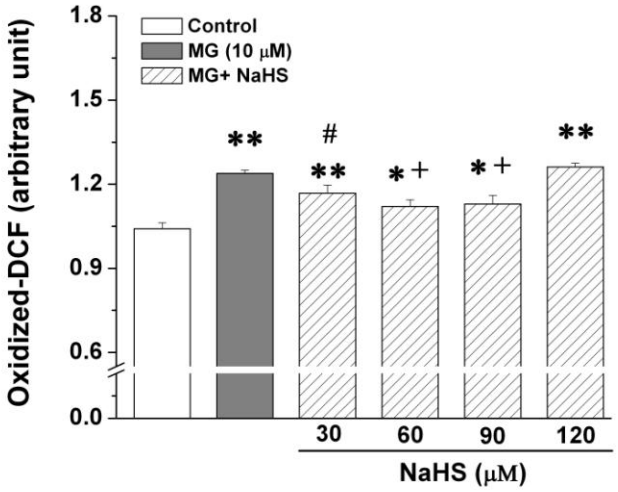
A


Control
NaHS (30 μM)
MG
MG+ NaHS (30 μM)
Oxidized-DCF (arbitrary unit)
1.4
1.2
1.0
0.8
0.6
0.0
10
10
30
30
50
50
MG (μM)

B


Control
MG (10 μM)
NaHS (30 μM)
MG + NaHS
Oxidized-DCF (arbitrary unit)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
8
16
24
Time (h)

C


Control
MG (10 μM)
MG+ NaHS
Oxidized-DCF (arbitrary unit)
1.8
1.5
1.2
0.9
0.6
0.0
30
60
90
120
NaHS (μM)

D

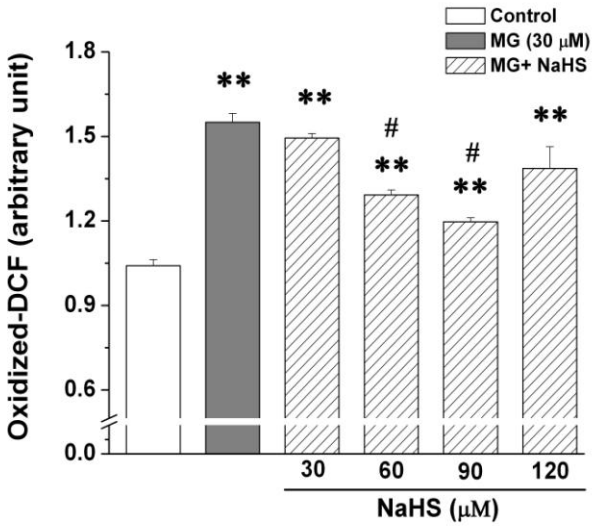

Control
MG (30 μM)
MG+ NaHS
Oxidized-DCF (arbitrary unit)
1.8
1.5
1.2
0.9
0.6
0.0
30
60
90
120
NaHS (μM)

Figure 3-4: Effect of NaHS on MG-induced ROS production in VSMCs. A. Oxidized DCF level in cells treated with MG at different concentrations in the presence NaHS (30 μM). B. Time-dependent effect of NaHS (30 μM) on MG (10 μM)-induced ROS production. C & D. Concentration-dependent effect of NaHS on MG (10 and 30 μM)-induced ROS production. n = 8 for each group. $^{*}P < 0.05$ or $^{**}P < 0.01$ *versus* untreated control; $^{\#}P < 0.05$ *versus* MG (10 μM) or *versus* MG (30 μM) + NaHS (30 μM); $^{+}P < 0.05$ *versus* MG (10 μM) + NaHS (30 μM).

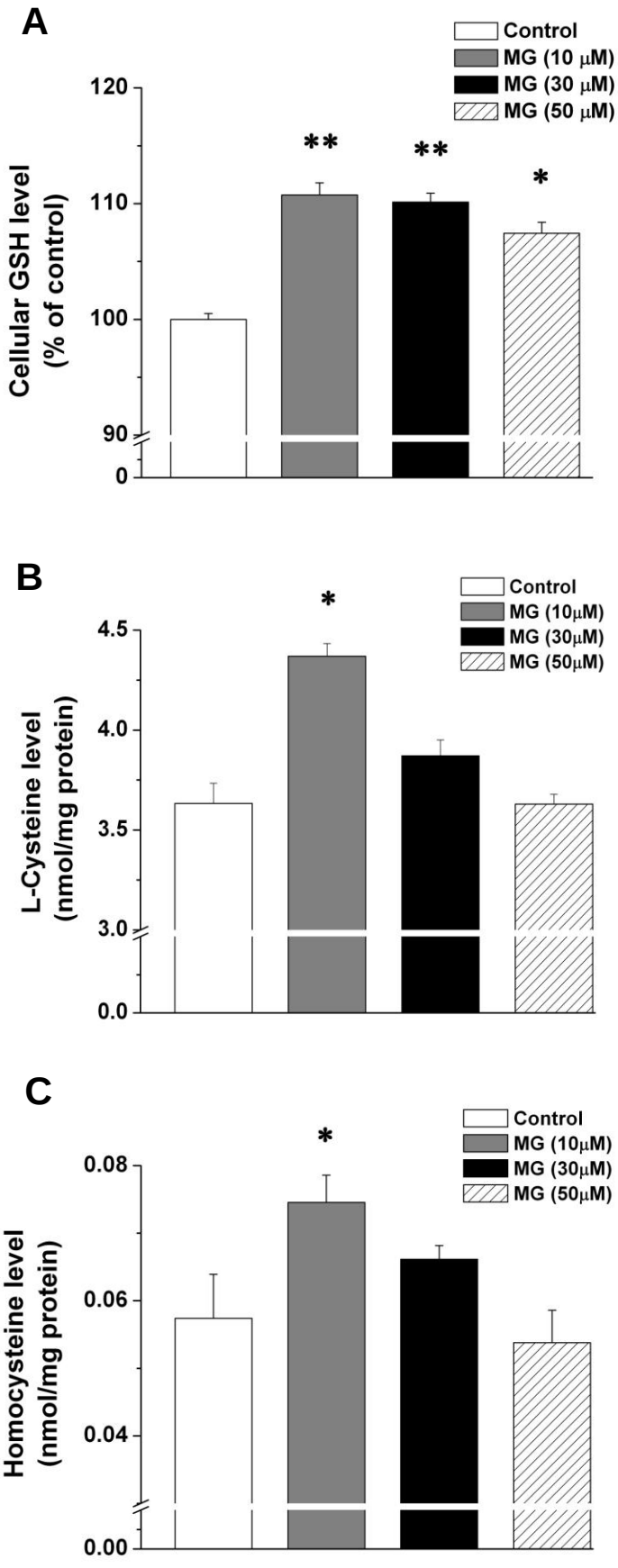
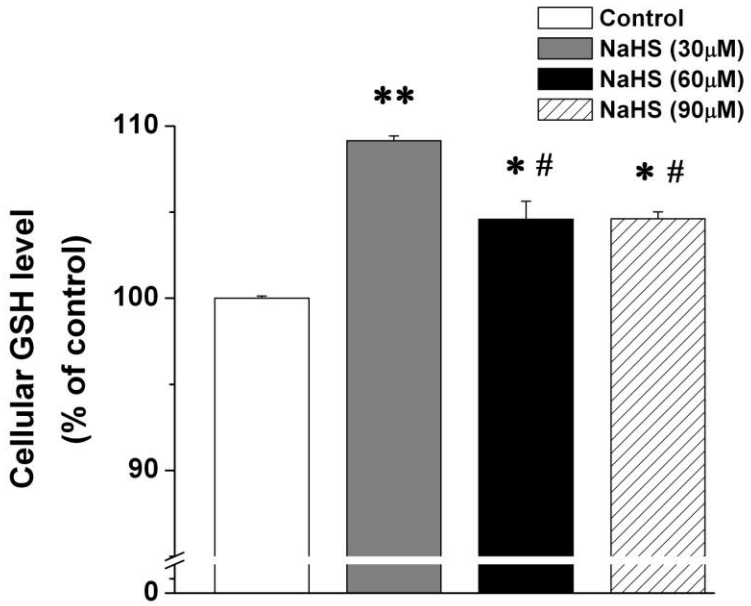

A
Control
MG (10 μM)
MG (30 μM)
MG (50 μM)
Cellular GSH level
(% of control)
120
110
100
90
0
**
**
*
B
Control
MG (10μM)
MG (30μM)
MG (50μM)
L-Cysteine level
(nmol/mg protein)
4.5
4.0
3.5
3.0
0.0
*
C
Control
MG (10μM)
MG (30μM)
MG (50μM)
Homocysteine level
(nmol/mg protein)
0.08
0.06
0.04
0.00
*

Figure 3-5: GSH, $_{L}$-cysteine, and homocysteine levels in MG-treated VSMCs. Cells treated with MG at different concentrations were harvested after 24 h to determine cellular GSH (A), $_{L}$-cysteine (B) and homocysteine (C) levels using HPLC method as described in Materials and Methods. *n* =4-7 for each group. **P* < 0.05 or ***P* < 0.01 *versus* untreated control.

A


Control
NaHS (30μM)
NaHS (60μM)
NaHS (90μM)
Cellular GSH level
(% of control)
110
100
90
0
**
* #
* #

B

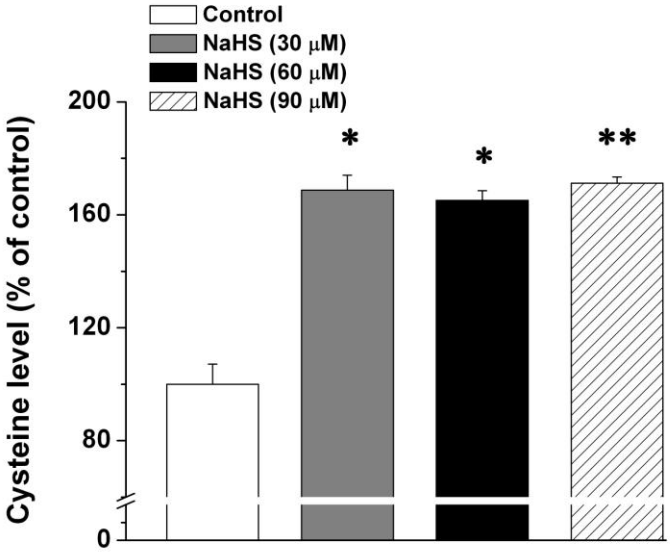
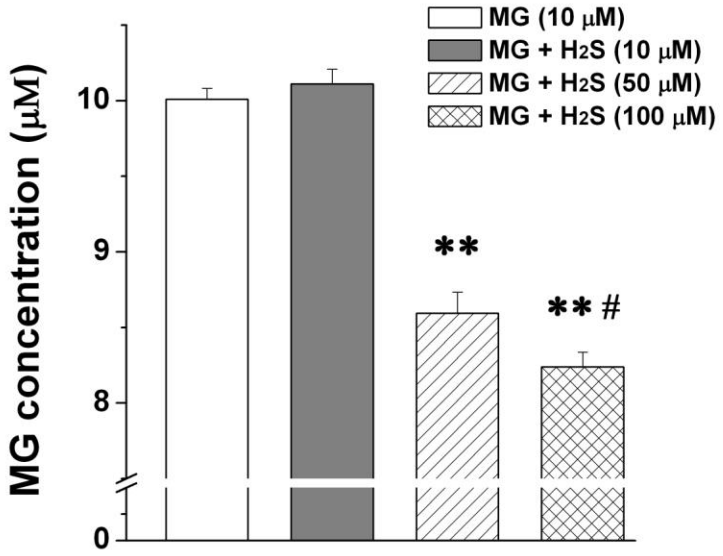

Control
NaHS (30 μM)
NaHS (60 μM)
NaHS (90 μM)
Cysteine level (% of control)
200
160
120
80
0
*
*
**

Figure 3-6: GSH and $_{L}$-cysteine levels in NaHS-treated VSMCs. Cells treated with NaHS at different concentrations were harvested after 24 h to determine cellular GSH (A) and $_{L}$-cysteine (B) levels using HPLC method as described in Materials and Methods. $n = 4$ for each group. *$P < 0.05$ or **$P < 0.01$ *versus* untreated control; $^{\#}P < 0.05$ *versus* NaHS (30 μM).

A


MG (10 μM)
MG + H2S (10 μM)
MG + H2S (50 μM)
MG + H2S (100 μM)
MG concentration (μM)
10
9
8
0
**
** #

B

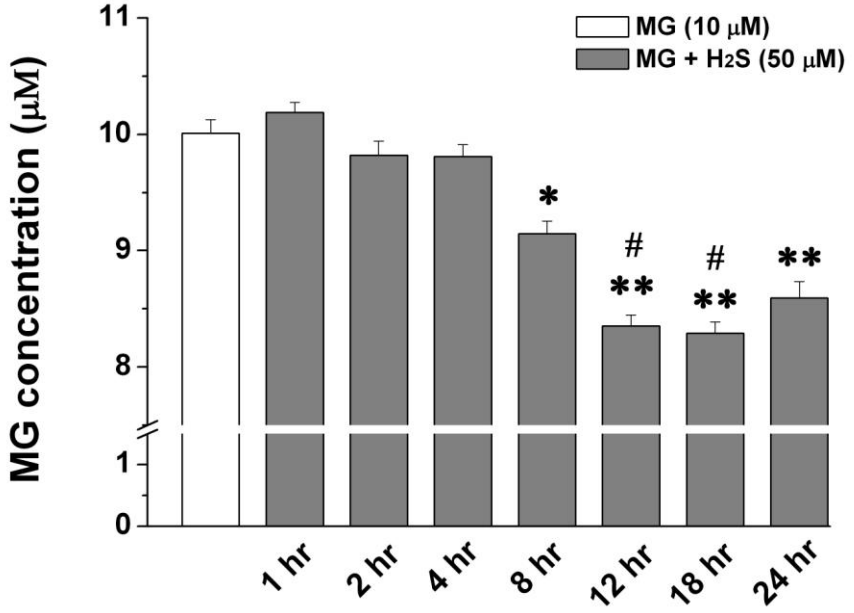

MG (10 μM)
MG + H2S (50 μM)
MG concentration (μM)
11
10
9
8
1
0
*
**
**
**
1 hr
2 hr
4 hr
8 hr
12 hr
18 hr
24 hr

Figure 3-7: Reaction of H_2S with MG. A & B. MG (10 μM) was mixed with H_2S at different concentrations (10, 50, and 100 μM) in PBS buffer and incubated at 37 °C for 24 h. Free MG in the mixtures was measured with HPLC as described in Materials and Methods. $n = 3$. $^{*}P < 0.05$ or $^{**}P < 0.01$ *versus* MG (10 μM); $^{\#}P < 0.05$ *versus* MG (10 μM) + H_2S (50 μM) or MG (10 μM) + H_2S (50 μM) 8 h after incubation.

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CHAPTER 4

INCREASED RENAL METHYLGLYOXAL FORMATION WITH DOWN-REGULATION OF PGC-1α-FBPase PATHWAY IN CYSTATHIONINE γ-LYASE KNOCKOUT MICE

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ABSTRACT

Background: We reported that hydrogen sulfide (H_2S), a gasotransmitter and vasodilator has cytoprotective properties against methylglyoxal (MG), a reactive glucose metabolite associated with diabetes and hypertension. Accordingly, we sought to determine whether MG levels and gluconeogenic enzymes are altered in kidneys of 6-22 week-old cystathionine γ-lyase ($CSE^{-/-}$; H_2S-inducing enzyme) knockout male mice.

Materials and Methods: MG levels were determined by HPLC. Plasma glucose levels were measured by an assay kit. Q-PCR was used to measure mRNA levels of peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α, estrogen-related receptor-α (ERRα), phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase, and (FBPase)-1 and -2. Coupled-enzymatic assays were used to determine FBPase or phosphofructokinase (PFK) activity, and fructose-6-phosphate (F-6-P) or triosephosphates levels.

Results: Plasma glucose levels were significantly decreased in all three age groups of $CSE^{-/-}$ mice, whereas plasma MG levels were significantly increased in 6-22 week-old $CSE^{-/-}$ mice. Significantly higher levels of MG and triosephosphates, along with lower levels of FBPase activity, FBPase-1 and -2 mRNA, and F-6-P were observed in kidneys of 6-22 week-old $CSE^{-/-}$ mice. No change was observed with renal PFK activity. Furthermore, we observed lower mRNA levels of PGC-1α, and its down-stream targets, PEPCK, and ERRα, in kidneys of the $CSE^{-/-}$ mice. In correlation, FBPase-1 and-2 mRNA levels were also decreased in aorta tissues from $CSE^{-/-}$ mice. Administration of NaHS, a H_2S donor, significantly up-regulated the gene expression of PGC-1α, FBPase-1 and -2, and ERRα in cultured A-10 cells.

Conclusions: In conclusion, overproduction of MG in $CSE^{-/-}$ mice is due to H_2S-mediated down-regulation of the PGC-1α-FBPase pathway, further suggesting the important role of H_2S in the regulation of glucose metabolism and MG generation.

Key words: Hydrogen sulfide ■ methylglyoxal ■ fructose-1,6-bisphosphatase-1 and -2 ■ peroxisome proliferator-activated receptor-γ coactivator-1α

INTRODUCTION

Hydrogen sulfide (H_2S) is the most recent addition to the endogenous gasotransmitter family that includes nitric oxide (NO) and carbon monoxide (CO). H_2S has remarkable vasodilatory [1], anti-inflammatory [2], and anti-oxidant properties [3-5]. This gasotransmitter is produced by cystathionine β-synthase, which is predominantly expressed in the brain and CNS, cystathionine γ-lyase (CSE), the predominant H_2S-producing enzyme in the cardiovascular system [6], and by a newly identified enzyme, 3-mercaptopyruvate sulfurtransferase localized in the brain [7] and endothelium [8]. Recently, we showed that CSE deficiency and reduced endogenous H_2S production in vascular tissues resulted in the development of hypertension in $CSE^{-/-}$ mice [1].

Methylglyoxal (MG) is a reactive glucose metabolite and has been linked to type 2 diabetes mellitus (T2DM) [9, 10], as well as hypertension [11-15]. As a member of the reactive carbonyl species, MG is formed mainly through the non-enzymatic conversion of triosephosphates, such as dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GA3P), under hyperglycemic conditions [16]. The triosephosphate pool, in turn, is regulated by both glycolytic and gluconeogenic pathways. Our most recent study demonstrated that MG lowers H_2S concentrations in vascular smooth muscle cells (VSMCs) both directly by scavenging H_2S and indirectly by down-regulating CSE expression [3], suggesting an important interaction between MG and H_2S. As a reciprocal, it is likely that low H_2S levels may result in elevated MG levels.

The kidney plays a vital role in BP regulation [17], but its role in glucose metabolism is often ignored [18]. Indeed, renal gluconeogenesis has been estimated to account for 20 ± 2% of total glucose release [18], where under diabetic conditions this is dramatically increased [18, 19]. The

rate of gluconeogenesis is mainly regulated by the activities of certain unidirectional enzymes, notably phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase), and glucose-6-phosphatase [20]. Peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α is a key regulator of energy metabolism [21] and is a strong coactivator of PEPCK, FBPase, and the orphan nuclear receptor estrogen-related receptor-α (ERRα), which in turn mediates PGC-1α activity [22]. Interestingly, NO has been shown to increase peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α expression in adipocytes and HeLa cells [23], and similar findings have been reported for CO in mouse hearts [24]. However, it has yet to be determined if H_2S can also alter PGC-1α expression.

The present study investigated whether MG level was altered in $CSE^{-/-}$ mice and its underlying mechanisms. To this end, we measured plasma and renal MG levels in both $CSE^{+/+}$ and $CSE^{-/-}$ mice at different age groups (6-22 weeks). We also evaluated the role of FBPase and related signaling pathway in the regulation of MG formation.

MATERIALS AND METHODS

Animals and Tissue Preparation

Male 6-22 week-old $CSE^{+/+}$ and $CSE^{-/-}$ mice were housed in a temperature-regulated animal facility, exposed to a 12 h light/dark cycle with free access to food and water. All animal experiments were conducted in accordance with protocols approved by the Animal Health Care Committee of the University of Saskatchewan, Canada. Prior to harvesting tissues, mice were starved for 16 h. Kidneys and aortas were isolated in ice-cold PBS, cleaned, and snap-frozen in

liquid nitrogen immediately. Tissues were pulverized with a Mikro-Dismembrator (B. Braun Biotech International, PA, USA) and stored at -80 °C until processing.

VSMC preparation

A rat thoracic aortic smooth muscle cell line (A-10 cells) was obtained from American Type Culture Collection and cultured in DMEM containing 10% bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, as described [3]. Cultured cells were grown to 60~80% of confluence before starved in serum-free DMEM for 24 h and then exposed to NaHS treatment for 24 h. Treated and untreated cells were washed with ice-cold PBS, harvested by trypsinization, and resuspended in cell lysis buffer supplied by RNeasy Mini Kit (Qiagen sciences, MD, USA).

Plasma glucose measurement

Plasma glucose levels were determined by using the QuantiChrom^{TM} Glucose Assay Kit (BioAssay Systems, USA), and followed accordingly to the manufacturer's instructions. Briefly, 5 μL of sample was mixed with 500 μL of Reagent, and then placed on a heating block set at 100 °C for 8 min. After cooled to room temperature, samples were transferred to 96-well plate and the absorbance was read at 630 nm in a Multiskan Spectrum (Thermo Labsystems). Samples were measured in triplicate and calibrated by comparison with the given manufacturer standards.

MG measurement

Quantitation of MG was performed by the widely accepted *o*-PD-based assay as described [25]. Kidney samples were prepared in 50 mM sodium phosphate monobasic buffer (pH 4.5) and

sonicated twice for 15 s on ice, then centrifuged at 12,000 rpm at 4 °C for 10 min. A portion of the supernatant was used for protein determination *via* the BCA procedure. The supernatant of kidney homogenate was incubated with a final concentration of 10 mM *o*-PD (derivatizing agent) and 0.45 N PCA with 50 μM EDTA for 24 h at room temperature and protected from light. The quinoxaline formed between dicarbonyl compounds and *o*-PD, as well as the internal standard (5-methylquinoxaline) were measured using a Hitachi D-7000 HPLC system (Hitachi Ltd., Ontario, Canada). A Nova-Pak C18 column was used (Waters, MA, USA). The mobile phase was composed of 8% (v/v) of 50 mM NaH_2PO_4 (pH 4.5), 17% (v/v) of HPLC grade acetonitrile and 75% of water. Samples were measured in triplicate and calibrated by comparison with a 2-methylquinoxaline standard.

Measurement of Enzyme Activities

To determine FBPase activity, kidney homogenates were added to an assay mixture that contained 40 mM glycine buffer (pH 9.1), 1.0 mM EDTA, 2.0 mM $MgCl_2$, 0.6 mM $NADP^+$, and 1.2 U/mL of both glucose-6-phosphate dehydrogenase and phosphoglucose isomerase. The reaction mixture was equilibrated for 10 min at 37 °C and initiated by the addition of 70 μM F-1,6-P and the increase in absorbance was measured at 340 nm in a Multiskan Spectrum (Thermo Labsystems), as described [26].

To determine PFK activity, kidney homogenates were added to an assay mixture that contained 50 mM Tris buffer (pH 8.0), 1.0 mM EDTA, 5.0 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.2 mM NADH, 1.0 mM F-6-P, 1.5 U/mL aldolase, and 1.0 U/mL of both triosephosphate isomerase and glycerophosphate dehydrogenase. The reaction was initiated by the addition of 0.5 mM ATP and

the decrease in absorbance was measured at 340 nm in a Multiskan Spectrum (Thermo Labsystems), as described [27].

RNA isolation and Real-time quantitative PCR

Total RNA was isolated using RNeasy Mini Kit (QIAGEN) and followed accordingly to the manufacturer's instructions. First strand cDNA was prepared from total RNA (1 µg) by reverse transcription using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA). Real-time quantitative PCR was performed on the iCycler iQ Real-time PCR Detection System (Bio-Rad, Nazareth). The following primers used in this study were specifically designed for mice (gene, forward primer/reverse primer, 5´ to 3´): FBPase-1, CAGGGACGTGAAGATG-AAGAAGAA/TTGTTGGCGGGGTATAAAAAGAT; FBPase-2, ACGTTATGGAAAAGG-GGCGACAGG/GCTCCCCGAAATCCCATACAGGTT; PEPCK, ATCTTTGGTGGCCGTAG-ACCT/GCCAGTGGGCCAGGTATTT; ERRα, ATCTGCTGGTGGTTGAACCTG/AGAAGC-CTGGGATGCTCTTG; PGC-1α, GGTACCCAAGGCAGCCACT/GTGTCCTCGGCTGAGC-ACT; and finally, β-actin, CCCATCTACGAGGGCTAT/TGTCACGCACGATTTCC. The PCR conditions and relative mRNA quantification are described [3, 28, 29].

Triosephosphates, F-1,6-P, and F-6-P analyses

Kidney homogenates were acidified with the addition of 1 N PCA (0.1 mM EDTA) for 5 min on ice, then centrifuged for 5 min at 12,000 rpm at 4 ºC. The supernatant was neutralized with 2.5 M K_2CO_3 and left to vent for 5 min on ice and later centrifuged for 2 min at 12,000 rpm at 4 ºC. The supernatant was used for the metabolite assays described below.

The assay mixture to determine renal triosephosphates (GA3P and DHAP) and F-1,6-P levels contained 0.4 M triethanolamine buffer (pH 7.6), 40 mM EDTA, 34 µM NADH, 0.095 U/mL aldolase, 2.0 U/mL triosephosphate isomerase, and 0.3 U/mL glycerophosphate dehydrogenase. The reaction was initiated by the addition of the appropriate enzyme sequence and the decrease in fluorescence was measured at 355/440 nm *via* Fluoroskan Ascent (Thermo Labsystems) at as described [30].

The assay mixture to determine the renal level of F-6-P contained 0.4 M triethanolamine buffer (pH 7.6), 1 mM EDTA, 5 mM $MgCl_2$, 0.6 mM $NADP^+$, 1.0 U/mL glucose-6-phosphate dehydrogenase, and 1.7 U/mL phosphoglucose isomerase. The reaction was initiated by the addition of the appropriate enzyme sequence and the increase in fluorescence was measured at 355/440 nm *via* Fluoroskan Ascent (Thermo Labsystems) at as described [31].

Chemicals and Data Analysis

All chemicals, primers, and enzymes used in this study were obtained from Sigma-Aldrich (Canada). The data are expressed as mean ± SEM. Statistical analyses were performed using Student's *t* test, and when applicable, the one-way ANOVA followed by a *post hoc* analysis (Tukey's test). Statistical significance was considered at $P < 0.05$.

RESULTS

Reduced plasma glucose levels in 6-22 week-old CSE$^{-/-}$ mice

The plasma glucose levels (mmol/L) were significantly reduced by 18.64% in 6-8 (4.01 ± 0.18, $P < 0.05$), 21.94% in 14-16 (4.35 ± 0.12, $P < 0.01$) and 17.96% in 20-22 (4.74 ± 0.19, $P < 0.05$)

week-old $CSE^{-/-}$ mice with comparison to their age-matched WT mice (4.92 ± 0.19, 5.57 ± 0.24, and 5.53 ± 0.23; respectively) under starvation conditions (16 h) (Figure 4-1). Although plasma glucose levels were significantly reduced, it was comparably higher in the 20-22 to the 6-8 week-old $CSE^{-/-}$, as well as $CSE^{+/+}$ mice ($P < 0.05$).

Elevated MG levels in 6-22 week-old $CSE^{-/-}$ mice

Plasma MG levels (μmol/L) were increased in $CSE^{-/-}$ mice by 28.28% in 6-8 (0.59 ± 0.03, $P < 0.05$), 33.80% in 14-16 (0.60 ± 0.02, $P < 0.001$), and 20.30% in 20-22 (1.85 ± 0.05, $P < 0.001$) week-old age groups with comparison to that from age-matched $CSE^{+/+}$ groups (0.46 ± 0.05, 0.42 ± 0.01, and 1.54 ± 0.04; respectively) (Figure 4-2A). Kidney MG levels (nmoL/mg) were elevated by 11.41% in 6-8 (0.78 ± 0.01, $P < 0.05$), 15.40% in 14-16 (0.76 ± 0.02, $P < 0.01$), and 25.67% in 20-22 (0.68 ± 0.01, $P < 0.001$) week-old $CSE^{-/-}$ mice compared to that from age-matched $CSE^{+/+}$ mice (0.70 ± 0.02, 0.66 ± 0.03, and 0.54 ± 0.01; respectively) (Figure 4-2B).

Impairment of FBPase activity and mRNA expression in 6-22 week-old $CSE^{-/-}$ mice

Total kidney FBPase activity (nmoL NADPH/min/mg) was decreased by 12.74% in 6-8 (0.30 ± 0.02, $P < 0.05$), 23.98% in 14-16 (0.22 ± 0.01, $P < 0.05$), and 36.12% in 20-22 (0.16 ± 0.01, $P < 0.01$) week-old $CSE^{-/-}$ mice compared to age-matched $CSE^{+/+}$ groups (0.34 ± 0.01, 0.29 ± 0.03, and 0.24 ± 0.01; respectively) (Figure 4-3A). Similarly, we found that the FBPase-1 mRNA levels were decreased by 25.80 ± 6.64% in 6-8 ($P < 0.05$), 51.10 ± 6.03% in 14-16 ($P < 0.001$), and 63.90 ± 4.20% in 20-22 ($P < 0.001$) week-old $CSE^{-/-}$ mice compared to that from age-matched $CSE^{+/+}$ group (Figure 4-3B). Although FBPase-1 was reported to be the dominant FBPase in the kidneys [32, 33], in one group of experiments we investigated whether FBPase-2

was expressed in $CSE^{+/+}$ or altered in $CSE^{-/-}$ mice. We observed that FBPase-2 mRNA levels were expressed in both $CSE^{+/+}$ and $CSE^{-/-}$ mice. The mRNA levels of FBPase-2 were not changed in 6-8, but down-regulated by 38.05 ± 9.12% in 14-16 ($P < 0.05$) and 34.33 ± 6.34% in 20-22 ($P < 0.05$) week-old $CSE^{-/-}$ mice compared to that from age-matched $CSE^{+/+}$ groups (Figure 4-3C).

Altered F-6-P, F-1,6-P, DHAP and GA3P levels in 6-22 week-old $CSE^{-/-}$ mice

The levels of F-6-P (nmoL NADPH/min/mg), the product of FBPase during gluconeogenesis, was decreased by 10.09% in 6-8 (1.51 ± 0.05, $P < 0.05$), 11.56% in 14-16 (1.49 ± 0.03, $P < 0.01$), and 16.07% in 20-22 (1.14 ± 0.03, $P < 0.001$) week-old $CSE^{-/-}$ mice in comparison with that from age-matched $CSE^{+/+}$ groups (1.68 ± 0.04, 1.69 ± 0.06, and 1.36 ± 0.04; respectively) (Figure 4-4A). In contrast, the levels of F-1,6-P (nmoL NADPH/10 min/mg), the substrate of FBPase, was increased by 22.21% in 6-8 (1.60 ± 0.06, $P < 0.01$), 27.14% in 14-16 (1.47 ± 0.08, $P < 0.01$), and 39.44% in 20-22 (1.50 ± 0.11, $P < 0.01$) week-old $CSE^{-/-}$ mice compared to that from age-matched $CSE^{+/+}$ group (1.31 ± 0.06, 1.16 ± 0.08, and 1.07 ± 0.05; respectively) (Figure 4-4B). Likewise, the renal DHAP and GA3P levels (nmoL NADH/10 min/mg), were also increased in $CSE^{-/-}$ mice by 24.10% in 6-8 (1.44 ± 0.06, $P < 0.001$), 29.38% in 14-16 (1.55 ± 0.09, $P < 0.001$), and 47.14% in 20-22 (1.55 ± 0.08, $P < 0.001$) weeks old in comparison with that from age-matched $CSE^{+/+}$ group (1.16 ± 0.04, 1.20 ± 0.04, and 1.05 ± 0.04; respectively) (Figure 4-4C). There was no change in the renal phosphofructokinase (PFK) activity, a glycolytic enzyme that coverts F-6-P to F-1,6-P, in both the $CSE^{-/-}$ and $CSE^{+/+}$ group at different age groups (Figure 4-4D).

Decreased mRNA expression of PGC-1α, PEPCK, and ERRα in 6-22 week-old CSE$^{-/-}$ mice

Since PGC-1α was reported to be a major contributor to gluconeogenesis *via* the up-regulation of FBPase and PEPCK [20], the gene expression level of PGC-1α was investigated in these CSE$^{-/-}$ mice. Interestingly, as shown in Figure 4-5A, the mRNA levels of renal PGC-1α were lowered in what appeared to be in an age-related manner, by 38.50 ± 5.99% in 6-8 ($P < 0.05$), 45.90 ± 6.55% in 14-16 ($P < 0.01$), and 68.78 ± 9.35% in 20-22 ($P < 0.01$) week-old age groups of CSE$^{-/-}$ mice with comparison to age-matched CSE$^{+/+}$ mice. Likewise, the renal mRNA levels of one of the down-stream targets of PGC-1α, PEPCK, were also decreased by 16.60 ± 4.52% in 6-8 ($P < 0.05$), 28.20 ± 7.84% in 14-16 ($P < 0.05$), and 42.70 ± 5.18% in 20-22 ($P < 0.01$) week-old CSE$^{-/-}$ mice in comparison to that from age-matched CSE$^{+/+}$ mice (Figure 4-5B). Moreover, PGC-1α was also suggested to be a strong coactivator of ERRα [22], thus we sought to determine whether there was a corresponding fall in ERRα mRNA levels. Indeed, we observed lower mRNA levels of ERRα in the CSE$^{-/-}$ mice by 27.70 ± 5.65% in 6-8 ($P < 0.05$), 32.40 ± 10.10% in 14-16 ($P < 0.05$), and 65.20 ± 10.93% in 20-22 ($P < 0.01$) weeks old compared to that from age-matched CSE$^{+/+}$ groups (Figure 4-5C).

Down-regulation of FBPase-1 and -2 mRNA levels in aorta of CSE$^{-/-}$ mice

Due to the observed down-regulation of the mRNA levels of FBPase-1 and -2 in the kidney, we investigated whether this similar phenomenon also occurred in aorta extracts from 14-16 week-old CSE$^{-/-}$ mice. In agreement, we observed a significant reduction in FBPase-1 by 36.91 ± 2.88% ($P < 0.01$) and FBPase-2 by 41.38 ± 14.16% ($P < 0.05$) mRNA expression levels in 14-16 week-old CSE$^{-/-}$ with comparison to CSE$^{+/+}$ mice (Figures 4-6A & B, respectively).

H_2S-induced up-regulation of PGC-1α, FBPase-1 and -2, and ERRα mRNA levels in A-10 cells

To determine if H_2S directly up-regulated the gene expression of PGC-1α, ERRα, and FBPase-1 and -2, rat VSMCs (A-10 cells) were subjected to excess amounts of NaHS, a H_2S donor. We observed a significant increase in the mRNA levels of PGC-1α by 53.03 ± 20.12% ($P < 0.05$) in 30 μM NaHS- and 69.21 ± 15.18% ($P < 0.01$) in 50 μM NaHS-treated cells in comparison to the untreated cells (Figure 4-7A). Correspondingly, we have also observed an up-regulation in the gene expression of FBPase-1 by 63.12 ± 6.01% ($P < 0.05$) in 30 μM NaHS- and 97.32 ± 26.22% ($P < 0.01$) in 50 μM NaHS-treated cells (Figure 4-7B), along with FBPase-2 by 78.32 ± 6.24% ($P < 0.05$) and ERRα by 61.23 ± 8.02% ($P < 0.05$) in 50 μM NaHS-treated A-10 cells (Figures 4-7C & D, respectively).

DISCUSSION

In the present study we report on what appears to be an age-dependent increase in MG formation with altered PGC-1α-FBPase signaling pathway in the genetic knockout CSE mice, a H_2S-generating enzyme. We observed significantly lower levels of plasma glucose in all three age groups of the $CSE^{-/-}$ mice. The most notable changes were significantly increased MG levels in plasma and kidney of 6-22 week-old $CSE^{-/-}$ mice, accompanied by increased renal triosephosphates (DHAP and GA3P), the immediate precursors for MG formation [34]. We observed lower mRNA levels of PGC-1α and FBPase-1 and -2, decreased total FBPase activity and its metabolic product (F-6-P), along with higher levels of its substrate (F-1,6-P) in the kidney of the CSE knockout mice. Furthermore, lower mRNA levels of FBPase-1 and -2 were observed in the aorta of the CSE deficient mice. NaHS-treatment induced a significant up-regulation in

the gene expression of PGC-1α, FBPase-1 and -2, and ERRα in cultured A-10 cells. Thus, our results suggest that elevated kidney MG levels were likely due to a H_2S-mediated down-regulation of PGC-1α-FBPase signaling pathway, which lead to the accumulation of triosephosphates in the $CSE^{-/-}$ mice.

It has been extensively studied that abnormally high levels of H_2S may be linked to T2DM and insulin-resistance [28, 35-37], which could mainly be accredited to its ability to inhibit both insulin secretion from pancreatic β-cells [29, 38-40] and glucose-uptake into adipocytes [41]. Consequently, one would assume that this would affect the overall circulating glucose levels, where high H_2S levels could lead to high systemic glucose levels. On the other hand, lower levels of H_2S in the circulation and specific tissues are expected in favour of reducing plasma glucose levels and postponing the development of diabetes. Indeed, we have observed significantly lower plasma glucose levels in all three age groups of the CSE knockout mice (Figure 4-1). Furthermore, our recent studies also indicated that $CSE^{-/-}$ mice that received streptozotocin injections exhibited a delayed onset of diabetic status (hyperglycaemia, hypoinsulinemia, and glucose intolerance), in comparison with wild-type mice [42]. Thus, these findings further support the involvement of the CSE/H_2S system in glucose regulation.

This is the first study to show that MG levels are elevated under reduced gluconeogenic conditions in plasma and renal tissues of the CSE knockout mice (Figures 4-2A & B, respectively). The elevated renal MG levels, in turn, appear to be accounted for by the elevated renal levels of the MG precursors, DHAP and GA3P (Figure 4-4C). This increased MG formation in kidneys of CSE knockout mice is important, as we have shown that elevated MG

levels in kidneys of spontaneously hypertensive rats lead to increased advanced glycation endproducts formation and oxidative stress [11,43]. The renal MG levels in the $CSE^{-/-}$ mice were increased significantly in all three age groups (6-8, 14-16, and 20-22 weeks) when compared to age-matched $CSE^{+/+}$ groups (Figure 4-2B).

FBPase is the rate-limiting enzyme in the gluconeogenic pathway and catalyzes the conversion of F-1,6-P to F-6-P [32]. There are two main isoforms of FBPase, FBPase-1 or liver FBPase, which is predominant in the liver and kidney [19,32,33] and FBPase-2 or muscle FBPase, which is predominant in skeletal muscle [44]. We observed lower activity of total FBPase in kidneys of 6-22 week-old $CSE^{-/-}$ mice (Figure 4-3A). The lower FBPase activity appears likely due to the down-regulation of the mRNA levels of FBPase-1 (Figure 4-3B), and to a lesser extent, the down-regulation of FBPase-2 (Figure 4-3C). As such, the decreased FBPase activity was accompanied by lower levels of its product, F-6-P, and higher levels of its substrate, F-1,6-P (Figures 4-4A & B, respectively). To rule out possible interference from the glycolytic system, PFK activity, the enzyme responsible for the conversion of F-6-P to F-1,6-P, were measured. No changes in PFK activity were observed (Figure 4-4D). These observations suggest that decreased FBPase activity was mainly responsible for the increased MG levels in renal tissues of 6-22 week-old $CSE^{-/-}$ mice. Moreover, the mRNA levels of FBPase-1 and -2 were also down-regulated in aorta extracts from CSE knockout mice (Figures 4-6A & B, respectively). Thus, this further indicates that the CSE deficient mouse shows a reduced gluconeogenic system with enhanced MG formation, not only in its kidney, but also in its aorta or possible other tissues.

The gene transcription of FBPase, along with PEPCK and ERRα, can be induced by PGC-1α [20]. PGC-1α is a critical regulator of genes related to energy metabolism [21]. As well, PGC-1α, along with ERRα, is abundant in human kidney, skeletal muscle, or tissues with high metabolic demand [45]. Interestingly, the endogenous gasotransmitters, NO [23] and CO [24], were shown to increase PGC-1α expression levels. Moreover, many reports have shown that NO can significantly up-regulate the endogenous level of H_2S [46-48], not just through the induction of CSE but by also enhancing CBS activity [49], leaving one to wonder the possible involvement of H_2S in the NO-induced up-regulation of PGC-1α. In line with these previous observations, we show here for the first time that decreased H_2S levels in renal tissues led to decreased PGC-1α gene expression (Figure 4-5A), which most likely resulted in significantly lowered FBPase-1 and -2 mRNA levels and impaired FBPase activity in renal tissues of 6-22 week-old $CSE^{-/-}$ mice (Figure 4-3). In fact, both PEPCK and ERRα mRNA levels were decreased in these renal tissues (Figures 4-5B & C, respectively). Theoretically, decreased gene expression of PEPCK would reduce the formation DHAP and GA3P, thus resulting in decreased MG formation. Our data of higher levels of DHAP, GA3P, and MG in renal tissues of 6-22 week-old $CSE^{-/-}$ mice further indicated that the specific PGC-1α-FBPase pathway rather than PGC-1α-PEPCK pathway contributed to the enhanced MG formation in these CSE deficient mice. In correlation to these findings, we have demonstrated that administration of 30 and 50 μM NaHS induced an increase in the mRNA expression levels of both PGC-1α and FBPase-1 in A-10 cells (Figures 4-7A & B, respectively). Additionally, we have also observed a significant increase in FBPase-2 and ERRα mRNA levels in 50 μM NaHS-treated A-10 cells (Figures 4-7C & D, respectively), further supporting the phenomenon of the involvement of H_2S in the regulation of PGC-1α and its transcription-inducing properties.

Indeed, there seems to be an age-related relationship between reduced renal FBPase activity and F-6-P levels, the mRNA levels of PEPCK, ERRα, and FBPase-1 and -2, and increased F-1,6-P and MG levels in both $CSE^{-/-}$ and $CSE^{+/+}$ mice. In parallel, renal PGC-1α mRNA levels were also decreased in what seems to be in an age-related fashion, thus further supporting the notion that reduced PGC-1α resulted in reduced FBPase activity and increased MG levels in the $CSE^{-/-}$ mice. Further molecular study is needed to determine how H_2S regulates the gene transcription of PGC-1α.

Our studies demonstrated that insufficient H_2S levels in $CSE^{-/-}$ mice caused an increase in MG formation *via* down-regulation of PGC-1α-FBPase pathway (Figure 8). Due to inadequate endogenous H_2S level, PGC-1α, an important regulator of energy metabolism, is down-regulated along with some of its downstream targets, ERRα, PEPCK, and FBPase-1 and -2 in renal tissues of 6-22 week-old $CSE^{-/-}$ mice. Because the major unidirectional gluconeogenic enzymes, PEPCK and FBPase, are down-regulated, circulating glucose level is lowered. Consequently, due to the lowered FBPase activity in the kidney of $CSE^{-/-}$ mice, this encouraged the accumulation of the MG precursors, DHAP and GA3P, which resulted in higher levels of MG in both renal tissues and in the systemic circulation. Henceforth, over generation of MG and MG-induced advanced glycation endproducts and oxidative stress would eventually contribute to the development of insulin resistance and metabolic syndrome.

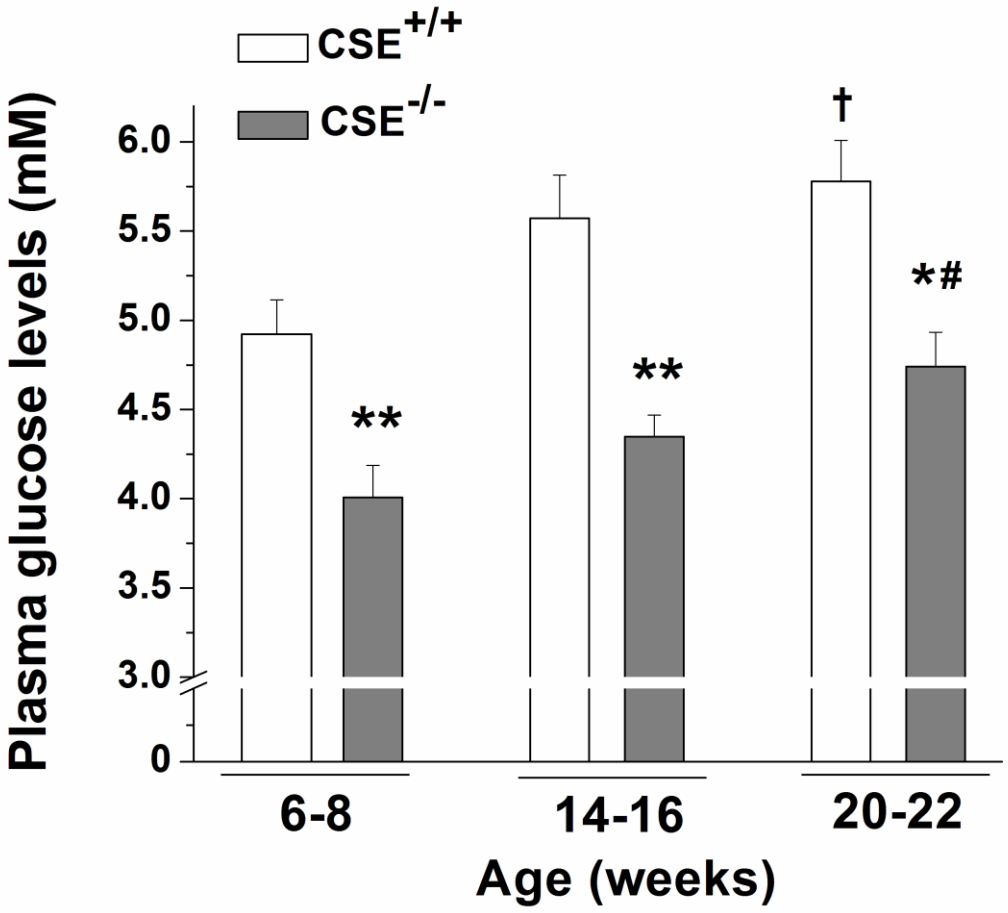


Figure 4-1: Plasma glucose levels in 6-22 week-old CSE$^{-/-}$ mice. Plasma glucose levels were measured in 6-8 (n = 5-7), 14-16 (n = 4-5), and 20-22 (n = 6-7) week-old CSE$^{-/-}$ and CSE$^{+/+}$ mice after starving for 16 h. *P < 0.05, **P < 0.01 *versus* corresponding age-matched CSE$^{+/+}$ mice; $^{\#}P$ < 0.05 *versus* 6-8 week-old CSE$^{-/-}$ mice; $^{\dagger}P$ < 0.05 *versus* 6-8 week-old CSE$^{+/+}$ mice.

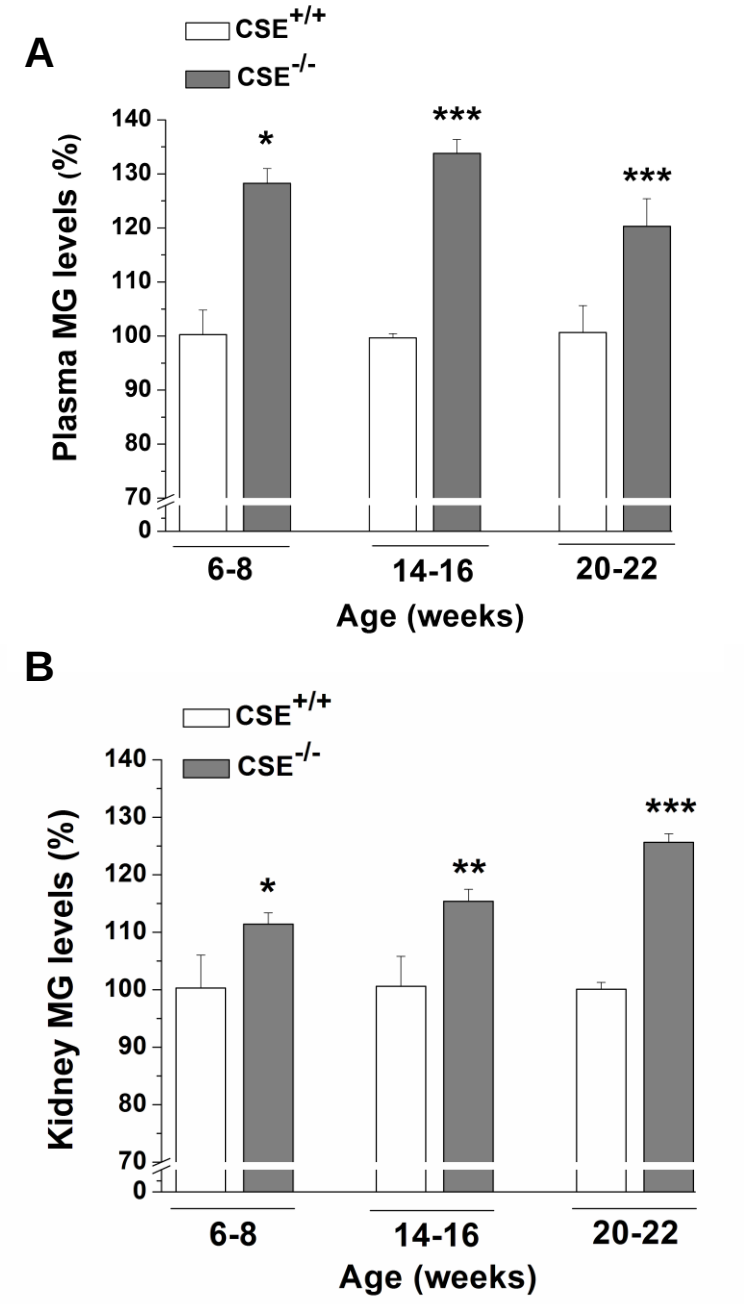
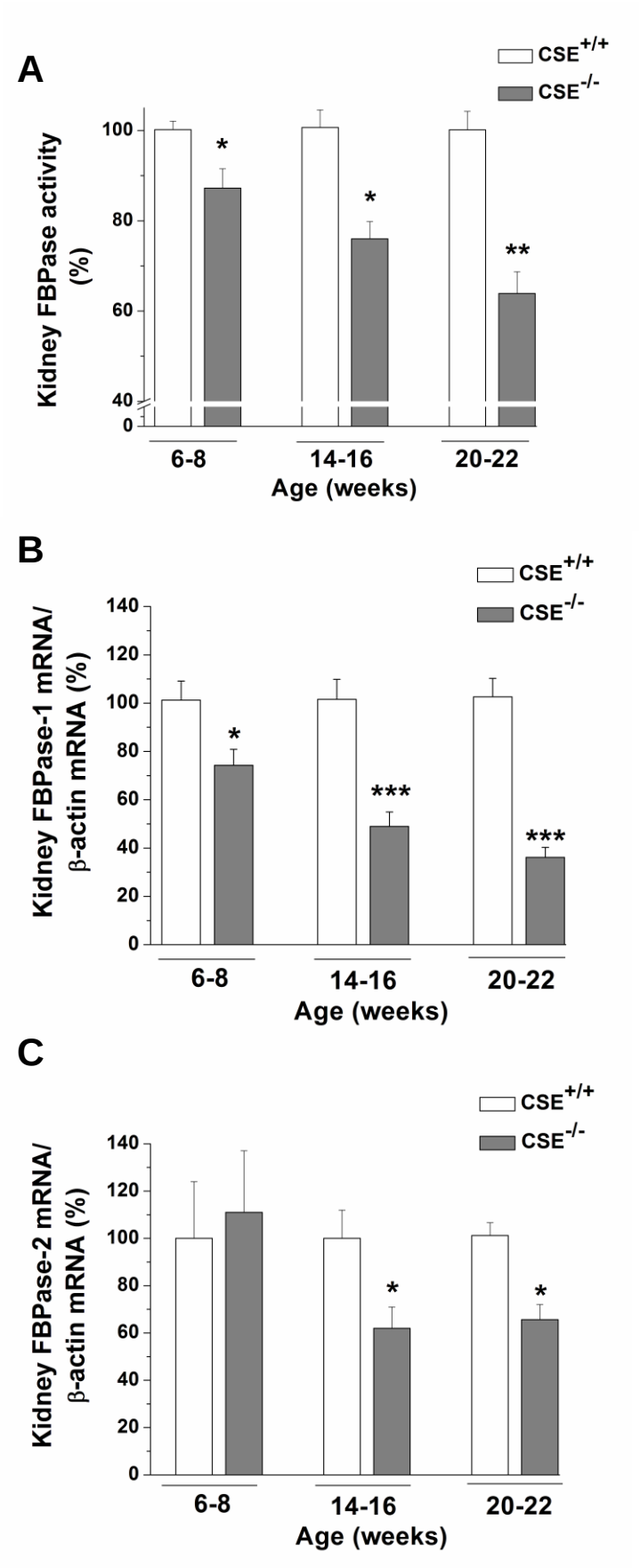


Figure 4-2: Methylglyoxal levels in plasma and kidneys of 6-22 week-old CSE$^{-/-}$ mice. A, Methylglyoxal (MG) levels in plasma of 6-8 (n = 7), 14-16 (n = 4), and 20-22 (n = 7) week-old mice. B, MG levels in renal tissues of 6-8 (n = 5), 14-16 (n = 6), and 20-22 (n = 7) week-old mice. MG values from CSE$^{-/-}$ mice are presented as a percentage of that in age-matched CSE$^{+/+}$ mice. *P < 0.05, **P < 0.01, and ***P < 0.001 *versus* corresponding age groups of CSE$^{+/+}$ mice.


A
CSE+/+
CSE-/-
Kidney FBPase activity (%)
100
80
60
40
0
*
*
**
6-8
14-16
20-22
Age (weeks)
B
CSE+/+
CSE-/-
Kidney FBPase-1 mRNA/ β-actin mRNA (%)
140
120
100
80
60
40
20
0
*
***
***
6-8
14-16
20-22
Age (weeks)
C
CSE+/+
CSE-/-
Kidney FBPase-2 mRNA/ β-actin mRNA (%)
140
120
100
80
60
40
20
0
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6-8
14-16
20-22
Age (weeks)

Figure 4-3: Total fructose-1,6-bisphosphatase activity and fructose-1,6-bisphosphatase-1 and -2 mRNA levels in renal tissues of $CSE^{-/-}$ mice. A, Total fructose-1,6-bisphosphatase (FBPase) activity in kidneys of 6-8 (n = 6), 14-16 (n = 6), and 20-22 (n = 4) week-old mice. B, Real-time PCR results of fructose-1,6-bisphosphatase (FBPase)-1 levels in renal tissues of $CSE^{-/-}$ mice ages 6-8 (n = 5), 14-16 (n = 7), and 20-22 (n = 5) weeks. C, Real-time PCR results of FBPase-2 mRNA levels in kidneys of 6-8 (n = 4), 14-16 (n = 4), and 20-22 (n = 5) week-old mice. FBPase activity, FBPase-1 and -2 mRNA values in $CSE^{-/-}$ mice are presented as a percentage of that in age-matched $CSE^{+/+}$ mice. *P < 0.05, **P < 0.01, and ***P < 0.001 *versus* corresponding age groups of $CSE^{+/+}$ mice.

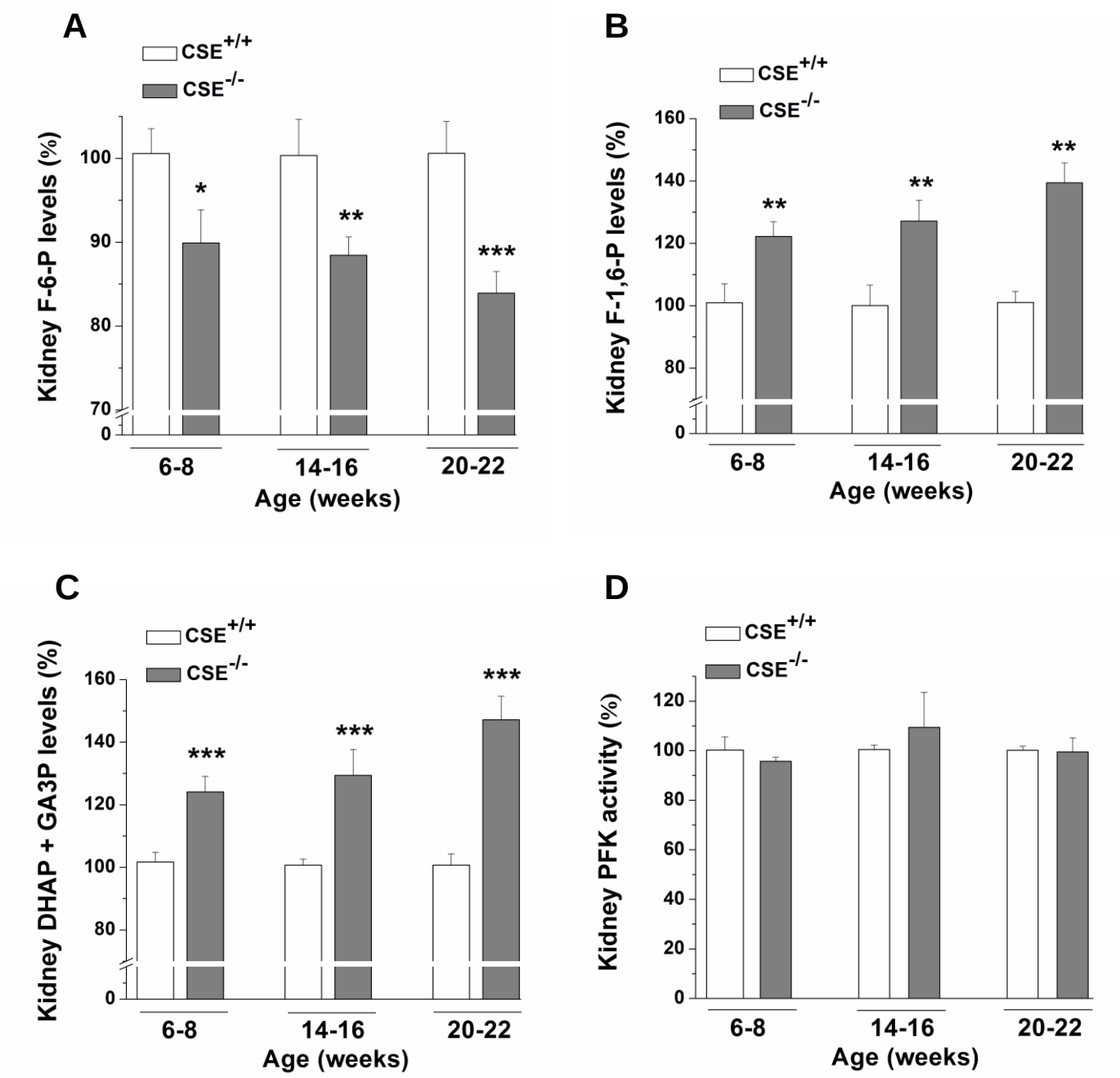

A
CSE+/+
CSE-/-
Kidney F-6-P levels (%)
100
90
80
70
0
*
**
***
6-8
14-16
20-22
Age (weeks)
B
CSE+/+
CSE-/-
Kidney F-1,6-P levels (%)
160
140
120
100
80
0
**
**
**
6-8
14-16
20-22
Age (weeks)
C
CSE+/+
CSE-/-
Kidney DHAP + GA3P levels (%)
160
140
120
100
80
0
***
***
***
6-8
14-16
20-22
Age (weeks)
D
CSE+/+
CSE-/-
Kidney PFK activity (%)
120
100
80
60
40
20
0
6-8
14-16
20-22
Age (weeks)

Figure 4-4: Fructose-6-phosphate, fructose-1,6-bisphosphate, and dihydroxyacetone phosphate and glyceraldehyde 3-phosphate levels in renal tissues of $CSE^{-/-}$ mice. A, Fructose-6-phosphate (F-6-P) levels were measured in kidneys of 6-8 (n = 6), 14-16 (n = 6), and 20-22 (n = 6-8) week-old mice. B, Fructose-1,6-bisphosphate (F-1,6-P) levels in kidneys were analyzed in 6-22 (n = 5) week-old mice. C, The triosephosphates, dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GA3P), were measured in kidneys of 6-22 (n = 5) week-old mice. D, Renal phosphofructokinase (PFK) activity was measured in 6-8 (n = 4), 14-16 (n = 4-5), 20-22 (n = 4-5) week-old mice. The values from $CSE^{-/-}$ are presented as a percentage of that in age-matched $CSE^{+/+}$ mice. *P < 0.05, **P < 0.01, and ***P < 0.001 *versus* corresponding age groups of $CSE^{+/+}$ mice.

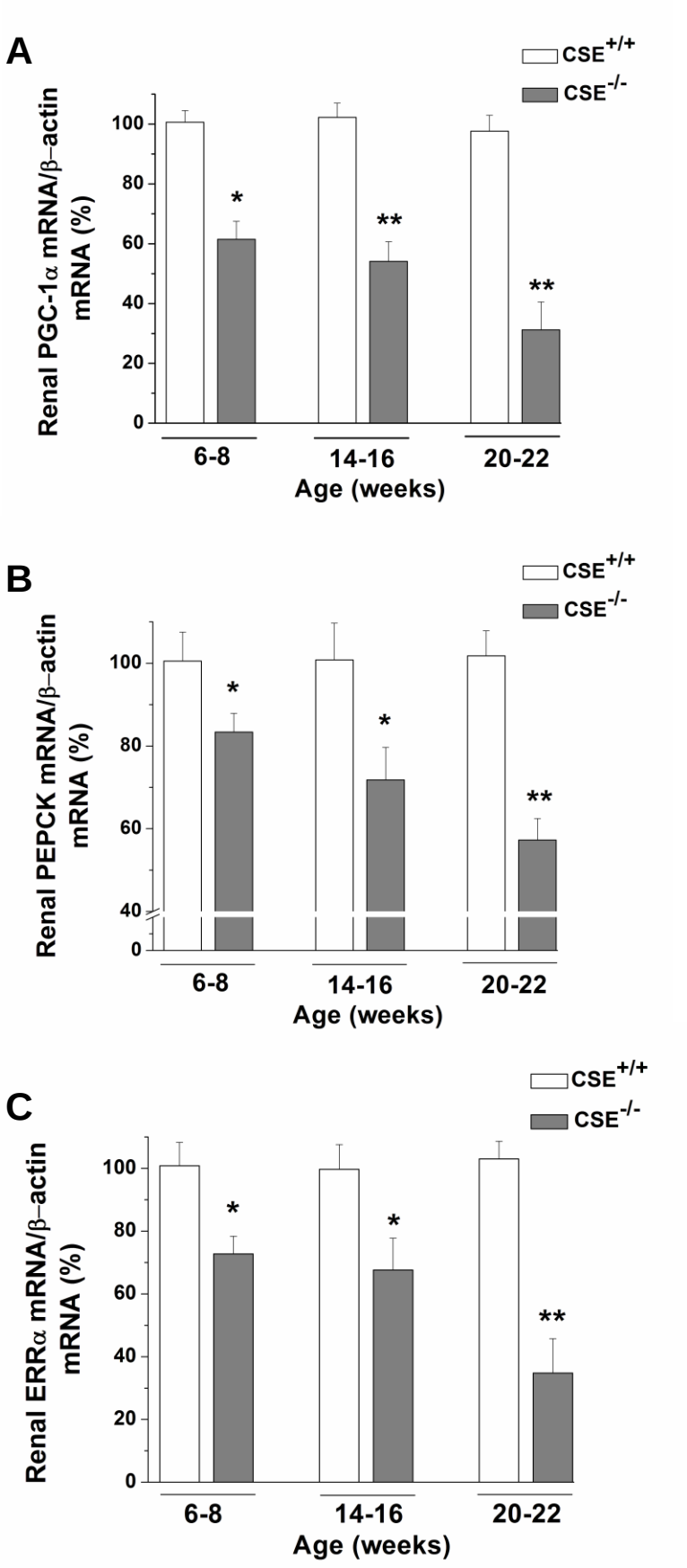
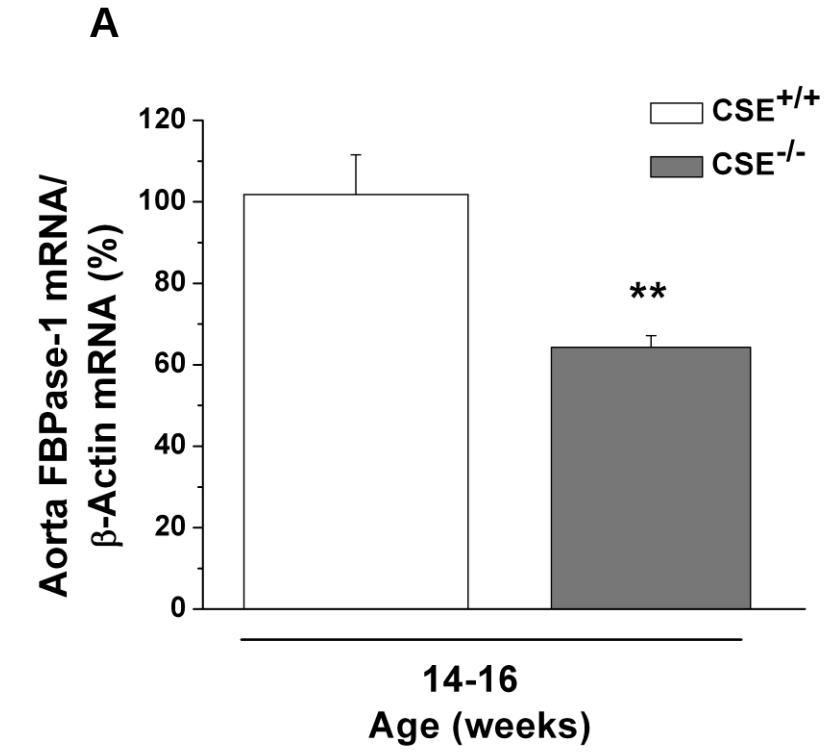

A
CSE+/+
CSE-/-
Renal PGC-1α mRNA/β–actin mRNA (%)
100
80
60
40
20
0
*
**
**
6-8
14-16
20-22
Age (weeks)
B
CSE+/+
CSE-/-
Renal PEPCK mRNA/β–actin mRNA (%)
100
80
60
40
0
*
*
**
6-8
14-16
20-22
Age (weeks)
C
CSE+/+
CSE-/-
Renal ERRα mRNA/β–actin mRNA (%)
100
80
60
40
20
0
*
*
**
6-8
14-16
20-22
Age (weeks)

Figure 4-5: mRNA levels of peroxisome proliferator-activated receptor-γ coactivator-1α, phosphoenolpyruvate carboxykinase, and estrogen-related receptor-α in renal tissues of $CSE^{-/-}$ mice. A, peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α mRNA levels in kidneys of $CSE^{-/-}$ mice in 6-8 (n = 5), 14-16 (n = 6), and 20-22 (n = 5) week-old mice. B, phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels in 6-8 (n = 5), 14-16 (n = 6), and 20-22 (n = 5) week-old mice. C, estrogen-related receptor-α (ERRα) mRNA levels in 6-8 (n = 5), 14-16 (n = 6), and 20-22 (n = 5) week-old mice. mRNA values in $CSE^{-/-}$ mice are presented as a percentage of that in age-matched $CSE^{+/+}$ mice. *P < 0.05 and **P < 0.01 *versus* corresponding age groups of $CSE^{+/+}$ mice.


A
CSE+/+
CSE-/-
Aorta FBPase-1 mRNA/
β-Actin mRNA (%)
120
100
80
60
40
20
0
**
14-16
Age (weeks)

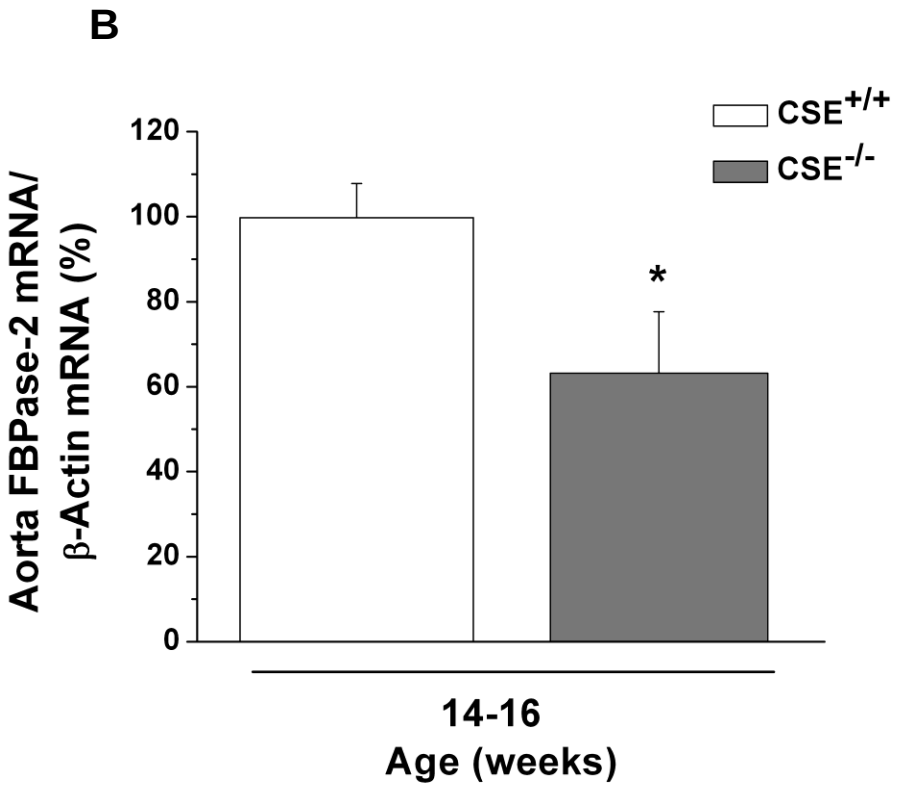

B
CSE+/+
CSE-/-
Aorta FBPase-2 mRNA/
β-Actin mRNA (%)
120
100
80
60
40
20
0
*
14-16
Age (weeks)

Figure 4-6: mRNA levels of FBPase-1 and -2 in the aorta of 14-16 week-old CSE$^{-/-}$ mice. FBPase-1 (A) and -2 (B) mRNA expression levels were measured in aortic extracts from 14-16 week-old CSE$^{-/-}$ mice (n = 3-4). The values from CSE$^{-/-}$ are presented as a percentage of that in age-matched CSE$^{+/+}$ mice. *P < 0.05 and **P < 0.01 *versus* 14-16 week-old CSE$^{+/+}$ mice.

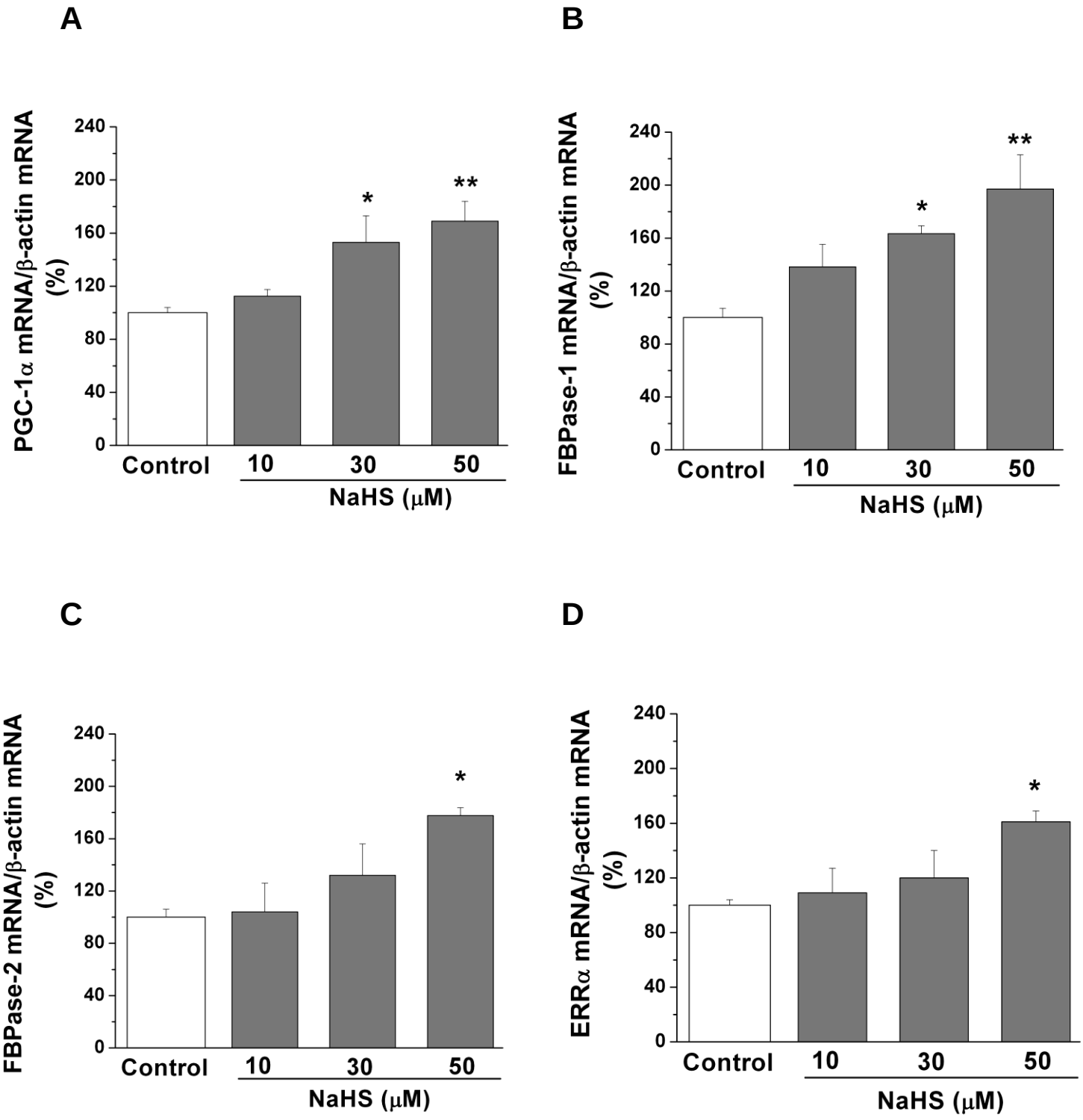

A
PGC-1α mRNA/β-actin mRNA (%)
240
200
160
120
80
40
0
*
**
Control
10
30
50
NaHS (μM)
B
FBPase-1 mRNA/β-actin mRNA (%)
240
200
160
120
80
40
0
*
**
Control
10
30
50
NaHS (μM)
C
FBPase-2 mRNA/β-actin mRNA (%)
240
200
160
120
80
40
0
*
Control
10
30
50
NaHS (μM)
D
ERRα mRNA/β-actin mRNA (%)
240
200
160
120
80
40
0
*
Control
10
30
50
NaHS (μM)

Figure 4-7: mRNA levels of peroxisome proliferator-activated receptor-γ coactivator-1α, fructose-1,6-bisphosphatase-1 and -2, and estrogen-related receptor-α in NaHS-treated A-10 cells. A-10 cells were treated with NaHS at different concentrations for 24 h to determine mRNA levels of peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α (A), fructose-1,6-bisphosphatase (FBPase)-1 (B), FBPase-2 (C), estrogen-related receptor-α (ERRα) (D) $n = 5$ for each group in A, B, C, and D. mRNA values obtained from NaHS-treated A-10 cells are presented as a percentage of that in control cells. $*P < 0.05$ and $**P < 0.01$ *versus* control group.

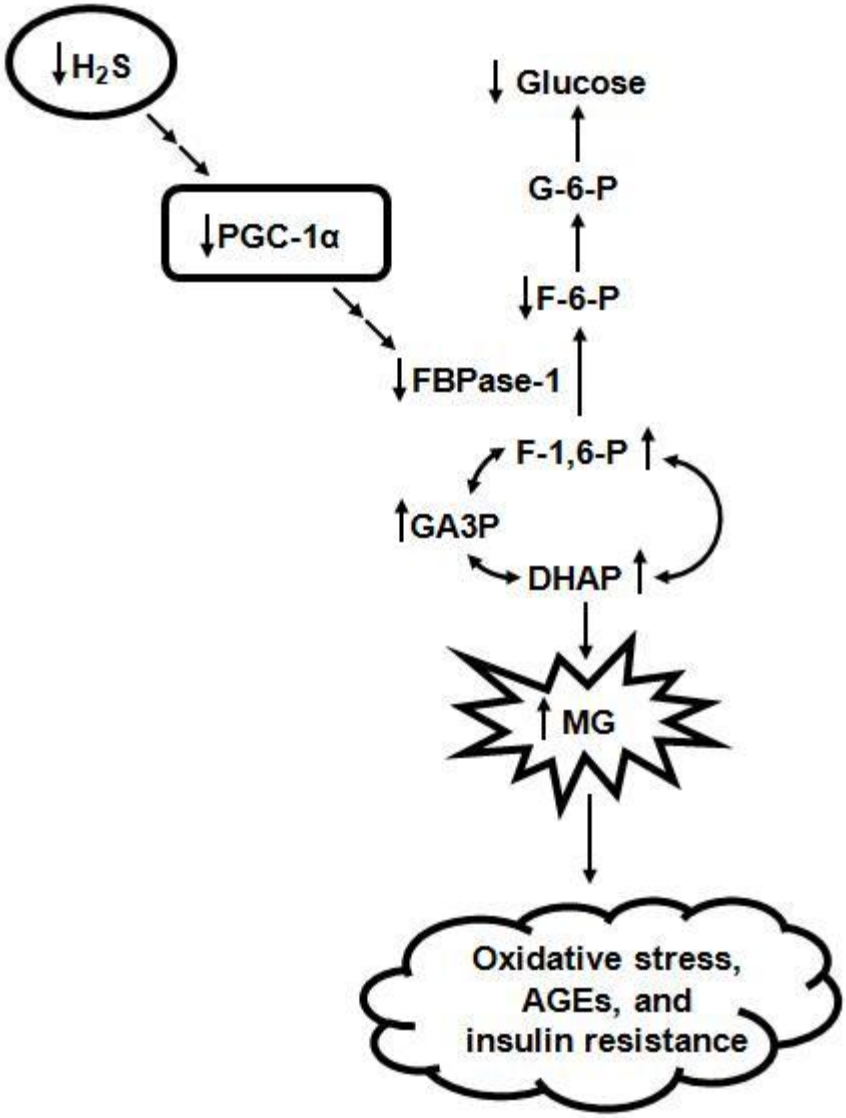


Figure 4-8: Schematic pathway for enhanced MG formation in CSE$^{-/-}$ mice. AGEs: advanced glycation endproducts; DHAP: dihydroxyacetone phosphate; F-1,6-P: fructose-1,6-phosphate; F-6-P: fructose-6-phosphate; FBPase: fructose-1,6-bisphosphatase; G-6-P: glucose-6-phosphate; GA3P: glyceraldehyde 3-phosphate; H_2S: hydrogen sulfide; MG: methylglyoxal; PGC-1α: peroxisome proliferator-activated receptor-γ coactivator-1α.

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CHAPTER 5

DISCUSSION AND CONCLUSION

General discussion

Our study has demonstrated that MG and H_2S are involved in opposing signaling pathways, suggesting the existence of a negative correlation between these two potent endogenous molecules. Chapter 3 presents the first line of evidence to demonstrate that MG and H_2S do in fact engage with and modulate each others' physiological outcomes. This includes the observation that H_2S has cytoprotective properties against MG, which was demonstrated by its direct scavenging of MG, abolishing MG-induced ROS production, as well as up-regulating GSH expression levels in A-10 cells. H_2S, on the other hand, may modulate MG levels in the vasculature by regulating gluconeogenesis.

Along with the liver, the kidney is another major organ that can significantly contribute to gluconeogenesis to help maintain plasma glucose levels in times of starvation or intense physical activity. The kidney was the tissue of choice for the studies presented in Chapter 4, because preliminary analysis showed that renal tissues have significantly higher levels of MG compared to the liver, which would provide us with an accurate assessment of any alterations in the MG formation pathway while at the same time studying gluconeogenesis. Indeed, in the 6-22 week-old male $CSE^{-/-}$ mice, we found that the mRNA levels of the main rate-limiting gluconeogenic enzymes, PEPCK and FBPase-1 and -2, along with the strong gluconeogenic regulator, PGC-1α, and its endogenous inhibitor, ERRα, were all significantly reduced in the kidney. A reduction in the renal gluconeogenic rate may help to explain the overall decrease in the plasma glucose levels in 6-22 week-old $CSE^{-/-}$ mice. Furthermore, decreased mRNA levels of FBPase-1 and -2 were also observed in the aorta of 14-16 week-old $CSE^{-/-}$ mice. The administration of 30 and 50 μM NaHS induced a significant increase in the mRNA expression levels of PGC-1α and FBPase-

1 in rat VSMCs, where in Chapter 3, concentrations at and higher than 30 μM significantly reduced MG levels in A-10 cells. Lastly, we have observed significantly lower levels of the MG precursors, DHAP and GA3P, as well as the renal and plasma MG levels in the 6-22 week-old $CSE^{-/-}$ mice. This gluconeogenic study in the CSE knockout mice suggests that the endogenous production of MG may be mediated through a H_2S-regulated PGC-1α-FBPase mechanism.

The vasodilatory ability of H_2S is greatly due to its activation of the K_{ATP} channels present on VSMCs (Yang et al. 2005; Zhao et al. 2001, 2003). In light of this, many researchers have pointed to the coupling phenomenon of the K_{ATP} channels with the activation of β-adrenergic receptors (AR) in vascular tissues (Randall and McCulloch 1995; Schackow and Ten Eick 1994; Jackson 1993; Narishige et al. 1994; Sheridan et al. 1997; Katsuda et al. 1996), thus challenging the traditional view that β-ARs are solely coupled to AC and cAMP production. In regards to gluconeogenesis, cAMP is associated with the enhancement of the gene expression level and activity of PGC-1α. In turn, cAMP levels are increased by the activation of β-AR through the coupling of G-linked proteins, leading to the activation of AC and thus increased production levels of cAMP. With this in mind, in Chapter 4 we show that the administration of 30 and 50 μM NaHS induced a significant increase in the mRNA expression levels of PGC-1α and FBPase-1, along with the up-regulation of ERRα and FBPase-2 in 50 μM NaHS-treated A-10 cells. Because the NaHS-treated A-10 cells exhibited significantly higher levels of PGC-1α, and its targets ERRα and FBPase-1and -2, this introduces the possible involvement of the β-adrenergic receptor/cAMP signaling cascade through the activation of K_{ATP} channels. Therefore, it is suggestive to assume the likelihood of H_2S and its indirect activation of the β-AR/cAMP

signaling cascade in the vasculature, *via* cAMP, further elaborating on the multifaceted physiological functions of H_2S.

This unique study calls upon further investigation to identify H_2S-related up-stream regulators that were responsible for the reduced gluconeogenic rate in the $CSE^{-/-}$ male mice. Overall, the elucidation of a physiological link between MG and H_2S could lead to more elaborate and effective therapeutic regimens to combat the complex array of the symptoms associated with the metabolic syndrome.

Possible mechanisms that demonstrate a physiological link between MG and H_2S in the cardiovasculature

Our studies showed, for the first time, that a physiological balance occurs between MG and H_2S in the cardiovasculature. The following mechanisms, by which MG and H_2S are physiologically linked, are proposed.

1.0 Direct interaction between MG and H_2S

Both MG and H_2S are highly reactive molecules, making the possibility of a direct interaction highly plausible. Indeed, we found there to be a concentration-dependent decrease of MG after 24 hours of exposure to 10, 50, and 100 μM H_2S in a cell-free mixture, demonstrating that H_2S reduces the bioavailability of MG. Likewise, we found that the interaction of MG and H_2S lowers their respective endogenous levels in a concentration-related manner in A-10 cells. Furthermore, an electrospray ionization mass spectrometry analysis of MG and H_2S mixture

indicated the formation of three possible MG-H_2S adducts (data not shown). This suggests that a direct chemical reaction occurred between MG and H_2S leading to the formation of three unknown chemical species. In order to chemically identify the MG-H_2S products, and if these products have any biological significance, further analysis is required.

2.0 MG-induced down-regulation of CSE and H_2S production

MG is the most reactive precursor for AGE formation, mainly by covalently binding to specific amino acids on the specifically-targeted protein. Likewise, we have also shown that in a concentration-dependent fashion, 30 and 50 µM MG can interact with and reduce the protein expression level of CSE, an important H_2S-production enzyme in the cardiovasculature. Indeed, 30 and 50 µM MG significantly decreased the enzymatic activity of CSE, where in combination to its H_2S scavenging ability, MG significantly lowered the endogenous levels of H_2S in VSMCs. Further investigation is required to determine the underlying mechanisms by which MG reduces the protein levels of CSE, and if it may interact with other H_2S-generating enzymes.

3.0 H_2S-induced inhibition of MG-generated ROS production

Extensive work has been documented on the anti-oxidant properties of H_2S (Chang et al. 2010; Yan et al. 2006; Kimura and Kimura 2004; Kimura et al. 2006; Whitman et al. 2004; Ali et al. 2006). To follow this up, we are the first group to show that H_2S can inhibit MG-induced ROS production in VSMCs. In fact, we have demonstrated that concentrations lower than 120 µM NaHS (H_2S donor) significantly reduced 10 and 30 µM MG-induced ROS formation in a concentration-dependent manner in A-10 cells. Furthermore, 30, 60, and 90 µM NaHS increased the endogenous levels of GSH, a potent anti-oxidant that can scavenge both MG and ROS. The

H_2S-induced increase in endogenous GSH could be due to H_2S-induced enhancement of the activity of γ-GC, an enzyme involved in $_L$-cysteine production, leading to increased GSH levels (Kimura et al. 2004; Kimura et al. 2006). Additionally, H_2S can enhance the cysteine/glutamate antiporter x_c^-, thus increasing available cysteine for GSH production (Kimura et al. 2004). Overall, H_2S-mediated inhibition of MG-induced ROS production represents an important cross-talk mechanism that allows the cell to regulate its production of ROS, where excess levels of ROS would be scavenged by H_2S.

4.0 H_2S-induced up-regulation of gluconeogenesis: lower MG formation

Many studies have indicated that hyperglycemia, including an increased rate of glycolysis, may be involved in the over-production of MG (Thornalley 1988, 1996, 2003; Wang et al 2006; Dhar et al 2010). However, we have demonstrated that in renal tissues from $CSE^{-/-}$ mice, mice with lower levels of H_2S, showed a significantly reduced rate of gluconeogenesis, which likely led to higher levels of MG. The 6-22 week-old male $CSE^{-/-}$ mice exhibited significantly lower mRNA levels of the main rate-limiting gluconeogenic enzymes, PEPCK and FBPase-1, along with lower mRNA levels of the gluconeogenic regulator, PGC-1α, and its regulator, ERRα, as well as reduced levels of circulating glucose. Total FBPase activity was significantly reduced, where we have also reported decreased levels of its product, F-6-P, and higher levels of its substrate, F-1,6-P, in the renal extracts of 6-22 week-old male $CSE^{-/-}$ mice. Additionally, 30 and 50 μM NaHS induced a significant increase in the mRNA expression levels of PGC-1α and FBPase-1 in A-10 cells, whereas, as was explained in Chapter 3, concentrations at and higher than 30 μM significantly reduced MG levels in these rat VSMCs. Moreover, 50 μM NaHS also significantly increased the gene expression levels of FBPase-2 and ERRα in A-10 cells. Plasma MG levels

were also significantly elevated in these mice. Therefore, the reduced rate of gluconeogenesis in mice with lower levels of vascular H_2S may have led to the significantly higher levels of MG and its precursors in the kidney. However, more work is needed in order to strongly establish H_2S as a gluconeogenic regulator.

CONCLUSION

This report demonstrates that MG and H_2S can interact with and modulate each others' physiological functions (Chapter 3). In a cellular environment, MG can down-regulate the cellular production rate of H_2S by decreasing the protein and activity level of CSE, the main H_2S-generating enzyme in the cardiovasculature. Additionally, in A-10 cells, H_2S can induce the inhibition of MG-generated ROS production in A-10 cells, which is in agreement with previous observations based on the anti-oxidant properties of H_2S (Yan et al. 2006; Kimura et al. 2006; Whitman et al. 2004; Ali et al. 2006).

Our data indicates that H_2S could regulate the rate of gluconeogenesis, in renal and aortic tissues from $CSE^{-/-}$ male mice, as well as in rat VSMCs (Chapter 4). The CSE knockout mice displayed significantly reduced plasma glucose levels, yet enhanced MG levels, accompanied by reduced mRNA levels of PGC-1α, along with its down-stream targets, ERRα, PEPCK, and FBPase-1 and -2 in renal extracts. The activity of FBPase was significantly down-regulated, along with higher levels of its substrate, F-1,6-P, and lower levels of its product, F-6-P. The MG precursors, DHAP and GA3P were significantly increased in the kidney of 6-22 week-old $CSE^{-/-}$ mice, thus explaining the elevated renal levels of MG. Moreover, administration of NaHS increased mRNA expression levels of PGC-1α, FBPase-1 and -2, and ERRα in A-10 cells. Overall, our results

suggest the involvement of H_2S a potent modulator of gluconeogenesis, leading to the negative regulation of MG production.

SIGNIFICANCE OF THE STUDY

Diagnosis of the metabolic syndrome can enhance the likelihood that an individual will develop cardiovascular diseases, such as hypertension and T2DM, where the prevalence of these diseases are only expected to increase (Ford et al. 2002; Alberti et al. 2005). In light of the complexity of the metabolic syndrome, this is the first study to introduce a link between MG, a known causative factor for hypertension and diabetes, and H_2S, a gasotransmitter linked to T2DM. The novelty of this discovery contributes to the growing knowledge of the elaborate underlying mechanisms of the gluconeogenic system, and its abnormalities associated with the metabolic syndrome.

This is the first study to suggest that H_2S could be connected to the regulation of a key gluconeogenic regulator, PGC-1α, and thus the regulation of plasma glucose levels. Due to the fact that gluconeogenesis is constantly ``turned on`` in diabetic patients, this raises concern of how to control hyperglycemia, which is the cause of many diabetic complications (Puigserver 2005). Indeed, one of the most successful anti-diabetic and anti-hyperglycemic drugs is a gluconeogenic suppresser, known as metformin (Hundal and Inzucchi 2003). Indeed, metformin has been available for treatment of T2DM for nearly 8 years, and it is the most widely prescribed anti-hyperglycemic agent (Hundal and Inzucchi 2003). This creates a window of opportunity for one to study the interaction of H_2S with PGC-1α and its effect on circulating glucose levels, as

well as the possible involvement of H_2S and the activation of the β-AR/cAMP signaling cascade and its influence on gluconeogenesis.

Additionally, this report is the first to identify the existence of an alteration of FBPase-1 and -2 in the gluconeogenic kidney. The fact that FBPase-1 and -2 were both down-regulated in the presence of reduced PGC-1α gene expression levels in the $CSE^{-/-}$ mice, further supports the notion that PGC-1α is a potent regulator of the rate-limiting enzyme FBPase-1 and -2. By identifying the involvement of the dominant kidney FBPase, FBPase-1, in the altered formation of the pro-oxidant, MG, these findings provide an incentive to study H_2S regulation of FBPase-2 and its influence on MG formation in skeletal muscles of insulin-resistance animal models. FBPase-2 is the dominant FBPase in skeletal muscles (Yañez et al. 2005). Overall, the significance of an altered regulation of FBPase-1 and -2 in the presence of decreased H_2S, could provide novel insight to one researching FBPase inhibitors as a therapeutic treatment for hyperglycemia in diabetic patients.

Lastly, this study demonstrated an alteration of MG levels in the plasma and renal extracts from 6-22 week-old $CSE^{-/-}$ mice. This further confirms previous observations that elevated levels of MG in the plasma and kidney are involved in increased BP and may be a causative factor for the development of hypertension in SHRs (Wang et al. 2004, 2005, 2008; Wu and Jurrlink 2002). Wang et al (2004, 2005) demonstrated that MG plasma levels in the SHRs progressively increased with age and were associated with increased BP in SHRs compared with the age-matched WKY rats. Likewise, we have also observed what appears to be an age-related increase in plasma MG levels in 6-22 week-old $CSE^{-/-}$ mice. This study, along with previous reports,

suggests the importance of researching and administrating MG scavengers as a therapeutic or prophylactic treatment for patients with hypertension.

Research for drug treatment for the metabolic syndrome is still in its infancy (Desai et al. 2011). However, substantial amount of work has been put into H_2S and its involvement in diabetes and hypertension (Desai et al. 2011). We have demonstrated that cross-talk occurs between a reactive glucose metabolite, MG and a gasotransmitter, H_2S, both linked to diabetes and hypertension, where this phenomenon has age-related changes. Further elucidation of this cross-talk phenomenon between MG and H_2S could lead to more elaborate and effective therapeutic regimens to combat metabolic syndrome and its related health complications.

LIMITATIONS OF THE STUDY

Although extensive work has been paid to the elaborate scheme of the altered MG formation in renal tissues of male $CSE^{-/-}$ mice (Chapter 4), most of this work was observational analysis. The extracted renal tissues from the $CSE^{-/-}$ and $CSE^{+/+}$ mice were not subjected to any sort of treatment that could have added more support for our theory that H_2S is in some way responsible for the altered PGC-1α mRNA levels, which resulted in a physiological-chain-reaction of changed FBPase-1 mRNA levels, affecting the total FBPase activity, leading to altered MG precursors and MG itself.

To further support the observed reduced gluconeogenic rate in the 6-22 week-old $CSE^{-/-}$ male mice, isolation of hepatocytes from these mice would greatly aid in the investigation of the changed PGC-1α-regulated FBPase mechanism. An attempt was made regarding this approach,

however, complications arose. Nonetheless, the information obtained from H_2S-treatment to isolated hepatocytes from the $CSE^{-/-}$ mice could provide substantial evidence of the significantly altered gluconeogenesis system and MG formation. As well, subcutaneous injections of H_2S to 6-22 week-old $CSE^{-/-}$ would also give us a better understanding of the role H_2S may have in the regulation of PGC-1α. Therefore, to follow-up on this hypothesis, a future direction, as discussed below, describes the recommended parameters be studied upon the induction of excess H_2S to both isolated hepatocytes and to the hypertensive male CSE deficient mice.

FUTURE DIRECTIONS

As a follow-up on this study, the suggested future directions are divided into two sections:

Part 1: Whole animal study involving the subcutaneous injections of H_2S to $CSE^{-/-}$ male mice

Study A: By giving injections of H_2S to 6-22 week-old $CSE^{-/-}$ male mice for three weeks, while $CSE^{+/+}$ and $CSE^{-/-}$ mice (no H_2S treatment) serve as controls, it would be important to identify:

1. The circulating levels of glucose, glucocorticoids, insulin, and glucagon.
2. The cAMP levels in the kidney, liver, and aorta tissues.
3. PGC-1α gene and protein expression levels in the kidney, liver, and aorta extracts, along with the mRNA expression levels of the main rate-limiting gluconeogenic enzymes, including FBPase-1 and PEPCK.
4. Lastly, the MG precursors, DHAP and GA3P, along with MG itself, in kidney, liver, and aorta extracts from H_2S-treated 6-22 week-old $CSE^{-/-}$ and $CSE^{+/+}$ mice.

Part 2: Isolated liver cells from 14-16 week-old $CSE^{-/-}$ and $CSE^{+/+}$ male mice

This approach is centered on determining the underlying mechanism of H_2S and its involvement on gluconeogenesis, by subjecting the isolated $CSE^{-/-}$ hepatocytes to NaHS treatment (in the fasting state), in order to identify:

1. AC activity and cAMP levels. Note: the β-adrenergic/cAMP cascade induces the up-regulation of PGC-1α and enhances its activity (Puigserver 2005).

2. The mRNA expression levels of PGC-1α, FBPase-1, PEPCK, along with hepatocyte nuclear factor (HNF4α) and cAMP response element-binding (CREB). Note: CREB is an important transcription factor for the regulation of PGC-1α gene expression level, and HNF4α is a down-stream target of PGC-1α that is highly expressed in the liver during starvation periods (Puigserver 2005; Finck and Kelly 2006).

3. Akt activity and forkhead box O1 (FOXO1) gene expression level. Note: Akt is involved in the degradation of FOXO1, a transcription factor that helps PGC-1α to bind to and localize to the promoter region of G6Pase, PEPCK, and FBPase (Finck and Kelly 2006).

4. Intracellular levels of glycogen and glucose in isolated $CSE^{-/-}$ hepatocytes.

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