

**Upregulation of Renin Angiotensin Aldosterone System (RAAS) by
Methylglyoxal: Role in Hypertension**

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By

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ABSTRACT

In 2008 the global prevalence of hypertension [high blood pressure (BP), systolic ≥ 140 mmHg and/or diastolic ≥ 90 mmHg] was around 40% in adults > 25 yrs of age, according to the 2013 WHO statistics. Hypertension is a major risk factor for myocardial infarction, heart failure and stroke. Currently, around 20% of the Canadian population is affected by hypertension. Hypertension is more closely associated with diabetes. More than two thirds of people with diabetes have hypertension, alongwith increased activity of the renin angiotensin aldosterone (RAAS) system. The RAAS plays a major role in maintaining fluid balance, vascular tone and BP. The components of the RAAS include the hormone renin, which cleaves angiotensinogen, a circulating inactive peptide into angiotensin I. Angiotensin converting enzyme (ACE) converts angiotensin I into the active peptide angiotensin II (Ang II). Ang II causes vasoconstriction, sodium reabsorption from the kidney tubules and also release of the hormone, aldosterone, from the adrenal cortex. The epidemic of hypertension, diabetes and obesity is widely attributed to a high carbohydrate diet, containing mainly high fructose corn syrup and sucrose. However, the underlying molecular mechanisms are far from clear. A high fructose diet increases BP in Sprague-Dawley (SD) rats; along with elevated plasma and aortic levels of methylglyoxal (MG). MG is a reactive dicarbonyl compound mainly formed as an intermediate during glycolysis. Small amounts of MG are also formed during amino acid (threonine) and fatty acid metabolism. MG reacts with certain proteins to form irreversible advanced glycation end products (AGEs). MG has high affinity for arginine, lysine and cysteine. Plasma MG levels are increased in hypertensive rats and diabetic patients. However, it is not yet clear whether MG is the cause or effect of hypertension. Moreover, safe and specific MG scavengers are not available.

The aim of the project was to determine the effect of MG and a high fructose diet on the

RAAS and the BP in male SD rats. The hypothesis that L-arginine, and its inactive isomer D-arginine, can efficiently scavenge MG *in vitro*, was also tested.

Male SD rats were treated with a continuous infusion of MG with a subcutaneous minipump for 4 weeks, or with a high fructose diet (60% of total calories) for 16 weeks. We also used isolated aortic rings from 12 week old normal male SD rats to study endothelial function. Organs / tissues, cultured human umbilical vein endothelial cells (HUVECs) and vascular smooth muscle cells (VSMCs) were used for molecular studies. HPLC, Western blotting and Q-PCR were used to measure MG, reduced glutathione (GSH), proteins and mRNA, respectively. siRNA for angiotensinogen and the receptor for advanced glycation endproducts (RAGE) were used to study mechanisms.

MG treated rats developed a significant increase in BP and plasma levels of aldosterone, renin, angiotensin and catecholamines. MG level, and protein and mRNA for angiotensin, AT₁ receptor, adrenergic α_{1D} receptor and renin were significantly increased in the aorta and/or kidney of MG treated rats, a novel finding. Alagebrium, a MG scavenger and AGEs breaker, attenuated the above effects of MG. Treatment of cultured VSMCs with MG or high glucose (25mM) significantly increased cellular MG, and protein and mRNA for nuclear factor kappa B (NF- κ B), angiotensin, AT₁ and α_{1D} receptors, which were prevented by inhibition of NF- κ B, and by alagebrium. Silencing of mRNA for RAGE prevented the increase in NF- κ B induced by MG. Silencing of mRNA for angiotensinogen prevented the increase in NF- κ B, angiotensin, AT₁ and α_{1D} receptors' protein. Fructose treated rats developed a significant increase in BP. MG level and protein and mRNA for angiotensin II, AT₁ receptor, adrenergic α_{1D} receptor and renin were significantly increased, whereas GSH levels were decreased, in the aorta and/or kidney of fructose fed rats. The protein expression of the receptor for AGEs (RAGE) and NF- κ B were also

significantly increased in the aorta of fructose fed rats. MG treated VSMCs showed increased protein for angiotensin II, AT₁ receptor, and α_{1D} receptor. The effects of fructose and MG were attenuated by metformin, a MG scavenger and AGEs inhibitor. In experiments to test the MG scavenging action of arginine, both D-arginine and L-arginine prevented the attenuation of acetylcholine-induced endothelium-dependent vasorelaxation by MG and high glucose. However, the inhibitory effect of the NOS inhibitor, N ω -nitro-L-arginine methyl ester, on vasorelaxation was prevented only by L-arginine, but not by D-arginine. MG and high glucose increased protein expression of arginase, a novel finding, and also of NADPH oxidase 4 and NF- κ B, and production of reactive oxygen species in HUVECs and VSMCs, which were attenuated by D- and L-arginine. However, D- and L-arginine did not attenuate MG and high glucose-induced increased arginase activity in VSMCs and the aorta. D- and L-arginine also attenuated the increased formation of the MG-specific AGE, N ϵ -carboxyethyl lysine, caused by MG and high glucose in VSMCs.

In conclusion, MG activates NF- κ B through RAGE and thereby increases renin angiotensin levels, a novel finding, and a probable mechanism of increase in BP. There is a strong association between elevated levels of MG, RAGE, NF- κ B, mediators of the RAAS and BP in high fructose diet fed rats. Arginine attenuates the increased arginase expression, oxidative stress, endothelial dysfunction and AGEs formation induced by MG and high glucose, by an endothelial NOS independent mechanism.

DEDICATIONS

to

Dr. Arti Dhar

*who groomed me and made me who I am today and is
my real life mentor, the biggest role model and a true blessing from God*

&

to the Almighty who made it all possible

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LIST OF ABBREVIATIONS

2-MQ	2-methyl quinoxaline
3-DG	3-Deoxyglucosone
5-MQ	5-methyl quinoxaline
AG	Aminoguanidine
AGEs	Advanced glycation end products
ALA	Alagebrium
ALD	Aldosterone
ANG	Angiotensin
ANG II	Angiotrensins II
ANOVA	One way analysis of variance
AT1	Angiotensin receptor subtype 1
DHAP	Dihydroxyacetone phosphate
DM	Diabetes mellitus
DMEM	Dulbeccos' modified eagle medium
EC	Endothelial cells
eNOS	Endothelial nitric oxide synthase
G-3-P	Glyceraldehyde-3-phosphate
GSH	Reduced glutathione
GSH-Red	Glutathione reductase
H ₂ O ₂	Hydrogen peroxide
HPLC	High performance liquid chromatography

HUVECs	Human umbilical vein endothelial cells
MG	Methylglyoxal
NAC	N-acetyl L-cysteine
NO	Nitric oxide
$O_2^{\cdot -}$	Superoxide
$ONOO^-$	Peroxynitrite
<i>o</i> -PD	Ortho phenylene diamine
PBS	Phosphate buffered saline
PCA	Perchloric acid
PI3K	Phosphoinositide 3 kinsase
RAAS	Renin angiotensin aldosterone system
RAECs	Rat aortic endothelial cells
RAGEs	Receptor for advanced glycation end products
ROS	Reactive oxygen species
SD	Sprague-Dawley
SSAO	Semicarbazide-sensitive amine oxidase
VSMCs	Vascular smooth muscle cells
α_{1D}	Alpha adrenergic receptor subtype D

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Hypertension

1.1.a Definition

Hypertension, also defined as high blood pressure, is a medical condition in which the blood pressure (BP) in the arteries stays persistently elevated. BP is the force of blood pushing up against the arterial vessel walls. The higher the pressure, the harder it requires the heart to pump the blood. BP is measured as systolic pressure i.e., peak pressure in the arteries, and diastolic pressure i.e., minimum pressure in the arteries. Normal BP is usually below 120/80 mmHg where 120 mmHg represents systolic and 80 mmHg represents diastolic pressure (Giles *et al.*, 2009; Chobanian *et al.*, 2003). A BP value between 120/80 mmHg and 139/89 mmHg is called prehypertension and BP above 140/90 mmHg is considered hypertension (Table 1-1). 90-95% cases of hypertension are characterized as primary (formerly also known as essential hypertension) with no apparent medical cause. 5-10% cases are secondary hypertension with a known cause such as kidney disease, tumors and heart disease (Carretero & Oparil, 2000). Although the exact cause/causes of hypertension is/are not known, there are several factors such as smoking, obesity, diabetes, lack of physical activity, high levels of salt intake, stress, aging, and tumors, which contribute to its development (Klaus *et al.*, 2009; Elley *et al.*, 2002) (Table 1-2). Hypertension is a major risk factor for stroke, ischemic heart disease, heart failure and chronic kidney disease (Coca *et al.*, 2008; Hatmi *et al.*, 2007).

Stages of Hypertension

BP		
Category	SBP (mmHg)	DBP (mmHg)
Normal	<120	<80
Prehypertension	120-139	80-89
Hypertension, Stage 1	140-159	90-99
Hypertension, Stage 2	≥160	≥100

Table 1-1: Stages of hypertension based on change in systolic and diastolic blood pressure. SBP – systolic blood pressure, DBP – diastolic blood pressure. Adapted from JNC 7, 2004. <http://www.nhlbi.nih.gov/guidelines/hypertension/phycard.pdf>

1.1.b Prevalence

In 2013, the World Health Organization chose hypertension as the theme for the World Health Day which was celebrated on April 7, 2013 (Campbell *et al.*, 2013) (<http://www.cdc.gov/features/worldhealthhypertension/>). The World Health Day highlights a topic that is a public health concern. The global prevalence of high BP in adults aged 25 years and above was around 40% (around 2.7 billion out of 6.7 billion people) in 2008. The number of people with uncontrolled hypertension was around 600 million in 1980 and rose to 1 billion by

the year 2008. High BP is estimated to cause 7.5 million deaths worldwide every year, which accounts for about 12.8% of the total number of deaths (http://www.who.int/gho/ncd/risk_factors/blood_pressure_prevalence_text/en/). Canada has the highest reported national rates of awareness, treatment, and control of high BP in the world. The prevalence of hypertension in Canada has remained fairly constant at around 20% of the 26 million people included in the retrospective study, for the past three decades (1985-2011) (Campbell *et al.*, 2013).

Lifestyle	Risk of hypertension
High dietary sodium	32%
Obesity	32%
Low dietary potassium	17%
Less physical activity	17%
High alcohol intake	3%

Table 1-2: Lifestyle causes of increase in the BP. Adapted from *Can J Cardiol.* 2013; 29:415-417.

1.1.c Classification of Hypertension

Hypertension can also be classified (Table 1-3) based on cardiovascular risk factors, early disease markers and target organ disease as follows:

Classification	Normal	Hypertension on Stage I	Hypertension Stage 2	Hypertension Stage 3
Description	Normal BP & no CVD	Intermittent or occasional BP elevation and early CVD	Sustained elevation of BP or progressive CVD	Marked elevation of BP or advanced CVD
Cardiovascular risk factors	-	Several risk factors present	Many risk factors	Many risk factors
Early disease markers	-	Present	Present	Present with progression
Target organ disease	-	-	Early signs	Present

Table 1-3: Classification of hypertension. Adapted from *J Clin Hypertens.* 2009 Nov;11(11):611-4.

Cardiovascular risk factors - Aging, obesity, dyslipidemia, insulin resistance, kidney disease etc.

Early disease markers - Loss of nocturnal BP dipping, left ventricular hypertrophy, artery stiffness, endothelial dysfunction, microalbuminuria, elevated serum creatinine.

Target organ disease - Left ventricular hypertrophy, peripheral arterial disease, stroke, heart failure and neuropathy

CVD – cardiovascular disease

1.1.d Regulation of BP

There are many factors which can influence BP like diet, exercise, disease condition and stress. One main physical factor involved in BP physiology is the volume of blood. When there is more blood present in the body, the rate of blood return to the heart is higher resulting in higher cardiac output (Freis, 1975). Another factor influencing BP is vascular resistance or better still, peripheral vascular resistance. Higher the resistance, higher will be the pressure upstream from the resistance to blood flow. Resistance is calculated by vessel radius, vessel length, and blood viscosity. Larger the vessel radius, lower the resistance, and longer the vessel, higher is the resistance. Smoothness of the vessel wall is affected by the buildup of fatty deposits on the arterial walls and thickness or viscosity of the fluid (Rosenson *et al.*, 2004). If the blood gets thicker, this results in an increase in arterial pressure. Certain medical conditions like anemia (low red blood cell count) reduce viscosity of the blood.

The short term regulation of BP is controlled by the sympathetic nervous system (SNS) and the renin angiotensin aldosterone (RAAS) system while long term BP is controlled by the kidney.

Normal BP is usually below 120/80 mmHg. Parameters influencing systolic pressure include stroke volume, systolic rate and arterial compliance. An increase in stroke volume, systolic rate and a decrease in compliance cause an increase in systolic pressure. Factors influencing diastolic pressure include vascular resistance, heart rate and systolic pressure. Diastolic pressure is directly proportional to these factors (Prasad, 2009).

Baroreceptor and chemoreceptor reflex

Baroreceptors, located in the carotid sinus at the bifurcation of the common carotid artery and aortic arch, respond to acute elevations in BP by causing a reflex vagal bradycardia. Cardio pulmonary receptors, located in the pulmonary vessels and in the walls of the right atrium and the ventricles of the heart, likewise respond to increases in atrial filling by causing bradycardia through inhibition of cardiac SNS, increasing atrial natriuretic peptide (ANP) release and inhibition of vasopressin release (Prasad, 2009).

Chemoreceptors, located at the bifurcation of the carotid artery and aortic arch, respond to changes in blood pO₂, pCO₂ and pH. Chemoreceptors are stimulated when either pO₂, pH, or the blood flow in the arteries is very low, or when the pCO₂ is high. In response to these changes, the chemoreceptors send impulses to cause vasoconstriction and increase the vascular resistance, heart rate and stroke volume *via* increased sympathetic activity (Prasad, 2009).

Sympathetic nervous system

The sympathetic nervous system (SNS) through renal sympathetic nerves also plays an important role in BP regulation by stimulating renin release from the juxtaglomerular apparatus (Joyner *et al.*, 2010). α_1 -adrenergic receptors, one of the subtypes of adrenergic receptors, belong to the G protein-coupled receptor (GPCR) family and mediate responses to norepinephrine and

epinephrine. α_1 -adrenergic receptors are further subdivided into α_{1A} , α_{1B} and α_{1D} using receptor binding and molecular cloning studies (Zhong & Minneman, 1999). All three α_1 -adrenergic receptor subtypes act *via* Gq/11 signaling pathway. Gq/11 signaling activation in turn leads to activation of phospholipase C, generation of inositol trisphosphate and diacylglycerol, and finally an increase in intracellular calcium levels (Piascik & Perez, 2001; Pupo & Minneman, 2001). Although all three α_1 adrenergic receptor subtypes activate the same Gq/11 protein signaling pathway, due to their different tissue distributions, they play distinct functional roles. α_{1A} adrenergic receptor is highly expressed in the prostate and is the dominant receptor controlling benign prostatic hypertrophy, and is thus an important therapeutic target for the treatment of benign prostatic hyperplasia (Nagarathnam *et al.*, 1998). α_{1D} is the major adrenergic receptor in the aorta and is responsible for the contraction of the aorta (Fan *et al.*, 2009). WB 4101[13], (+)-niguldipine (Boer *et al.*, 1989), and 5-methylurapidil (Gross *et al.*, 1988) have been used as α_{1A} selective antagonists, and BMY7378 (Goetz *et al.*, 1995) is used to characterize α_{1D} adrenergic receptors. However, little progress has been made to identify α_{1B} adrenergic receptor selective compounds. Three α_2 receptor subtypes have been identified, $\alpha_{2A/D}$, α_{2B} and α_{2C} . Most of the classical effects of α_2 adrenergic stimulation are mediated by pre-synaptic α_{2A} receptors, which inhibit the release of norepinephrine, and cause a decrease in BP. α_{2B} adrenergic receptors are mostly found at postsynaptic sites where they produce vasoconstriction and thus have effects opposite to those of α_{2A} receptors. α_{2C} adrenergic receptors may mediate a hypothermic response and do not have cardiovascular effects (Joyner *et al.*, 2010).

Renin angiotensin aldosterone system

The activity of the RAAS plays an important role in BP regulation. Renin is a 340 amino acid protein with a molecular weight of 37 kDa. As shown in (Fig 1-1), renin is secreted by the kidney from granular cells of the juxtaglomerular apparatus in response to a decrease in the BP, low salt levels in the nephron and an increase in the SNS activity. Renin breaks down angiotensinogen, produced by the liver, to an inactive decapeptide, angiotensin I. Angiotensin I is further converted into the active octapeptide angiotensin II (Ang II) by the angiotensin converting enzyme (ACE) within the capillaries ([Atlas, 2007](#)).

The amount of renin in the plasma acts as a limiting factor for Ang II production. Apart from Ang II generated in the circulation, blood vessels capture circulating renin and angiotensinogen and produce Ang II at the surface of their wall. Many tissues like blood vessels, heart, kidney, and brain possess all components of the renin angiotensin system and generate Ang II inside their cells. Ang II is involved in electrolyte balance, blood volume and pressure homeostasis. The most important action of Ang II is vasoconstriction produced directly as well as by increasing epinephrine/norepinephrine release and increasing central sympathetic outflow. Vasoconstriction involves arterioles and venules and occurs in all vascular beds. Ang II induced vasoconstriction promotes movement of fluid from the vascular to the extravascular compartment. Ang II increases the force of myocardial contraction by increasing Ca^{2+} influx.

Aldosterone is a steroid hormone belonging to the mineralocorticoid family. It is produced by the zona glomerulosa of the adrenal cortex in the adrenal gland. Aldosterone plays a central role in BP regulation by acting on the distal tubules and collecting ducts of the nephron,

increasing reabsorption of sodium ions and water in the kidney, to cause the conservation of sodium, secretion of potassium and increased water retention (Booth *et al.*, 2002).

ADH, also known as antidiuretic hormone, is synthesized in the hypothalamus and stored in the posterior pituitary. ADH release is increased by Ang II. ADH plays a role in water reabsorption in the renal tubules, which in turn causes increased fluid volume, cardiac output and later on increased vascular resistance and then finally increased arterial pressure (Prasad, 2009).

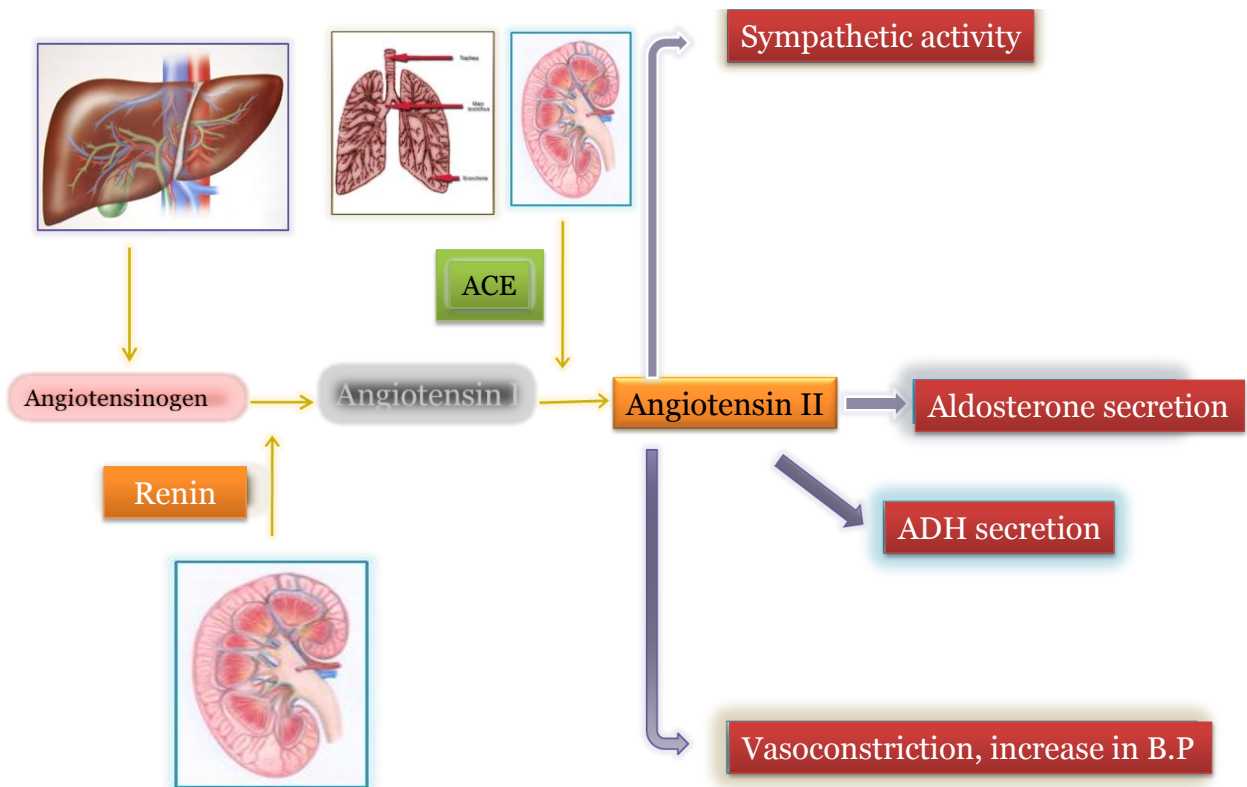


Figure 1-1. Pathway of regulation of BP by the renin angiotensin aldosterone system.

Adapted from *Nature Rev Drug Disc.* 2002;1:621-636. <http://www.acibademinternational.com/>

Although the pharmacological inhibition of the RAAS using ACE inhibitors and AT₁ receptor blockers can be beneficial in certain conditions including hypertension, a growing body of evidence suggests that blocking the RAAS at its initial step, *i.e.*, renin inhibition may provide an effective RAAS suppression. Aliskiren is an orally active direct renin inhibitor and was approved by the U.S. Food and Drug Administration in 2007 for the treatment of primary hypertension. Aliskiren, by inhibiting renin, prevents angiotensin I generation from angiotensinogen, and thus subsequently prevents the activation of the rest of the RAAS (Wood *et al.*, 2003).

Angiotensin I can also be converted to angiotensin (1-9) by angiotensin converting enzyme 2 (ACE2) and angiotensin (1-9) can be converted to angiotensin (1-7) by ACE. Angiotensin (1-7) is an active member of the RAAS system and has functions opposite to those of Ang II. Angiotensin (1-7) acts *via* a G protein-coupled receptor called Mas (Santos *et al.*, 2003; Santos *et al.*, 2005). AVE0991 is a non-peptide angiotensin (1-7) receptor Mas agonist, and its administration in coronary artery ligated rats attenuated post-ischemic heart failure induced by myocardial infarction (Ferreira *et al.*, 2007a). AVE0991 also prevented isoproterenol-induced impairment in heart function/remodeling in the rat (Ferreira *et al.*, 2007b). It has been reported recently that angiotensin (1-9) antagonizes pro-hypertrophic signaling in cardiomyocytes *via* angiotensin type 2 (AT₂) receptors (Flores-Muñoz *et al.*, 2011). Renin, ACE2, angiotensin (1-7) and angiotensin (1-9) are potential therapeutic targets and need further investigation for the prevention and treatment of cardiovascular disorders (Balakumar *et al.*, 2011).

1.1.e Pathogenesis of Hypertension

In general, hypertension is caused by both genetic and environmental factors (Oparil *et al.*, 2003). The heritability of blood pressure in humans has been estimated at 30-50% (Ehret & Caulfield., 2013).

Although several factors contribute to the pathogenesis of hypertension, renal mechanisms play a fundamental role. Mean arterial pressure is the product of cardiac output and systemic vascular resistance and patients with hypertension may either have an increase in cardiac output, an increase in systemic vascular resistance, or both. Regulation of normal BP is dependent on the balance between cardiac output and peripheral vascular resistance and most patients with primary hypertension have a normal cardiac output but raised peripheral resistance (Beevers *et al.*, 2001). In young patients the cardiac output is often elevated, while in older patients increased systemic vascular resistance and increased vascular stiffness are the contributing factors. The tone of the vessel may be elevated due to increased release of peptides (angiotensin, endothelin) or increased alpha receptor stimulation. The final outcome is an increase in cytosolic calcium in vascular smooth muscle cells (VSMCs) causing vasoconstriction (Foex & Sears, 2004). Hypertension (Fig 1-2) is usually a result of complex interaction of genetic, environmental and demographic factors. Hypertension also causes vascular and cardiac remodeling and hypertrophy which in turn leads to a change in the structure and function of the heart and vessel wall (Mayet & Hughes, 2003). Enhanced peripheral sensitivity to norepinephrine and decreased sensitivity to baroreceptor reflexes has also been observed in hypertensive patients (Foex & Sears, 2004). Increased activity of the RAAS has also been

suggested as one of the contributing factors in the pathogenesis of hypertension (Oparil *et al.*, 2003).

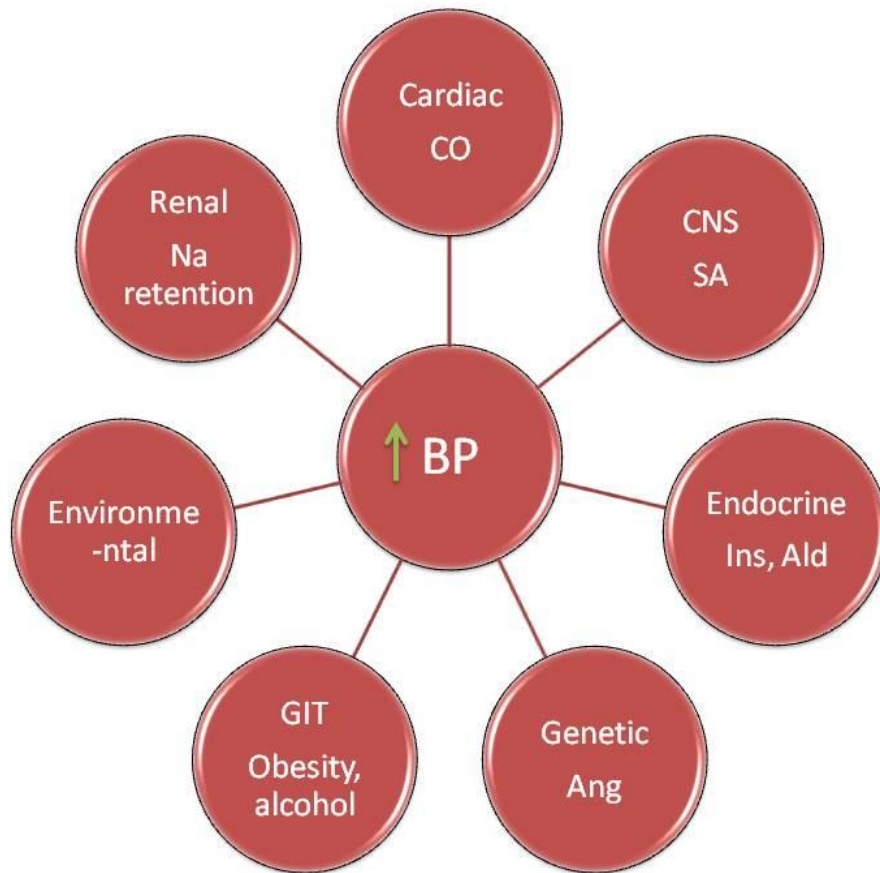


Figure 1-2. Pathogenesis of hypertension. Adapted from *Ann Intern Med.*2003;139:761-776.

Abbreviations: Ald - aldosterone; Ang - angiotensinogen; CO – cardiac output; GIT – gastrointestinal tract; Ins - insulin; SA – sympathetic activation.

SNS over activity increases BP *via* stimulation of the heart, peripheral vasculature, and kidney thus causing increased cardiac output, increased vascular resistance, and fluid retention

(Mark, 1996). Chronic stimulation of the SNS induces vascular remodeling and left ventricular hypertrophy, by direct action of norepinephrine on its own receptors and indirectly *via* release of various trophic factors such as transforming growth factor, insulin-like growth factor 1, and fibroblast growth factors (Brook & Julius, 2000). Sympathetic stimulation is also increased in the kidney in hypertensive patients compared with normotensive control patients. Psychological stress is also one of the contributing factors to increases in BP. It has been reported that exposure to stress increases sympathetic outflow, and constant stress induces vasoconstriction which in turn may lead to vascular hypertrophy, increases in peripheral resistance and BP (Light, 2000). Hyperuricemia, characterized by abnormally high uric acid levels in the blood, is also associated with hypertension in humans, but whether it is an independent risk factor or only a marker for associated CVD risk factors is not clear (Rich, 2000; Culleton *et al.*, 1999). In human patients hyperuricemia is associated with renal vasoconstriction (Messerli *et al.*, 1980) and is positively correlated with plasma renin activity in hypertensive patients (Saito *et al.*, 1978), thus indicating that uric acid could have adverse effects that are mediated by an activated RAAS system.

1.1.f Hypertension in Diabetes

Approximately 70% of patients with diabetes have hypertension and it is twice more common in patients with diabetes than without it (Arya, 2003). Hypertension in diabetic patients causes a significant risk of other complications like retinopathy, neuropathy, nephropathy and ischemic vascular disease (Arya, 2003). Diabetes mellitus is also an independent risk factor for coronary artery disease and the risk is significantly higher when hypertension is present. In patients with type 1 diabetes, diabetic nephropathy is the commonest cause of hypertension in

patients. In patients with type 2 diabetes hypertension commonly occurs without abnormal renal function and is often associated with obesity. Diabetes and insulin resistance can aggravate hypertension by stimulating the renin angiotensin system, the SNS and sodium retention (Lago *et al.*, 2007; Campbell *et al.*, 2011). Increased VSMC proliferation has also been observed under diabetic conditions. High BP and elevated blood glucose levels can impair vascular endothelial cell function which in turn leads to increased oxidative stress (Kolluru *et al.*, 2012; Giacco & Brownlee, 2010). Patients with diabetes also have increased vascular reactivity (Chittari *et al.*, 2011).

The pathogenesis of hypertension in diabetes is multifactorial and complex. Interactions between genetic, environmental and biological factors such as sedentary behavior, unhealthy diet, sodium retention, obesity, autonomic derangements, arterial stiffening, and endothelial dysfunction seem to play an important role (Campbell, 2011). Insulin resistance plays a pivotal role in the pathogenesis of hypertension. In many randomized controlled trials patients with diabetes have benefited from a more aggressive treatment of hypertension. Insulin resistance plays a role in the development of hypertension *via* defects in the insulin signaling pathway and associated cardiovascular and metabolic disorders. Insulin resistance and its accompanying hyperglycemia can lead to increased sympathetic activity, increased RAAS activity, suppressed atrial natriuretic peptide activity, and increased sodium retention with volume expansion, cardiac hyperactivity, and dyslipidemia. Chronic hyperglycemia causes increased oxidative stress (Sampanis & Zamboulis, 2008). Pregnant women with hypertension and diabetes are at increased risk of developing pre-eclampsia (Allison *et al.*, 2013), and children with diabetes and hypertension are vulnerable to end organ disease (Lago *et al.*, 2007).

1.1.g Glucose and Fructose Induced Hypertension

The intake of refined sugar in the diet increased dramatically in the latter part of the twentieth century after it was widely manufactured from sugar cane (Yudkin, 1967). In the 1970s the introduction of high-fructose corn syrup, a mixture of glucose and fructose in varying proportions, further increased the carbohydrate load in the diet because high fructose corn syrup is widely used in soft drinks, fruit punches, bread, and processed foods (Yudkin, 1967). In 1960s Yudkin proposed that the sugar in the diet, and not fat, is the primary cause of increased incidence of diabetes and various cardiovascular diseases (Yudkin, 1964). Epidemiologic studies show an association of soft drink consumption with obesity, hypertension, and diabetes (Schulze *et al.*, 2004; Bray *et al.*, 2004).

Two thirds of the patients with primary hypertension have an abnormal blood glucose metabolism (Garcia-Puig *et al.*, 2006). Several studies have shown that administration of a high carbohydrate diet significantly increases BP in animals and humans. In a clinical study, the administration of sucrose significantly increased body weight, serum triacylglycerols, and systolic BP (Raben *et al.*, 2002). Similarly, healthy adults fed a diet of 33% sucrose for 6 weeks showed an increase in BP (Israel *et al.*, 1983). Oral administration of 10% D-glucose to normal rats for 3 weeks induced hypertension (Tom *et al.*, 2011). Male Sprague-Dawley (SD) rats fed a glucose-rich diet developed a 7% increase in BP compared to rats fed a normal diet. Higher norepinephrine excretion in glucose fed rats suggests that increased SNS activity might participate in the glucose-induced rise in BP (Kaufman *et al.*, 1991). Fructose-fed SD rats have been widely used as a model of insulin resistance (Hwang *et al.*, 1987; Jia & Wu, 2007) and these rats also develop hypertension. For example, SD rats fed with a diet containing 66%

fructose as a percentage of total calories for approximately 2 weeks developed a significant increase in systolic blood pressure from 124 ± 2 to 145 ± 2 (SEM) mmHg, whereas no change occurred in the control group. In addition, hyperinsulinemia and hypertriglyceridemia were associated with hypertension in fructose-fed rats (Hwang *et al.*, 1987). In other studies male SD rats fed a 60% fructose diet for 8-11 weeks developed a significant increase in systolic BP, other features of the metabolic syndrome (Nakagawa *et al.*, 2006) and pathologic changes in the kidney (Sanchez-Lozada *et al.*, 2007; Gersch *et al.*, 2007). We have shown that fructose-fed SD rats have elevated levels of MG (Wang *et al.*, 2008), a product of fructose metabolism. Even lower doses of fructose (10% in drinking water) given to SD rats for 8 weeks induced a significant increase in systolic BP and microvascular changes in the kidney (Sanchez-Lozada *et al.*, 2007). High dietary carbohydrate-induced hypertension is associated with an increase in uric acid concentrations (Sanchez-Lozada *et al.*, 2007; Gersch *et al.*, 2007) with a consequent increase in oxidative stress (Sautin *et al.*, 2007) and inhibition of NO production (Khosla *et al.*, 2005). One study has reported that fructose-induced hypertension is mediated by an increase in salt re-absorption by the intestine and kidneys, and these effects are mediated by transporters Slc26a6 and Slc2a5 (Singh *et al.*, 2008).

1.1.h Treatment of Hypertension

Treatment for hypertension can be achieved with lifestyle changes along with medications. There are different classes of drugs available for the prevention and management of hypertension.

ACE inhibitors are often the first choice drugs in all grades of primary hypertension as well as renovascular hypertension. They include captopril, enalapril, lisinopril etc. (Ajayi *et al.*, 1985). ACE inhibitors increase plasma kinin levels such as bradykinin and potentiate the hypotensive action of bradykinin. ACE inhibitors induce hypotension by decreasing total peripheral resistance. The arterioles dilate and compliance of large arteries is increased. Both systolic and diastolic BP fall without change in cardiac output. They are the most appropriate drugs for diabetic nephropathy, left ventricular hypertrophy, congestive heart failure and angina pectoris (Brown & Vaughan, 1998).

Over the years several nonpeptide orally active AT₁ receptor antagonists have been developed. Losartan, candesartan and telmisartan are included in this class. AT₁ receptor antagonists are now first line antihypertensive drugs alternative to ACE inhibitors in efficacy, with the advantage of not inducing cough, and with a low incidence of angioedema. AT₁ receptor antagonists cause competitive antagonism of Ang II and are 10,000 times more selective for AT₁ than AT₂ receptors. They block all the actions of Ang II including vasoconstriction, central and peripheral sympathetic stimulation, release of aldosterone and vasopressin release (Fabia *et al.*, 2007; Unger, 2001).

A large number of chemically diverse calcium channel blockers (CCBs) with different pharmacological profiles have been produced. Three important classes are: verapamil - a phenylalkylamine hydrophilic papaverine congener, nifedipine - a dihydropyridine (lipophilic), diltiazem - a hydrophilic benzothiazepine. The dihydropyridines are the most potent CCBs. CCBs cause smooth muscle (vascular) relaxation by decreasing intracellular availability of Ca²⁺

by blocking L-type Ca^{2+} channels. They lower BP by decreasing peripheral resistance without compromising cardiac output (Sica, 2007; Schoen & Tanzi, 2008).

Diuretics have been the standard antihypertensive drugs over the last 4 decades. These include thiazide diuretics like chlorthalidone, which act by decreasing extracellular fluid volume and ultimately leading to a decrease in total peripheral resistance and fall in BP. Another class of drugs in this category is the high ceiling diuretics like furosemide which act by decreasing plasma volume and cardiac output (Ernst & Moser, 2009; Musini *et al.*, 2009).

Beta adrenergic receptor blockers are mild antihypertensives and do not lower the BP in normotensives. Alpha adrenergic receptor blockers or antagonists like prazosin dilate both resistance and capacitance vessels, with the former effect predominating. They cause a reduction in total peripheral resistance and mean BP with a slight decrease in venous return and cardiac output (Cruickshank, 2010; Stokes & Marwood, 1985).

Another group of antihypertensive drugs is central sympatholytics like clonidine, which is a partial agonist and has a high affinity for α_{2A} receptors in the brainstem. The major effect of α_{2A} receptor stimulation is a decrease in the sympathetic outflow resulting in a fall in BP and bradycardia, which is also due to enhanced vagal tone (Vongpatanasin *et al.*, 2011).

1.2 Methylglyoxal

1.2.a Source and Chemistry of Methylglyoxal

Methylglyoxal (MG) (Fig 1-3), also called 2-oxopropanal or pyruvaldehyde, is an aldehyde with two carbonyl groups, and hence is also called a dicarbonyl compound. It is a yellow liquid with a pungent odour and has a molecular weight of 72.06. MG is both an aldehyde

and a ketone. Since MG has two α,β carbonyl groups, this makes it a highly reactive compound, reacting with exposed arginine, lysine and cysteine residues of proteins, and with other macromolecules such as DNA, in the body.

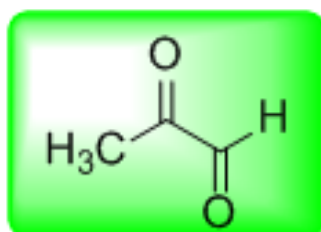


Figure 1-3. Chemical structure of methylglyoxal

1.2.b Synthesis and Degradation of MG

MG was first synthesized from iso-nitrosoacetone in the presence of dilute sulfuric acid along with the formation of glycol, formaldehyde and formate by Von Penchmann ([Von Penchmann, 1887](#)), who first developed the method for the synthesis of MG. MG can also be formed from acid hydrolysis of methylglyoxal dimethylacetal and from the oxidation of acetone with selenium dioxide followed by distillation under nitrogen atmosphere ([Kalapos, 1999](#)).

In living organisms (Fig 1-4), MG is an endogenous metabolite and has been proposed to be primarily formed during glycolysis from the triose phosphate intermediates. Other important precursors of MG, believed to produce lower amounts of MG, include aminoacetone, produced from amino acids L-threonine and glycine during protein catabolism, and ketone bodies such as acetone from fatty acid oxidation. MG formation from these sources involves various enzymatic

and nonenzymatic reactions, but the main enzymes which catalyze MG formation from these precursors include: MG synthase, semicarbazide-sensitive amine oxidase (SSAO) and mono-oxygenase (MO). MG synthase catalyzes the conversion of triose phosphate intermediate, dihydroxyacetone phosphate (DHAP), into MG, under inadequate availability of inorganic phosphate. SSAO catalyzes the deamination of aminoacetone to produce aldehyde, ammonia and hydrogen peroxide. Two enzymes, i.e. acetone and acetol mono-oxygenase (AMO), catalyze the conversion of acetone to acetol and acetol to MG, respectively, during fatty acid oxidation. SSAO is primarily found in the cytoplasm of adipose tissue, VSMCs, plasma and endothelial cells (Thornalley, 1996; Desai & Wu, 2007).

MG formed is rapidly detoxified to D-lactic acid by the glyoxalase system, which consists of 2 enzymes, glyoxalase I and II, and uses reduced glutathione (GSH) as a cofactor. GSH binds to MG and presents it to glyoxalase I, which first converts MG irreversibly to (S)-D-lactoylglutathione and then by glyoxalase II to D-lactate. Hence, adequate availability of cellular GSH is crucial for normal catabolism of MG in the body. Also, GSH-related enzymes, including glutathione peroxidase and glutathione reductase are equally important as they are involved in the synthesis and recycling of GSH (Thornalley, 1990).

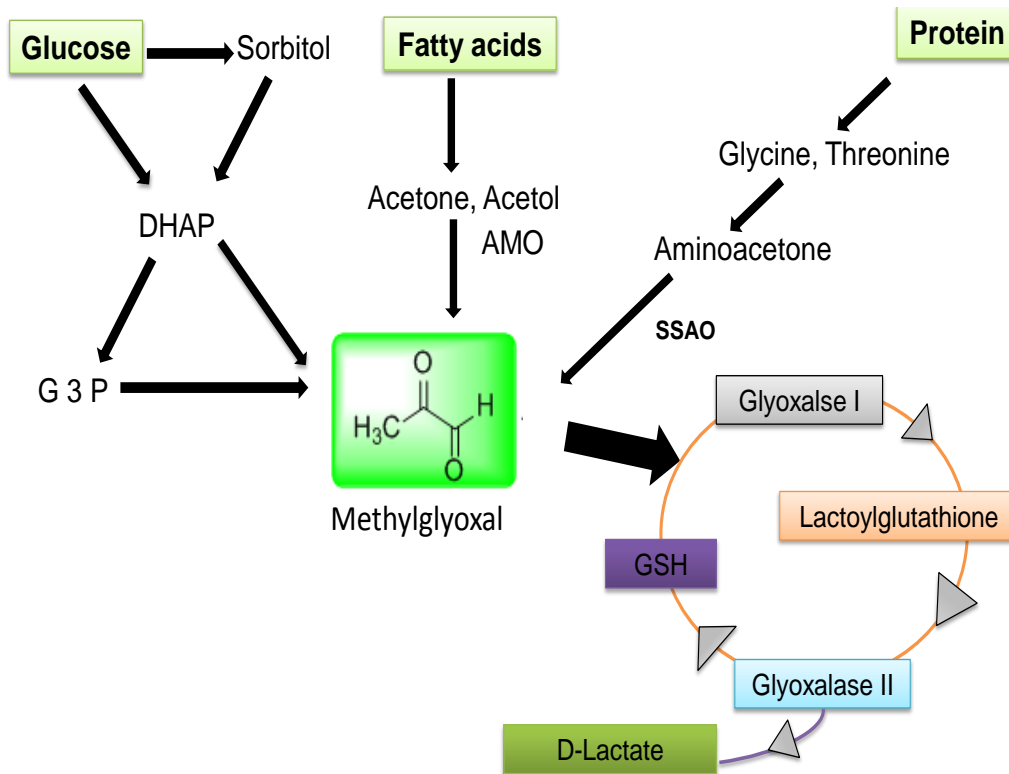


Figure 1-4. Metabolic pathways of MG formation and degradation. Adapted from *Metabolism*. 2008; 57(9):1211-20.

Schematic showing pathways, substrates and enzymes involved in the formation of MG in the body.

Abbreviations: AMO – acetol/acetone monooxygenase; DHAP – dihydroacetone phosphate; F-1,6-di-P – fructose-1,6-diphosphate; G-3-P – glyceraldehyde-3-phosphate; GSH – reduced glutathione; SSAO – semicarbazide-sensitive amine oxidase; ROS – reactive oxygen species.

1.2.c Plasma and Tissue Levels of MG

Under normal conditions, the plasma levels of MG are between 0.5 and 1.5 μM (Jia & Wu, 2007; Wang *et al.*, 2007) and the levels increase as much as 2 to 4 fold under hypertensive and diabetic conditions (Wang *et al.*, 2007; Beisswenger *et al.*, 1999). An increased body of evidence suggests that there is a link between increased MG formation and hypertension but the exact mechanism is far from clear. Previous studies have reported higher MG levels in the plasma of spontaneously hypertensive rats (SHR) of 13.8 μM at 8 weeks of age and 33 μM at 20 weeks as compared to Wistar Kyoto (WKY) rats at the same age (9.1 and 14.2 μM). In the aorta and kidney of SHR, the MG levels were 2.5 nmol/mg protein and 0.3 nmol/mg protein, respectively, as compared to the WKY rats (1.5 nmol/mg protein and 0.2 nmol/mg protein, respectively) at the same age (Wang *et al.*, 2004; Wang *et al.*, 2005). In Sprague-Dawley rats fed with a high fructose diet for 9 weeks, there was a significant increase in serum MG levels to 3.2 μM compared to 1.8 μM in the control rats and the effect was attenuated by the MG scavenger N-acetyl cysteine (NAC) (Jia and Wu, 2007). Rat VSMCs incubated with a high concentration of glucose or fructose lead to increased MG formation along with an increase in oxidative stress (Dhar *et al.*, 2008). High MG levels are related to diabetic microvascular complications including retinopathy, nephropathy and neuropathy, and diabetic macrovascular complications such as atherosclerosis.

1.2.d MG and Advanced Glycation Endproducts

Advanced glycation endproducts (AGEs) are harmful chemical entities and are complex, heterogeneous compounds produced from chemical modification of physiologic molecules of the

body during metabolic processes and have been implicated in diabetes related complications (Desai & Wu, 2007).

AGEs as shown in (Fig 1-5) are formed when reducing sugars such as glucose, react non-enzymatically with the amino group of proteins at their N terminal, and with lipids and nucleic acids. The non-enzymatic reaction is known as glycation and is an outcome of excess glucose in the body. It starts with the carbonyl group of sugar reacting with the protein to form a Schiff base, an unstable intermediate (Oya *et al.*, 1999; Desai & Wu, 2007). The Schiff base later undergoes an arrangement to form a stable Amadori product and this stable Amadori product can undergo oxidation, degradation or rearrangement to form AGEs. This process is also known as the Maillard reaction or “browning reaction”, when amino acids form a brown coloured product while heated in the presence of reducing sugars (Maillard, 1916).

If Amadori products undergo oxidation then the end products formed are known as glycoxidation products such as AGEs like pentosidine and carboxymethyl-lysine (CML) (Ahmed *et al.*, 1986; Sell & Monnier, 1989). Amadori rearrangement and sugar molecule fragmentation leads to the formation of reactive intermediate products known as α -dicarbonyls or oxoaldehydes and include 3-deoxyglucose (3-DG), MG and glyoxal. The accumulation of the dicarbonyls leads to carbonyl stress causing damage and oxidative stress in the body (Chellan & Nagaraj, 1999).

These AGEs affect the normal function of various proteins and enzymes by forming covalent cross-links between proteins (Reddy *et al.*, 1995) and thus lead to severe damage including diabetic retinopathy, nephropathy and aging.

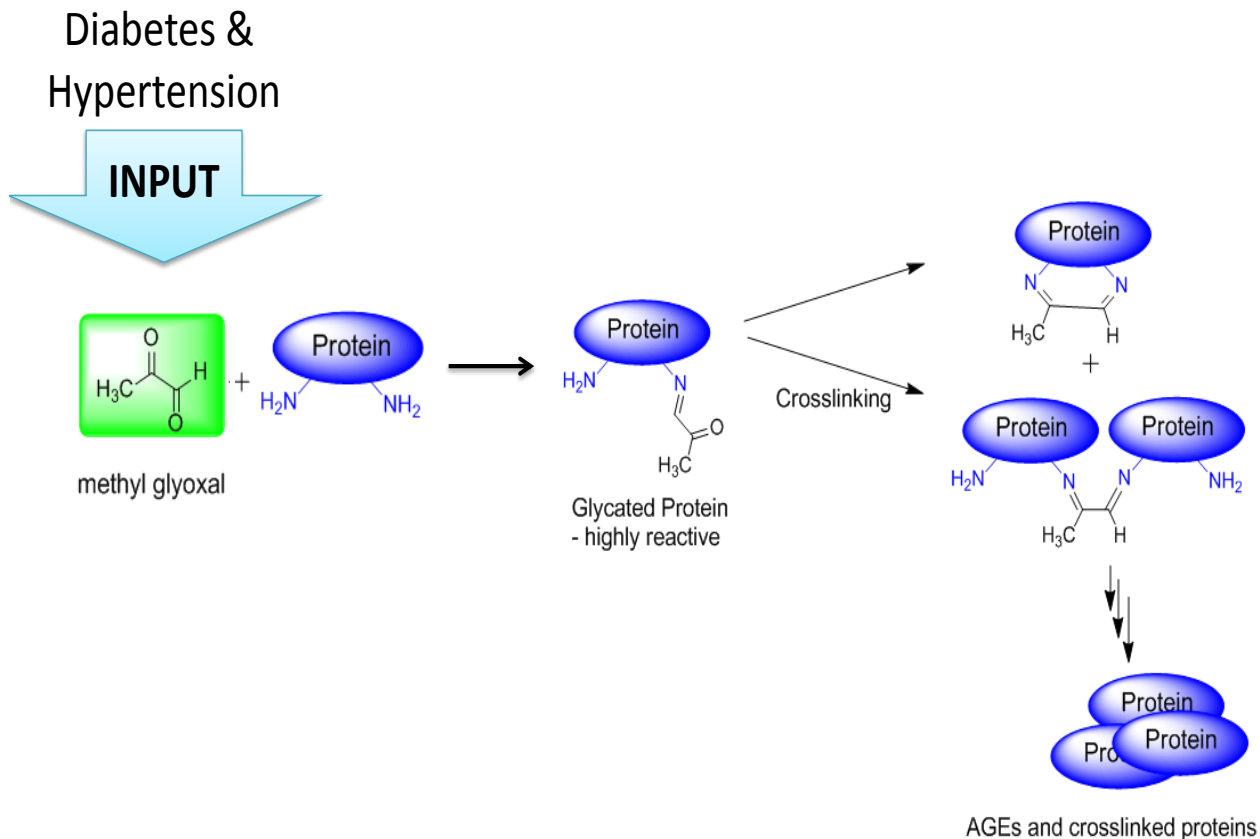


Figure 1-5. Pathway of AGEs formation by MG.

MG can also lead to AGEs formation. MG and two other oxoaldehydes (3-DG and glyoxal) are considered to be the major source of intracellular AGEs formation. The non-enzymatic reaction of MG, not degraded by the glyoxalase system, with arginine or lysine residues of proteins forms irreversible AGEs, leading to cross linking and denaturation of proteins. The irreversible reaction of MG with arginine forms the AGEs, hydroimidazolone, N ϵ -(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine and argpyrimidine, whereas the reaction between MG and lysine forms the AGEs N ϵ -carboxyethyllysine (CEL) and N ϵ -carboxymethyllysine (CML). Carbonyl precursors (MG, 3-DG and glyoxal) also form non-oxidative AGEs, which are non-fluorescent, such as methylglyoxal-lysine dimer (MOLD),

glyoxal-lysine dimer (GOLD) and deoxyglucosone-lysine dimer (DOLD). These AGEs can be detected by HPLC, ELISA and immunohistochemistry (Desai & Wu, 2007).

1.2.e MG and Oxidative Stress

Oxidative stress is the imbalance between reactive oxygen species (ROS) production and antioxidant defense mechanisms in the body, caused by either excess free radical or ROS generation or impaired anti-oxidant enzymes. Free radicals are atoms or molecules with an unpaired electron in the outer orbit. Oxygen derived free radicals such as superoxide anion, hydroxyl radical, and non-radicals such as hydrogen peroxide that do not have an unpaired orbiting electron, are known as reactive oxygen species (ROS) (Desai & Wu, 2008). ROS are chemically highly reactive.

The role of MG (Fig 1-6) in inducing oxidative stress in various cell types is well known. MG activates several pathways to increase oxidative stress. MG, both directly and *via* AGEs formation, increases the activity of p38 MAPK and expression of JNK. The increased activity of these enzymes increases the proinflammatory cytokines, such as interleukin-1 β , interleukin 6, interleukin 8 and tumor necrosis factor (Kikuchi *et al.*, 1999). MG has also been shown to increase the superoxide anion, hydrogen peroxide and peroxynitrite formation, and prooxidant enzyme activity such as NADPH oxidase in various cell types such as VSMCs, endothelial cells, rat kidney mesangial cells, rat hepatocytes, platelets, SH-SY5Y neural cells. Incubation of VSMCs with 25 mM glucose or fructose for 3 h increases MG production 3.5 or 3.9 fold, respectively, and increases oxidative stress (Dhar *et al.*, 2008). Excess superoxide anion so

formed can react with nitric oxide (NO) to form peroxynitrite which is a strong oxidant and nitrating agent (Takahashi *et al.*, 2010; Dhar *et al.*, 2008; Chen & Wu, 2008).

MG can also increase oxidative stress by reducing antioxidants such as glutathione peroxidase, glutathione reductase and GSH. Glutathione peroxidase detoxifies hydrogen peroxide to water with the help of GSH, which is converted to reduced glutathione and then back to GSH with the help of glutathione reductase. Thus, reduced availability of anti-oxidants impairs MG detoxification and begins a cycle which further causes oxidative damage (Blakytyn and Harding, 1992; Paget, 1998). MG-modified albumin treatment in monocytes resulted in increased ROS production, thus suggesting that MG, by activating monocytes, induced apoptosis of neutrophils, caused platelet–neutrophil aggregation, then thrombosis and inflammation (Desai & Wu, 2008).

The major source of energy production and major site of ATP in the cells is the mitochondrion, which is also a major source of free radical generation especially superoxide anion. The intracellular superoxide anion is formed when single electrons escape the mitochondrial chain reaction, mainly from complex I and complex III and interact with molecular oxygen to form superoxide anion. In *in vitro* studies, MG has been shown to increase superoxide anion production. Rat aortic VSMCs treated with MG (30 μ M) significantly increased superoxide anion production, which was prevented by the AGEs breaker, alagebrium (50 μ M) (Chang *et al.*, 2005; Wang *et al.*, 2008b). Recently, we have shown that VSMCs and endothelial cells treated with MG (30 – 100 μ M) for 24 h significantly increased ROS production in a time dependent manner which was prevented by arginine (Dhar *et al.*, 2012).

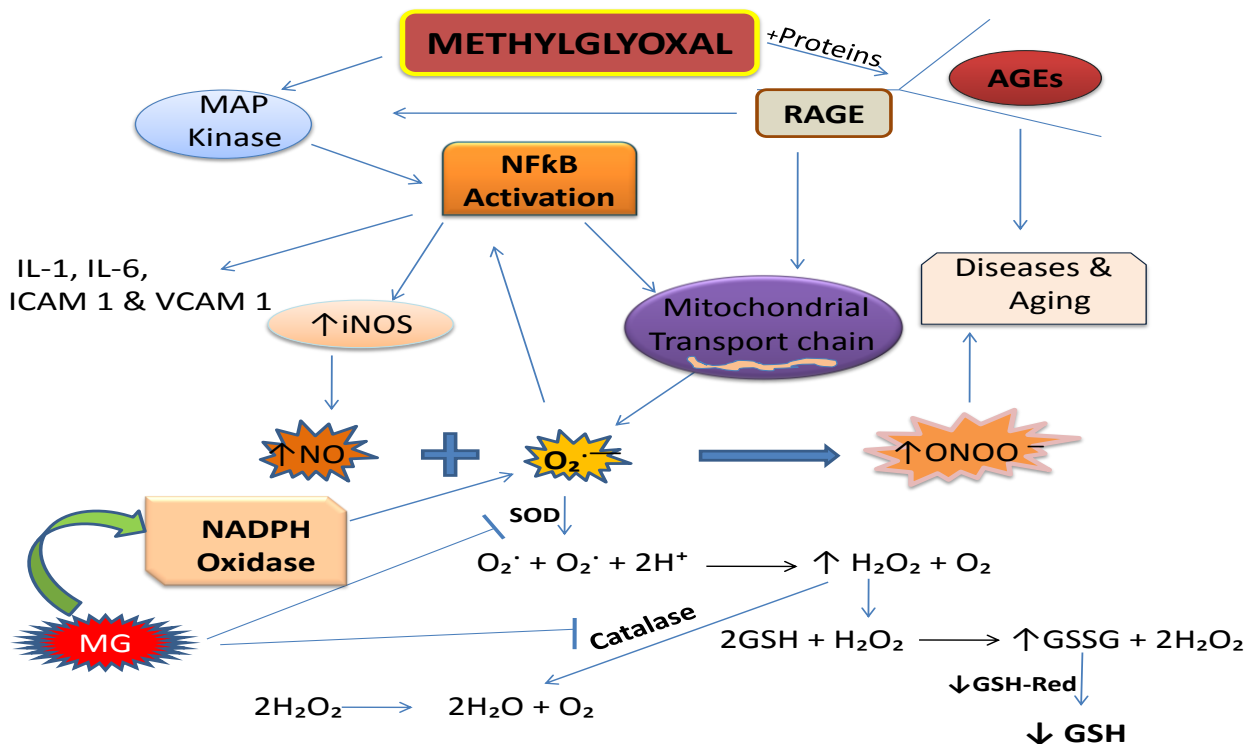


Figure 1-6: Mechanisms of MG induced oxidative stress. Adapted from *Drug Metabol Drug Interact.* 2008;23(1-2):151-73.

MG induces oxidative stress through multiple mechanisms and pathways as shown in the figure. *Abbreviations:* AGEs, advanced glycation end products; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; GSH-Red, glutathione reductase; GSSG, oxidized glutathione; H₂O₂, hydrogen peroxide; ICAM-1, intercellular adhesion molecule-1; IFN γ , interferon γ ; IL-1, interleukin-1; JNK, JUN N-terminal kinase; MG, methylglyoxal; NF- κ B, nuclear factor-kappaB; NO, nitric oxide; O₂^{•-}, superoxide anion; ONOO⁻, peroxynitrite; p38 MAPK, p38 mitogen activated protein kinase; RAGE, receptor for advanced glycation endproduct; SOD, superoxide dismutase; VCAM 1, vascular cell adhesion molecule 1.

In *in vivo* studies the effect of MG on oxidative stress is associative. For example, we have shown that elevated plasma and aortic MG levels in the aorta of 13 week old SHR with elevated blood pressure is associated with increased superoxide anion levels and significantly reduced GSH levels, and glutathione peroxidase and glutathione reductase activities, compared to age-matched WKY rats. In diseases such as diabetes and hypertension, the elevation of MG levels is associated with increased oxidative stress (Wang *et al.*, 2005). MG through AGEs formation can also cause oxidative stress. The excess of CEL, CML, and argpyrimidine formed by reaction of MG with lysine and arginine, are associated with oxidative stress. These AGEs and glycated proteins induce oxidative stress through production of cytokines and growth factors. Moreover, AGEs interact with the receptor for AGEs (RAGE) to induce oxidative stress in various cell types such as VSMCs, endothelial cells and phagocytes. AGE-RAGE interaction in the endothelial cells increases NF- κ B activity to increase oxidative stress (Desai & Wu, 2008).

1.2.f MG and Endothelial Dysfunction

L-arginine (L-Arg), but not D-arginine (D-Arg), is a substrate for nitric oxide synthase (NOS), which catalyzes the formation of nitric oxide (NO) and L-citrulline (Palmer *et al.*, 1988). There are three isoforms of NOS, endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) (Moncada *et al.*, 1991). eNOS is mainly found in the endothelial cells and mediates endothelium-dependent agonist induced vessel relaxation (Moncada *et al.*, 1991). Reduced production or availability of NO is a common feature of endothelial dysfunction (De Vriese *et al.*, 2000; Potenza *et al.*, 2009), which is commonly defined as reduced endothelium-dependent vascular relaxation, and is a feature of aging, as well as diabetes, atherosclerosis,

hypertension and several other conditions (Berkowitz *et al.*, 2003; Zhanget *al.*, 2001). Arginase is an enzyme of the urea cycle that converts L-Arg, but not D-Arg, to urea and ornithine. There are two isoforms, arginase I and arginase II (Haraguchi *et al.*, 1987; Morris *et al.*, 1997). Arginases are expressed in the endothelium (Wei *et al.*, 2000) and can cause endothelial dysfunction (Zhang *et al.*, 2001; Berkowitz *et al.*, 2003). High glucose (25 mM) has been shown to increase arginase I activity, but not protein expression, in bovine coronary endothelial cells, and arginase I is increased in the aorta of streptozotocin diabetic rats (Romero *et al.*, 2008), causing endothelial dysfunction (Ishizaka *et al.*, 2007; Romero *et al.*, 2008).

We have recently shown that in cultured rat aortic and human umbilical vein endothelial cells MG and high glucose reduced basal and bradykinin-stimulated NO production, cyclic guanosine monophosphate levels, and serine-1177 phosphorylation and activity of eNOS without affecting threonine-495 and Akt phosphorylation, and total eNOS protein (Dhar *et al.*, 2010b). Evidently, safe and specific MG scavengers have the potential to prevent several different pathological conditions such as endothelial dysfunction and AGEs formation. Currently, there is a lack of safe and specific MG scavengers. Several investigators have used different compounds as MG scavengers, which have other actions and thus are non-specific and can even produce toxicity. These compounds include aminoguanidine (Brownlee *et al.*, 1986; Edelstein *et al.*, 1992).

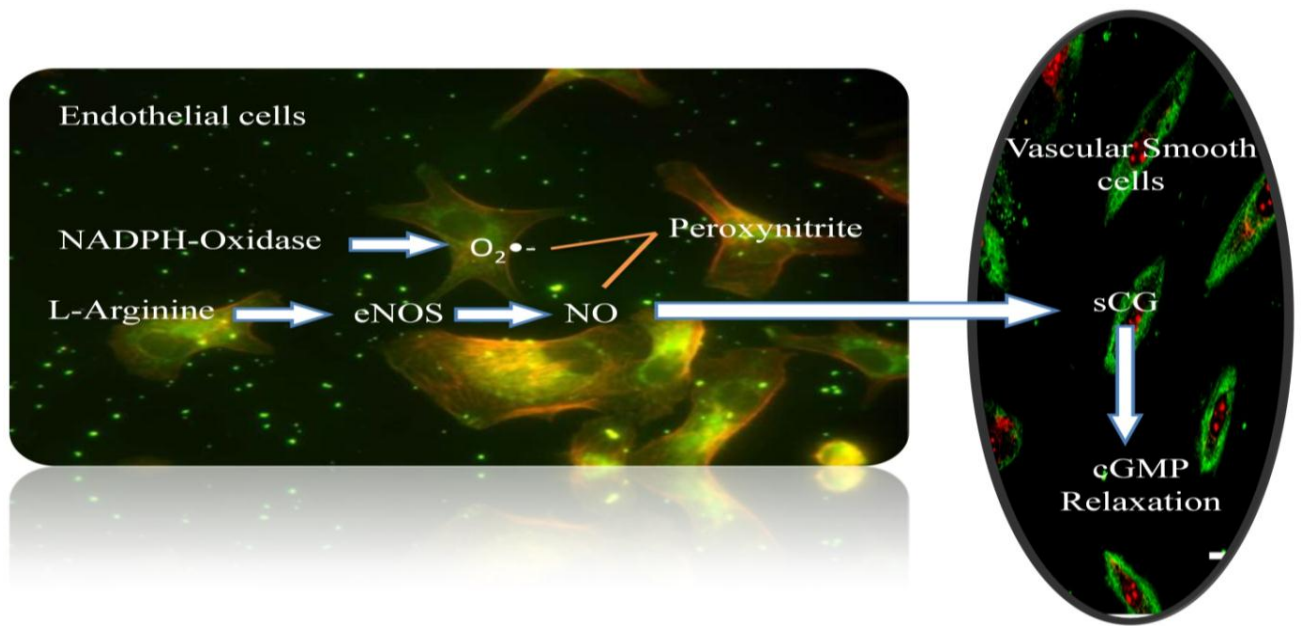


Figure 1-7. Mechanism of nitric oxide mediated endothelium-dependent relaxation and endothelial dysfunction. Adapted from *Biochem Pharmacol.* 1989;38(11): 709-15.

The figure (Fig 1-7) shows an endothelial cell and the adjacent VSMC. Stimulants like shear stress, bradykinin, insulin and vascular endothelial growth factor (VEGF) activate eNOS by causing an increase in intracellular calcium (Ca^{2+}) and produce NO from the substrate L-Arg. NO diffuses to the VSMC, activates soluble guanylate cyclase (sGC), increases cyclic guanosine monophosphate (cGMP), reduces intracellular calcium and causes relaxation (Evora and Nobre, 1999). Under pathological conditions like diabetes and hypertension, increased superoxide anion ($\text{O}_2\bullet^-$) is produced, mainly from NADPH oxidase, which reacts with NO and forms peroxynitrite. This in turn reduces the bioavailability of NO and causes endothelial dysfunction. Reduced eNOS activity can also cause reduced NO production (Endemann and Schiffrin, 2004).

1.2.g MG Scavengers

The MG scavengers available are also known as anti-MG or anti-AGE and are usually non-specific and have multiple effects, which limits their efficacy. Some of the common MG scavengers are aminoguanidine, metformin, N-acetyl cysteine, alagebrium and arginine.

Aminoguanidine

Aminoguanidine (AG) is the most commonly used MG scavenger and AGEs formation inhibitor (Brownlee *et al.*, 1986). It reacts with the carbonyl group of glucose and prevents it from reacting with proteins and thus prevents AGEs formation. It also neutralizes reactive aldehydes such as MG, glyoxal and 3-DG which are the major precursors of AGEs formation, thus acting as a scavenger (Brownlee *et al.*, 1986; Edelstein *et al.*, 1992). Aminoguanidine is a hydrazine derivative like hydralazine and has many properties similar to hydrazine (Nilsson, 1999). AG is non-specific and besides acting as an AGEs inhibitor and MG scavenger it has other effects as well. AG prevents NO and MG production in the body by inhibiting NOS, which catalyzes the conversion of L-Arg to NO (Moncada *et al.*, 1991), and SSAO, an enzyme which catalyzes the conversion of aminoacetone to MG, respectively. AG prevents deamination of diamines such as histamine by potently inhibiting diamine oxidase, thereby raising histamine concentration (Schuler 1952; Evy *et al.*, 1956). AG increases the synthesis of polyamines by binding to the enzyme S-adenosylmethionine decarboxylase (Stjernborn & Persson, 1993). AG can cause vitamin B6 deficiency by binding to pyridoxal, leading to its adverse effects (Miyata & Van, 2003). AG has an inhibitory effect on AGEs formation as well as other mixed effects as shown in various *in vitro* and *in vivo* studies (Brownlee *et al.*, 1986; Edelstein *et al.*, 1992; Sajithlal *et al.*, 1994; Conman *et al.*, 1998). The *in vivo* dose of AG used in rodents ranges from

25 mg/kg/day (Brownlee *et al.*, 1986; Seyer-Hansen *et al.*, 1991) to 100 mg/kg/day (Inoue *et al.*, 1998). Besides acting as an anti-oxidant *in vitro*, AG lowers total cholesterol, LDL and triglycerides in diabetic subjects. Studies have shown that AG reduces diabetes related complications like neuropathy, retinopathy and nephropathy in diabetic animal models. However, in clinical trials AG has shown safety concerns and unexpected results. In a clinical trial conducted on 690 patients with diabetic nephropathy, AG did not prevent the renal damage progression and did not show a statistically significant difference compared to the placebo group, apart from lowering serum creatinine (Bolton *et al.*, 2004; Freedman *et al.*, 1999). AG being like hydrazine, has many toxic effects. For example it may have effects like, antinuclear cytoplasmic antibody production, drug induced systemic lupus erythematosus and abnormal liver function. The patients treated with AG have flu-like symptoms, vasculitis and anaemia, thus limiting its therapeutic potential (Freedman *et al.*, 1999). Moreover, AG through hydrogen peroxide formation in the presence of Fe^{3+} can also cause DNA damage (Suji & Sivakami, 2006).

Metformin

Metformin is a commonly used drug for type 2 diabetes and comes under the class of biguanides. Chemically it is dimethylbiguanide and it is also a MG scavenger and AGEs formation inhibitor (Beisswenger *et al.*, 1999). One study has shown that metformin scavenges MG by neutralizing it, *via* its guanidine group, which actually binds to MG and forms the inactive product triazepinone (Beisswenger & Ruggiero-Lopez, 2003; Ruggiero-Lopez *et al.*, 1999; Baynes, 1991; Ota *et al.*, 2007). Furthermore, metformin by increasing MG detoxification also reduces MG levels. Due to its MG scavenging effect, type 2 diabetes patients treated with high doses of metformin, ranging from 1,500 to 2,500 mg have significantly reduced MG levels

(Beisswenger *et al.*, 1999). We have also shown that SD rats fed with a high fructose diet have significantly high levels of MG in the plasma, hydrogen peroxide and MG-derived AGE, CEL, in the aorta, and high BP, which were attenuated by metformin (Wang *et al.*, 2008) Also, in another study diabetic rats treated chronically with metformin had reduced AGEs formation in the lens, kidney and nerves (Tanaka *et al.*, 1999). However, its use as a MG scavenger and AGEs inhibitor is limited due to its multiple effects, and it is currently used for type 2 diabetes as an insulin sensitizing drug.

N-acetyl cysteine

N-acetyl cysteine (NAC) is a well-known anti-oxidant and a MG scavenger (Millea, 2009; Vasdev *et al.*, 1998). It is also known by other names such as acetylcysteine or N-acetyl-L-cysteine. NAC is the acetyl derivative of the amino acid cysteine, containing a thiol group, and MG has high binding affinity for cysteine (Vasdev *et al.*, 1998; Lauterberg *et al.*, 1983). NAC can also increase GSH levels (Lauterberg *et al.*, 1983), the prime MG scavenger and antioxidant (Jia *et al.*, 2006; Millea, 2009; Vasdev *et al.*, 1998); thereby further acting as a MG scavenger and an anti-oxidant. It is also sold as a food supplement for protecting against liver abnormalities. It is clinically used in the management of acetaminophen overdose and cough (Millea, 2009; Lauterberg *et al.*, 1983).

Alagebrium (ALA)

Alagebrium (ALA), also known formerly as ALT 711, is 4, 5-dimethylthiazolium (Wolffenbittel *et al.*, 1998) (Fig 1-8). ALA is an AGEs breaker and a MG scavenger. It acts as an AGEs breaker by breaking the covalent cross-links and thus is believed to be more important compared to AGEs inhibitors which are ineffective against formed AGEs (Desai & Wu, 2007).

ALA also causes reduction in oxidative stress and profibrotic cytokines (Susic *et al.*, 2004). Moreover, AGEs induced oxidative stress is inhibited by ALA (Vasan *et al.*, 2001). ALA has several beneficial actions on aging as well as on pathological conditions such as increased ventricular and arterial stiffness as observed in different animal models (Asif *et al.*, 2000). ALA decreased ventricular stiffness and improved cardiac function in aged dogs treated with ALA for one month (Asif *et al.*, 2000). ALA (10 mg/kg for 16 weeks) reduced oxidative stress and increased enzyme activity of glutathione peroxidase and superoxide dismutase in aging rats (Guo *et al.*, 2009). Altered cardiac function was improved in older rhesus monkeys treated with ALA (Vaitkevicius *et al.*, 2001). Clinical studies conducted on ALA showed it to be safe and revealed favourable results. ALA improved arterial compliance in a clinical study involving elderly patients with vessel stiffness, high systolic blood pressure and large artery compliance (Kass *et al.*, 2001). In addition, other clinical studies showed that ALA reduced vascular fibrosis in elderly patients with systolic hypertension (Zieman *et al.*, 2007) and decreased left ventricular mass in patients with diastolic heart failure (Little *et al.*, 2005; Bakris *et al.*, 2004).

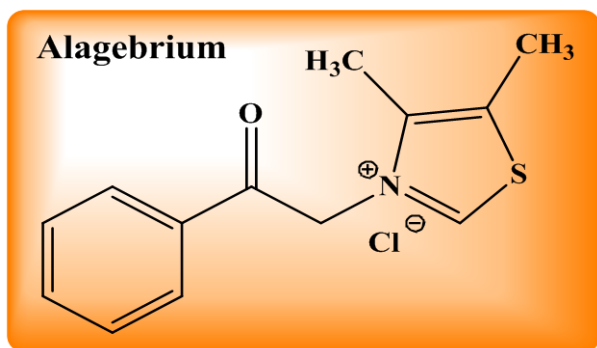


Figure 1-8 Chemical structure of alagebrium.

Increased DNA deletion, AGEs formation, reduced antioxidant capacity in aging heart and decreased superoxide dismutase activity and glutathione peroxidase activity in both aging heart and cardiomyocytes were all reversed on treatment with ALA, which also improved cardiac function (Guo *et al.*, 2009). Furthermore, ALA does not inhibit thiamine diphosphokinase, which catalyses deamination of thiamine, and it therefore does not interfere with thiamine metabolism (Krautwald *et al.*, 2011).

ALA also has acute preventive effect against MG as shown in our study in cells and SD rats. In SD rats administered MG (tested after 6 h exposure to MG), ALA significantly attenuated increased MG levels in the plasma and different organs and also attenuated MG-induced glucose intolerance. Moreover, ALA attenuated the increased MG levels in cells treated with MG, indicating its acute MG scavenging effect (Dhar *et al.*, 2010).

Arginine

Arginine (Fig 1-9) is an amino acid and exists in two isomeric forms, L-Arg and D-Arg. L-arginine is a substrate for several enzymes including nitric oxide synthase (NOS), which converts it into NO and L-citrulline (Palmer *et al.*, 1988). NO is a physiological mediator and a neurotransmitter with several physiological and pathological functions such as vascular and gastrointestinal smooth muscle relaxation, immune response, excessive vasodilation in septic shock etc (Moncada *et al.*, 1991). The enzyme arginase uses L-arginine to produce ornithine and enable ammonia detoxification through the urea cycle (Haraguchi *et al.*, 1987; Morris *et al.*, 1997; Morris, 2009). L-arginine, alongwith glycine, is also converted by arginine:glycine amidinotransferase to ornithine and guanidinoacetate, the latter is then metabolized to creatine (Levillain, 2012; Van Pilsum *et al.*, 1972). This helps with Na/K ATPase activity,

neurotransmitter release and other functions in the central nervous system. The enzyme arginine decarboxylase uses L-arginine as a substrate to produce agmatine, which is further converted to putrescine and urea by agmatinase (Satriano, 2003; Grillo & Colombatto, 2004). Thus, L-arginine is a part of several metabolic pathways.

L-arginine supplements have been reported to be useful in various conditions (Coman *et al.*, 2008; Tapiero *et al.*, 2002) including in preterm infants (Wu *et al.*, 2004). It has been used for improving endothelium-dependent vasodilation in humans with hypercholesterolemia (Creager *et al.*, 1992; Clarkson *et al.*, 1996) and to lower the blood pressure in hypertension (Dong *et al.*, 2011; Rajapakse *et al.*, 2008). It was shown to reduce AGEs in diabetic rats (Pai *et al.*, 2010). D-arginine has been reported to have no significant toxicity in mice besides having some non-specific effects (Navarro *et al.*, 2005).

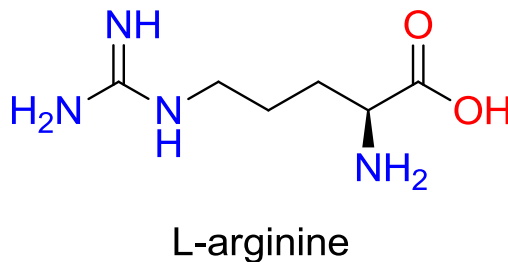


Figure 1-9 Chemical structure of L-arginine.

Due to its high affinity for MG, arginine can have great value as a safe MG scavenger. Since L-Arg is a substrate for 5 different enzymes, D-Arg can be a more specific and effective MG scavenger.

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

2.1 Rationale for hypothesis

MG is a reactive aldehyde produced mainly from glucose and fructose in the body. Plasma MG levels are elevated in conditions of hyperglycemia. An excess of MG, not degraded by the glyoxalase enzymes, reacts with exposed arginine, lysine and cysteine residues of proteins and enzymes and causes pathology. An ongoing investigation of proteins, enzymes and other macromolecules modified by MG is revealing the pathological effects of excess MG. For example we have recently shown that treatment of cultured rat aortic and human umbilical vein endothelial cells with MG or high glucose reduces eNOS activity and causes endothelial dysfunction (Dhar *et al.*, 2010b). We have also shown that administration of MG for 4 weeks as a continuous infusion by a subcutaneous minipump induces features of type 2 diabetes in male SD rats (Dhar *et al.*, 2011).

Dr Wu's lab investigated two animal models which have hypertension, the SHR and high fructose diet fed rats (Hwang *et al.*, 1987), and found that the plasma and tissue levels of MG were significantly elevated by 2-4 fold in both of these animal models, compared to their respective control groups (Wang *et al.*, 2005; Wang *et al.*, 2008). However, elevated plasma and tissue levels of MG under hypertensive condition were associative findings in these studies and did not establish a cause-effect relationship. In order to establish a direct role of MG in causing hypertension, it is necessary to administer MG exogenously in healthy animals and observe its effect on the BP and on body systems known to regulate the BP such as the RAAS and the sympathetic nervous system. The *in vivo* effects of exogenously administered MG have not been studied to evaluate its role in the pathogenesis of hypertension. Based on this rationale I used exogenous administration of MG in normal male SD rats in this project. The results obtained by

exogenous administration of MG can be corroborated by studies in which endogenous production of MG is increased. One way of increasing endogenous MG production in the body is by feeding animals with a high carbohydrate diet, either a high glucose or a high fructose diet, both of which are known precursors of MG formation (Dhar *et al.*, 2008). Moreover, high carbohydrate diet fed animals are known to develop hypertension (Hwang *et al.*, 1987; Kaufman *et al.*, 1991; Nakagawa *et al.*, 2006; Sanchez-Lozada *et al.*, 2007; Tom *et al.*, 2011; Wang *et al.*, 2008). Therefore, I also used high fructose diet fed male SD rats in my project to study the role of endogenous MG in hypertension and on the body systems regulating the BP such as the RAAS.

The effects of an exogenous or endogenous compound in the body can be additionally confirmed by the use of co-treatment with a known inhibitor of the compound, which should attenuate or prevent the effects of the compound. Studies involving MG become limited by lack of availability of specific MG scavengers. Some of the MG scavengers used by researchers include aminoguanidine (Brownlee *et al.*, 1986; Edelstein *et al.*, 1992), N-acetyl cysteine (Jia *et al.*, 2006; Millea, 2009; Vasdev *et al.*, 1998), metformin (Beisswenger *et al.*, 1999; Beisswenger & Ruggiero-Lopez, 2003; Ruggiero-Lopez *et al.*, 1999; Baynes, 1991; Ota *et al.*, 2007) and alagebrium (Dhar *et al.*, 2010). However, none of these are specific as MG scavengers, and aminoguanidine also has toxic effects (Freedman *et al.*, 1999; Suji & Sivakami, 2006). One of the aims of my project was to identify a novel specific and safe MG scavenger. MG has great affinity for arginine (Takahashi, 1977; Lo TW *et al.*, 1994). As discussed in the introduction, L-arginine is a substrate for at least five different enzymes in the body so it can have other effects when it is used as a MG scavenger. On the other hand D-arginine is not a substrate for

those enzymes and it may act specifically as a MG scavenger only with no or minimal other effects. It is also possible that L-arginine, besides scavenging MG can be an attractive oral supplement due to its reported beneficial effects on endothelial dysfunction and the BP (Creager *et al.*, 1992; Clarkson *et al.*, 1996; Dong *et al.*, 2011; Rajapakse *et al.*, 2008).

Based on the above rationale the following hypotheses were formed:

2.2 Hypotheses:

- (i) A pathological elevation of methylglyoxal levels in the body, resulting from exogenous administration, or produced from a high fructose diet, upregulates the renin angiotensin aldosterone system and causes hypertension.**
- (ii) Arginine can scavenge methylglyoxal and attenuate its deleterious effects on endothelial function and oxidative stress.**

2.3 Objectives and experimental approach:

2.3.a To investigate whether exogenous MG, administered by a continuous infusion for 4 weeks, is a causative factor in the pathogenesis of hypertension: A study of the underlying signaling pathways and molecular mechanisms in Sprague-Dawley rats and cultured cells.

I performed experiments to determine whether MG is one of the causative factors in the development of hypertension. Since the RAAS plays a major role in the regulation of BP, I focused my attention on the RAAS. Ang II acts on VSMC AT₁ receptors to cause vasoconstriction, therefore I also looked at the expression of AT₁ receptors. Since Ang II also facilitates peripheral noradrenergic neurotransmission and releases catecholamines from the adrenal medulla, I also determined the expression of adrenergic α_{1D} receptors.

The effects of MG, and alagebrium, a MG scavenger, on the RAAS and the BP were investigated in 12 week old male SD rats. SD rats were treated with a continuous infusion of MG with a subcutaneously implanted minipump for 4 weeks. Organs / tissues and cultured VSMCs were used for molecular studies. HPLC was used to measure MG levels, western blotting was used to measure proteins for adrenergic α_{1D} receptor, AT₁ receptor, angiotensin, renin, Erk 1/2 (p-Erk 1/2), NFATc and NFkB. Q-PCR was used to measure mRNA levels for α_{1D} receptor, AT₁ receptor, angiotensin and renin in cultured cells, kidney and aorta of MG treated rats. Possible mechanisms were explored through gene silencing of angiotensinogen and RAGE.

2.3.b To investigate the effects of a high fructose diet, a precursor for MG formation, on the pathogenesis of hypertension and its attenuation by a MG scavenger: A study of the underlying signaling pathways and molecular mechanisms in Sprague-Dawley rats.

High fructose corn syrup and sugar are the main ingredients of a high carbohydrate diet, which is believed to be the root cause of the current epidemic of obesity, type 2 diabetes and the associated cardiovascular pathology. The aim of my study was to investigate whether MG is a

mediator of fructose-induced hypertension and the underlying molecular mechanisms, since fructose is a known precursor of MG generation (Dhar *et al.*, 2008). Male SD rats were treated with a high fructose diet (60% of total calories) for 16 weeks. The thoracic aorta and cultured VSMCs were used to study the signaling pathways and the molecular mechanisms. HPLC was used to measure MG and GSH levels, western blotting was used to measure proteins for adrenergic α_{1D} receptor, AT₁ receptor, angiotensin, renin, Erk 1/2 (p-Erk 1/2) and NFATc. Q-PCR was used to measure mRNA levels for α_{1D} receptor, AT₁ receptor, angiotensin, renin in the kidney and the aorta of fructose treated rats.

2.3.c To investigate whether arginine can attenuate methylglyoxal- and high glucose-induced endothelial dysfunction and oxidative stress in rat isolated aortic rings and cultured cells.

Specific and safe MG scavengers are not available. Since MG has great affinity for arginine (Takahashi, 1977; Lo TW *et al.*, 1994), I investigated whether L-arginine and D-arginine can attenuate MG and high glucose-induced endothelial dysfunction and oxidative stress. Aortic rings isolated from 12 week old male SD rats were used for contractility studies. Cultured human umbilical vein endothelial cells (HUVECs) and VSMCs were used to define molecular pathways and mechanisms. HPLC was used to measure MG levels and western blotting was used to measure proteins for NF- κ B, NADPH oxidase 4, arginase I and II. Dichlorofluorescein (DCF) probe was used to measure oxidative stress. Immunostaining was used to measure MG-induced AGE (CEL).

CHAPTER 3

GENERAL METHODOLOGY

3.1 Animals

Male 11-week old Sprague-Dawley rats from Charles River Laboratories (Saint-Constant, Quebec, Canada) were used according to a protocol approved by the Animal Care Committee at The University of Saskatchewan, following guidelines of the Canadian Council on Animal Care. After one week of acclimatization, the rats were anesthetized with an inhalation of isoflurane (Forane, 4% in oxygen), delivered through a precision vaporizer and circle absorption breathing system (Ohio, 30/70 Proportioner Anesthesia Machine, Madison, Wisconsin). When anesthesia reached sufficient depth as determined by the absence of the leg flexor response and the eyelid reflex, the thorax was quickly opened by a midline incision and the rat was killed by cutting open the heart and causing exsanguination, an accepted method of euthanasia by the Animal Care Committee. The aorta was quickly removed without damaging the endothelium and placed in a beaker filled with Krebs's solution and bubbled with 95% O₂ + 5% CO₂.

In one separate batch of 28 male 12 week old SD rats a subcutaneous mini-osmotic pump (Alzet® 2ML4) (Durect Corporation, Cupertino, CA, USA) was implanted to deliver either saline (0.9%) or MG (24 mg/day) by continuous infusion for 28 days. The rats were randomly divided into the following treatment groups ($n = 7$ in each group): 1. Control (0.9% saline by pump), 2. MG (24 mg/day by pump), 3. MG + alagebrium (ALA, 30 mg/kg/day in drinking water), 4. ALA (30 mg/kg/d in drinking water) + 0.9% saline by pump. In a second batch of 24 male 12 week old Sprague-Dawley rats a different MG scavenger, aminoguanidine, had to be used due to unavailability of ALA. The following treatments were provided to 4 groups ($n = 6$ each): 1. Control (0.9% saline by pump), 2. MG (24 mg/day by pump), 3. MG + aminoguanidine

(AG, 100 mg/kg/day in drinking water), 4. AG (100 mg/kg/d in drinking water) + 0.9% saline by pump

For high fructose diet study 32 male 5 week old Sprague-Dawley rats were divided into the following groups ($n = 8$ in each group): 1. Control chow, 2. High fructose (60% in diet), 3. High fructose + metformin (500 mg/kg/day in drinking water), 4. Metformin (500 mg/kg/day in drinking water + control chow). The treatments were for 16 weeks.

At the end of study fructose treated (16 weeks) and MG pump treated (28 days) rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight) which was given intraperitoneally. BP was again measured by carotid artery cannulation for 30 min. Blood was collected from the carotid artery and plasma was separated from the samples after centrifugation at 12,000 g for 10 min at 4°C. Tissues cleaned in ice-cold phosphate buffer saline were immediately frozen in liquid nitrogen and stored at -80°C until processing.

3.2 Cell culture

Rat thoracic aortic smooth muscle cell line (A-10 cells) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37° C in a humidified atmosphere of 95% air and 5% CO₂, as described in previous studies from our lab (Dhar *et al.*, 2008). A-10 cells were seeded either in 100 mm dishes for MG measurement or in 96-well plates for other assays, with an equal amount of cells (10⁶/mL) in each well, and cultured to confluence. For immunocytochemistry staining, cells were seeded on cover glass slides (2×10⁶/mL). Cells were starved in FBS-free DMEM for 24 h before exposure to different metabolic precursors.

Human umbilical vein endothelial cells from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Kaighn's F12K medium containing 10% fetal bovine serum (FBS), 0.1 mg/mL heparin and 0.03-0.05 mg/mL endothelial cell growth supplement (Dhar *et al.*, 2010b).

3.3 Methlyglyoxal assay

MG was measured by a specific and sensitive HPLC method as described previously (Dhar *et al.*, 2009). MG was derivatized with *o*-phenylenediamine (*o*-PD) to specifically form 2-methylquinoxaline. The samples were incubated in the dark for 24 h with 0.45 N perchloric acid (PCA) and 10 mM *o*-PD at room temperature. Samples were centrifuged at 12000 rpm for 10 min. 2-methylquinoxaline and quinoxaline internal standard (5-methylquinoxaline) were quantified on a Hitachi D-7000 HPLC system (Hitachi, Ltd., Mississauga, ON, Canada) *via* Nova-Pak® C18 column (3.9×150 mm, and 4 µm particle diameter, Waters Corporation, Milford, MA, USA) (Dhar *et al.*, 2009).

3.4 Western blotting

Cell lysates were prepared as described earlier (Dhar *et al.*, 2010b; Dhar *et al.*, 2011) and the protein concentration in the supernatant was determined by the BCA protein assay (Sigma-Aldrich Canada Co., Oakville, ON, Canada). Aliquots of cell lysates (40-50 µg of protein each) were separated on 6-10% SDS-PAGE, electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, Canada), blocked with 5% nonfat milk in TBS-Tween buffer for 2 h at room temperature, and incubated overnight at 4° C

with the primary antibodies to arginase I, arginase II, NADPH oxidase 4 (NOX4), AT₁ receptor (AT₁R), α_{1D} receptor (α_{1D} R), renin, angiotensin, ACE, (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), receptor for AGEs (RAGE), nuclear factor κ B (NF- κ B) (Abcam, Cambridge, MA, USA), and anti- β -actin (diluted 1:1000) (Sigma-Aldrich Canada Ltd., Mississauga, ON, Canada) followed by incubation with horse radish peroxidase conjugated secondary antibodies (Life Science, Hercules, CA, USA) (diluted 1:3000) for 2 h at room temperature and then with horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at room temperature. After extensive washing, the immunoreactive proteins were detected with an Enhanced Chemiluminescence Detection System (ECL; Amersham Biosciences Corp., Piscataway, NJ, USA) (Dhar *et al.*, 2010b; Dhar *et al.*, 2011).

3.5 Real time Quantitative PCR (RT-PCR)

Total RNA from the cultured cells, kidney and aorta was isolated using RNA isolation kit (Qiagen, Germantown, MD, USA). The pre-designed primers for angiotensin, AT₁R, α_{1D} R, renin, aldosterone receptor (MR) and RAGE were purchased from Qiagen, (Germantown, MD, USA). The real-time PCR was performed in an iCycler iQ apparatus (Life Science, Hercules, CA, USA) associated with the ICYCLER OPTICAL SYSTEM software (version 3.1) using SYBR Green PCR Master Mix (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, Canada).

3.6 Arginase activity assay

Arginase activity was measured using an arginase assay kit (BioAssay Systems, Hayward, CA, USA). Arginase catalyses the conversion of arginine to ornithine and urea.

Briefly cell lysates were mixed with substrate arginine buffer and incubated for 2 h. Urea reagent was added to stop the reaction and optical density (OD) was measured at 430 nm.

3.7 Measurement of reactive oxygen species

Confluent cells were loaded with a membrane-permeable, nonfluorescent probe 2',7'-dichlorofluorescein diacetate (CM-H2DCFDA, 5 μ M) for 2 h at 37° C in FBS-free medium in the dark. The cells treated with MG (30 μ M) or glucose (25 mM) for 24 h were assayed for fluorescent oxidized dichlorofluorescein (DCF) as an indicator of production of ROS as described earlier (Dhar *et al.*, 2008; Dhar *et al.*, 2010b). The protein content of the homogenate was measured by BCA Protein assay (Bio-Rad, Hercules, CA, USA).

3.8 Immunocytochemistry

A-10 cells were seeded on glass cover slips followed by incubation with different test compounds for 24 h, and subjected to staining for the MG-induced AGE, N ϵ -carboxyethyl lysine (CEL). As described previously (Dhar *et al.*, 2008; Dhar *et al.*, 2011), the treated cells were fixed in 4% paraformaldehyde for 30 min at room temperature and washed twice with 0.01 N phosphate buffered saline (PBS). After permeation with 0.1% Triton X-100 for 5 min and two washes with PBS, the cells were incubated with normal goat serum (diluted 1:30 in 0.1 N PBS) for 1 h to block non-specific binding sites. After shaking off the goat serum the slides were incubated with the CEL antibody (1:100; a generous gift from Novo Nordisk, A/S, Denmark) overnight at room temperature. Cells were washed twice in PBS (0.01 N) for 5 min and incubated with secondary fluorescein isothiocyanate (FITC) conjugated anti-CEL antibody

(Molecular Probes) for 2 h. After washing thrice with PBS the slides were mounted in glycerol:PBS (3:7), coverslipped and observed under a fluorescence microscope. Staining intensity was quantified using the Metamorph image analysis software (v. 7, Molecular Devices). Slides from four different experiments were analyzed with 5 fields per slide observed and averaged.

3.9 Isometric tension studies on aortic rings

A group of 24 SD rats was used. Isometric tension studies were carried out on rat aortic rings as described (Dhar *et al.*, 2010b). Briefly, 3-4 mm thoracic aortic rings from the rat aorta were mounted under a 2 g load in four separate 10 mL organ baths containing Krebs solution with 5 mM glucose, maintained at 37° C and bubbled with 95% O₂ + 5% CO₂. After a 90 min equilibration period the rings were pre-contracted with phenylephrine (1 μM) and cumulative concentration-dependent relaxation in response to acetylcholine (ACh) was obtained before (Control) and 2 h after incubation with either glucose (25 mM) or MG (100 μM) (Dhar *et al.*, 2010b), or the nitric oxide synthase inhibitor, N ω -nitro-L-arginine methyl ester (L-NAME, 10 μM). In initial experiments the responses to ACh were repeated before and 2 h after incubation with normal Krebs solution to confirm reproducibility of responses to ACh. Some sets of rings were co-incubated with either L-Arg (300 μM) or D-Arg (300 μM) for 2 h. Treatment with each compound was tested in rings from at least 5 different rats. Isometric tension was measured with isometric force transducers with the 'Chart' software and Powerlab equipment (AD Instruments Inc., Colorado Springs, CO, USA).

3.10 Plasma assays

Assay kits for quantitative determination of plasma renin (AnaSpec, Fremont, CA, USA), angiotensin II (Biovendor LLC, Candler, NC, USA), aldosterone (Cayman Chemical Co., AnnArbor, MI, USA), and catecholamines (Rocky Mountain Diagnostics Inc., Colorado Springs, CO, USA) were used according to the manufacturer's instructions.

3.11 Short interfering (siRNA) transfection

In a six well tissue culture plate, 2×10^5 cells per well were grown in 2 mL antibiotic-free normal growth medium supplemented with FBS. Cells were incubated at 37° C in a CO₂ incubator until 60-80% confluent. For each transfection, 2-8 µL of siRNA duplex (Solution A, 0.25-1 µg or 20-80 pmols siRNA) was diluted with 100 µL siRNA transfection medium (Cat # sc-36868, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). For each transfection, 2-8 µL of siRNA transfection reagent (Solution B, Cat # sc-29528, Santa Cruz Biotechnology Inc.) was diluted with 100 µL siRNA transfection medium (Cat # sc-36868). siRNA duplex solution (Solution A) was directly added to the dilute transfection reagent (Solution B) using a pipette. The solution was mixed gently by pipetting up and down, following which it was incubated for 15-45 min at room temperature. The cells were washed once with 2 mL of siRNA transfection medium (Cat # sc-36868). For each transfection, 0.8 mL siRNA transfection medium was added to each tube containing the siRNA transfection reagent mixture (Solution A + Solution B). After gentle mixing the mixture was added to the washed cells, and incubated for 5-7 h at 37° C in a CO₂ incubator. After the required incubation, 1 mL of normal growth medium containing 2 times the normal serum and antibiotics concentration (2x normal growth medium) was added without

removing the transfection mixture. The cells were additionally incubated for another 18-24 h. After 24 h, the medium was aspirated and replaced with fresh 1x normal growth medium. The cells were assayed 48-72 h later after the addition of fresh medium.

3.12 Measurement of reduced glutathione (GSH)

The reduced glutathione (GSH) levels in tissues was determined by derivation with 5, 5'-dithio-bis (2-nitrobenzoic acid), and reverse-phase HPLC using ultra-violet detection (Kamencic *et al*, 2000). An equal volume of 10% 5-sulfosalicylic acid containing 0.2 mM EDTA was added to the tissue or cell homogenate to precipitate the proteins. After centrifugation at 10,000 g for 15 min the supernatants were collected for GSH analysis. The reaction mixture was made by mixing 0.5 mL 50 mM Tris-HCl buffer (pH 8.9), 0.13 mL sample or standard, 0.02 mL internal standard [400 μ M D(2)-penicillamine in cold 5% sulfosalicylic acid containing 0.1 mM EDTA], and 0.35 mL 10 mM 5,5-dithiobis(2-nitrobenzoic acid) made in 0.5 mM K_2HPO_4 (pH 7.2). After 5 min incubation, the mixture was acidified with 0.05 mL 7 M H_3PO_4 . 50 μ l of the mixture was injected for elution at 37°C using a Hitachi D-7000 HPLC system (Hitachi, Ltd., Mississauga, ON, Canada) *via* Nova-Pak® C18 column (150 x 4.6 mm, 4 μ m particle diameter, Waters Corporation, Milford, MA, USA). The mobile phase was made by mixing 12.5% methanol (v/v) and 100 mM KH_2PO_4 (pH 3.85). The flow rate was set at 1.1 mL/min. Sulfhydryl-DTNB derivatives were detected by ultraviolet absorbance at 330 nm. Standards contained L-cysteine, cysteinyl glycine, glutathione, and DL-homocysteine.

Publications and their relation to the thesis

The results of this thesis have been published as three manuscripts.

Chapter 4 describes the effects of exogenously administered MG on the RAAS and BP. These experiments were carried out to investigate our hypothesis (i) that a pathological elevation of MG, administered exogenously or produced from a high fructose diet in the body, causes hypertension. The experimental protocols were designed in accordance with objective 2.2.a described in chapter 2. The roles of the authors listed in this manuscript were as follows: Indu Dhar – I performed all the experiments, helped to design the experimental protocols and suggested some ideas to the supervisor. I wrote the first draft of the manuscript and revised the manuscript as suggested by the supervisor, co-supervisor and peer reviewers. Arti Dhar – a senior graduate student and a postdoctoral fellow taught me some of the techniques and supervised me in the lab at the beginning. Lingyun Wu – my co-supervisor suggested some ideas, and provided critical inputs towards my work, manuscript and discussions. Kaushik Desai – my supervisor suggested the idea for the work and provided critical inputs towards my work, manuscript and discussions.

Chapter 5 describes the effects of a high fructose diet and the role of endogenously produced MG on the RAAS and BP. These experiments were carried out to investigate our hypothesis (i) that a pathological elevation of methylglyoxal, administered exogenously or produced from a high fructose diet in the body, causes hypertension. After showing the role of exogenous MG in up regulating the RAAS and increasing the BP, the next logical step was to see if endogenously produced MG had similar effects on the RAAS and the BP, as exogenously

administered MG. Fructose is a precursor for MG formation (Dhar *et al.*, 2008) and a major component of the high carbohydrate diets in humans. High fructose diet is believed to be responsible for the current epidemic of type 2 diabetes and the associated cardiovascular conditions (Johnson *et al.*, 2007; Selvaraju *et al.*, 2012; Stanhope, 2012). Therefore, rats were fed a high fructose diet, which has been shown previously to generate increased MG (Wang *et al.*, 2008). The experimental protocols were designed in accordance with objective 2.2.b described in chapter 2. The roles of the authors listed in this manuscript were as follows: Indu Dhar – I performed all the experiments, helped to design the experimental protocols and suggested some ideas to the supervisor. I wrote the first draft of the manuscript and revised the manuscript as suggested by the supervisor, co-supervisor and peer reviewers. Arti Dhar – a senior graduate student and a postdoctoral fellow taught me some of the techniques, and reviewed and suggested corrections for my manuscript. Lingyun Wu – my co-supervisor suggested some ideas, and provided critical inputs towards my work, manuscript and discussions. Kaushik Desai – my supervisor suggested the idea for the work and provided critical inputs towards my work, manuscript and discussions.

Chapter 6 describes the effects of arginine in preventing or attenuating the deleterious effects of MG. These experiments were carried out to investigate our hypothesis (ii) that arginine can scavenge MG and attenuate its deleterious effects on endothelial function and oxidative stress. One major problem we encountered in our project work was finding a specific, safe and effective MG scavenger to prevent its harmful effects. One of the principles of pharmacological experiments is to demonstrate that effects of a given agent A can be prevented by another agent B, which can be a specific antagonist, or a scavenger of agent A. This strategy is used to

establish a cause effect relationship and to discover new drugs. Since there are no safe and specific MG scavengers our intention was to determine whether arginine, an amino acid and a commonly used nutritional supplement, can act as an effective MG scavenger. This idea was based on the known affinity of arginine for MG (Takahashi, 1977). The experimental protocols were designed in accordance with objective 2.2.c described in chapter 2. The roles of the authors listed in this manuscript were as follows: Indu Dhar – I performed all the experiments and helped to design the experimental protocols. I wrote the first draft of the manuscript and revised the manuscript as suggested by the supervisor, co-supervisor and peer reviewers. Arti Dhar – a senior graduate student and a postdoctoral fellow taught me some of the techniques including isolated aortic ring experiments, and reviewed and suggested corrections for my manuscript. Lingyun Wu – my co-supervisor suggested some ideas, and provided critical inputs towards my work, manuscript and discussions. Kaushik Desai – my supervisor suggested the idea for the work and provided critical inputs towards my work, manuscript and discussions.

CHAPTER 4

Methylglyoxal, a reactive glucose metabolite, increases renin angiotensin aldosterone and blood pressure in male Sprague-Dawley rats

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Non-standard abbreviations: AGEs - advanced glycation endproducts; ALA – alagebrium; Erk
1/2 - extracellular signal related kinases 1/2; MG – methylglyoxal; NF-κB - nuclear factor kappa
B; RAAS - renin angiotensin aldosterone system; RAGE - receptor for AGEs; VSMCs - vascular
smooth muscle cells

4.1 Abstract

The majority of people with diabetes develop hypertension, along with increased activity of the renin angiotensin system. Methylglyoxal, a reactive glucose metabolite, is elevated in diabetic patients. We investigated the effects of methylglyoxal, and alagebrium, a methylglyoxal scavenger, on the renin angiotensin system and blood pressure. Male Sprague-Dawley rats were treated with a continuous infusion of methylglyoxal with a minipump for 4 weeks. Organs / tissues and cultured vascular smooth muscle cells (VSMCs) were used for molecular studies. HPLC, Western blotting and Q-PCR were used to measure methylglyoxal, proteins and mRNA, respectively. siRNA for angiotensinogen and the receptor for advanced glycation endproducts (RAGE) were used to study mechanisms. Methylglyoxal treated rats developed a significant increase in blood pressure and plasma levels of aldosterone, renin, angiotensin and catecholamines. Methylglyoxal level, and protein and mRNA for angiotensin, AT₁ receptor, adrenergic α_{1D} receptor and renin were significantly increased in the aorta and/or kidney of methylglyoxal treated rats, a novel finding. Alagebrium attenuated the above effects of methylglyoxal. Treatment of cultured VSMCs with methylglyoxal or high glucose (25mM) significantly increased cellular methylglyoxal, and protein and mRNA for NF- κ B, angiotensin, AT₁ and α_{1D} receptors, which were prevented by inhibition of NF- κ B, and by alagebrium. Silencing of mRNA for RAGE prevented the increase in NF- κ B induced by methylglyoxal. Silencing of mRNA for angiotensinogen prevented the increase in NF- κ B, angiotensin, AT₁ and α_{1D} receptors' protein. Methylglyoxal activates NF- κ B through RAGE and thereby increases renin angiotensin levels, a novel finding, and a probable mechanism of increase in blood pressure.

4.2 Introduction

Type 2 diabetes and hypertension are major global health issues. Two-thirds of the people with diabetes have hypertension [blood pressure (BP): systolic ≥ 140 mmHg or diastolic ≥ 90 mmHg] (Ferrannini *et al.*, 2012). The mechanism of the pathogenesis of hypertension in diabetes is not well established. The renin-angiotensin aldosterone system (RAAS) plays a major role in maintaining fluid balance, vascular tone and BP (Crowley *et al.*, 2012; Fournier *et al.*, 2012). Angiotensin II (Ang II) also increases activity of the sympathetic nervous system (Crowley *et al.*, 2012). Increased activity of the RAAS is seen in type 2 diabetics with hypertension (Hsueh *et al.*, 2011). Increased activity of the RAAS has also been proposed to be one of the etiologic factors for type 2 diabetes mellitus (Goossens, 2012; Luther *et al.*, 2011; Zhou *et al.*, 2012).

Glucose is one of the main precursors for the formation of methylglyoxal (MG), a reactive aldehyde (Cooper *et al.*, 1970; Dhar *et al.*, 2008). Normally, MG is rapidly degraded mainly by the glyoxalase enzymes with the help of reduced glutathione (GSH) (Cooper, 1984). In conditions of excess MG formation, such as hyperglycemia, the glyoxalase enzymes get overwhelmed and the excess MG reacts with other proteins and enzymes and disrupts their normal function (Vander Jagt *et al.*, 2003). Plasma MG levels are elevated in people with diabetes (McLellan *et al.*, 1994; Wang *et al.*, 2007a). We have recently shown that administration of MG for 4 weeks as a continuous infusion by a subcutaneous minipump induces features of type 2 diabetes in Sprague-Dawley rats (Dhar *et al.*, 2011). MG is a major precursor for the formation of advanced glycation endproducts (AGEs) (Ahmed *et al.*, 1997; Vander Jagt *et al.*, 2003). MG causes increased oxidative stress (Desai *et al.*, 2008; Wu *et al.*, 2002), which in turn is believed to cause the pathophysiological changes in diabetes, hypertension, and aging

(Ceriello, 2008; Ceriello *et al.*, 2004; Harman, 1998). For example, MG activates nuclear factor kappa B p65 (NF- κ B p65), and inactivates antioxidant enzymes like glutathione reductase and glutathione peroxidase, in rat vascular smooth muscle cells (VSMCs), and causes oxidative stress (Wu *et al.*, 2002). On the other hand increased oxidative stress can deplete GSH and slow down degradation of MG, thereby increasing its levels and harmful effects (Desai *et al.*, 2008; Vander Jagt *et al.*, 2003). Specific and safe scavengers of MG will have great therapeutic value to prevent hyperglycemia and high carbohydrate diet-induced pathology resulting from excess MG formation. Elevated plasma levels of MG have been reported in spontaneously hypertensive rats and fructose-fed rats (Wang *et al.*, 2005; Wang *et al.*, 2008), but it is not yet known whether MG is the cause or effect of hypertension. We hypothesized that MG upregulates the RAAS and increases BP in rats, as the basis of the work reported here.

4.3 Methods

Animals

Male Sprague-Dawley rats from Charles River Laboratories (Quebec, Canada) were used according to guidelines of the Canadian Council on Animal Care. All animal protocols were approved by the University of Saskatchewan's Animal Research Ethics Board. 28 male 12 week old Sprague-Dawley rats with closely matching body weights were used. A miniosmotic pump (Alzet® 2ML4, Durect Corp., Cupertino, CA, USA) was surgically implanted subcutaneously on the back under sterile conditions (Dhar *et al.*, 2011). This pump holds a fixed volume (2 mL) of drug and releases a continuous small amount of MG (40% solution, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) into the body at a rate of 1 mg/h or 24 mg/day or 672 mg over 28 days.

Hence each rat received a fixed dose (24 mg/day) and therefore it is not expressed as mg/kg. To control for this fixed dose the rats were from the same batch, of the same age and similar in weight. We have successfully used this minipump previously to deliver MG to rats for 28 days (Dhar *et al.*, 2011). Pumps were implanted in all rats to deliver either MG or normal saline (0.9% NaCl). Anesthesia was provided by a continuously monitored inhalation of isoflurane (Forane) (2-4%) in oxygen, delivered through a precision vaporizer and circle absorption breathing system (Ohio, 30/70 Proportioner Anesthesia Machine, Madison, Wisconsin), with the rat placed on a heated pad to maintain a rectal temperature of 37°C. Buprenorphine injection (0.025 mg/kg s.c. twice a day) was used as a preanesthetic and post-surgical recovery analgesic for two days following implantation.

The rats were divided into the following treatment groups ($n = 7$ each): 1. Control (0.9% saline by pump), 2. MG (24 mg/day by pump), 3. MG + alagebrium (ALA, a MG scavenger (Dhar *et al.*, 2010a), 30 mg/kg/day in drinking water), 4. ALA + 0.9% saline by pump. At the end of the study, the rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight, i.p.). After 15 min stabilization the BP was measured by carotid- artery cannulation for 30 min (Desai *et al.*, 2006; Laight *et al.*, 2000). Blood was collected from the carotid artery and plasma was separated and stored at -80°C. Organs / tissues cleaned in ice-cold phosphate buffer saline were immediately frozen in liquid nitrogen and stored at -80°C until processing.

Biochemical assays

Assay kits for quantitative determination of plasma renin (AnaSpec, Fremont, CA, USA), angiotensin (Cayman Chemicals, Ann Arbor, USA), aldosterone (Cayman Chemical Co.,

Ann Arbor, MI, USA) and catecholamines (Rocky Mountain Diagnostics, Inc., Colorado Springs, CO, USA) were used according to the manufacturer's instructions.

Cell culture

Rat thoracic aortic smooth muscle cells (A-10 cells, CRL-1476, American Type Culture Collection, Manassas, VA, USA) were cultured as described previously (Dhar *et al.*, 2008). The cells were seeded either in 100 mm dishes for MG measurement or 6 well plates for other assays, with an equal amount of cells (10^6 /mL) in each well, and cultured to confluence. Cells were starved in FBS-free DMEM medium for 24 h prior to exposure to different treatments alone or in combination: MG (30 μ M based on preliminary results), glucose (5, 25 mM, based on preliminary results), ALA (100 μ M) and a specific NF- κ B inhibitor, CAY10512 (0.1 μ M) (Cayman Chemical Co., Ann Arbor, MI, USA).

Methylglyoxal measurement

MG was measured by a specific and sensitive high-performance liquid chromatography (HPLC) method (Dhar *et al.*, 2009). MG was derivatized with *o*-phenylenediamine to form the quinoxaline, 2-methylquinoxaline, which is very specific for MG. The 2-methylquinoxaline and the internal standard 5-methylquinoxaline were quantified on a Hitachi D-7000 HPLC system (Hitachi, Ltd., Mississauga, ON, Canada) *via* a Symmetry C18 column (3.9 x 150 mm, and 4 μ m particle diameter; Waters, Milford, MA, USA).

Western blotting

Total proteins from cultured cells, aortic tissue and kidney were subjected to Western blot analysis as previously described (Dhar *et al.*, 2011; Dhar *et al.*, 2012). The following primary antibodies were used: Ang II AT₁ receptor (AT₁R), adrenergic α_{1D} receptor (α_{1D} R), renin, angiotensin, angiotensin converting enzyme (ACE) (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), receptor for advanced glycation endproducts (RAGE), nuclear factor κ B (NF- κ B) (Abcam, Cambridge, MA, USA), and anti- β -actin (diluted 1:1000) (Sigma-Aldrich Canada Ltd., Mississauga, ON, Canada). Horse radish peroxidase conjugated secondary antibodies (Life Science, Hercules, CA, USA) (diluted 1:3000) were used. The proteins were visualized with ECL chemiluminescence reagent (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and captured on X-ray film (GE Healthcare Life Sciences, Pittsburgh, PA, USA) (Dhar *et al.*, 2011).

Real time quantitative PCR (RT-PCR)

Total RNA from the cultured cells, kidney and aorta was isolated using RNA isolation kit (Qiagen, Germantown, MD, USA) (Dhar *et al.*, 2011). The pre-designed primers for angiotensin, AT₁R, α_{1D} R, renin, aldosterone receptor (MR) and RAGE were purchased from Qiagen (Germantown, MD, USA). The real-time PCR was performed in an iCycler iQ apparatus (Life Science, Hercules, CA, USA) associated with the ICYCLER OPTICAL SYSTEM software (version 3.1) using SYBR Green PCR Master Mix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) as previously described (Dhar *et al.*, 2011).

siRNA transfection for RAGE and angiotensinogen

In a six well cell culture plate, 2×10^5 cells per well were grown in 2 mL antibiotic-free normal growth medium supplemented with FBS. The cells were incubated at 37° C in an incubator until 60-80% confluent. For each transfection, 2-8 μ L of siRNA duplex (Solution A, 0.25-1 μ g or 20-80 pmols siRNA) was diluted with 100 μ L siRNA transfection medium (Cat # sc-36868, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). For each transfection, 2-8 μ L of siRNA transfection reagent (Solution B, Cat # sc-29528, Santa Cruz Biotechnology Inc.) was diluted with 100 μ L siRNA transfection medium (Cat # sc-36868). siRNA duplex solution (Solution A) was directly added to the dilute transfection reagent (Solution B) and mixed by pipetting the solution up and down, and incubated for 15-45 min at room temperature. The cells were washed once with 2 mL of siRNA transfection medium (Cat # sc-36868). For each transfection, 0.8 mL siRNA transfection medium was added to the siRNA transfection reagent mixture (Solution A + Solution B), mixed gently and added to the washed cells. The cells were incubated for 5-7 h at 37° C, followed by addition of 1 mL of normal growth medium containing 2 times the normal serum and antibiotics concentration, to the transfection mixture. The cells were incubated further for 18-24 h. The medium was aspirated and replaced with fresh 1x normal growth medium and the cells were assayed 48-72 h later.

Materials

Glucose, methylglyoxal and other reagents were purchased from Sigma Aldrich Canada Ltd. (Mississauga, ON, Canada) or VWR International (Mississauga, ON, Canada). Alagebrium (previously known as ALT-711, N-phenacyl-4,5-dimethyl-1,3-thiazolium) was a generous gift

from Synvista Therapeutics Inc. (Montvale, NJ, USA) (formerly Alteon Corp.). Synvista ceased operations in 2009 ([BioSpace, 2009](#)).

Statistical analysis

Data obtained from separate experiments are expressed as mean \pm SEM. Statistical analysis was performed using ANOVA with post hoc Bonferroni's test. A *P* value of less than 0.05 was considered to be statistically significant.

4.4 Results

Acute or chronic methylglyoxal treatment increases cellular, aortic and renal methylglyoxal levels

Incubation of cultured VSMCs for 24 h or 5 days with MG (30 μ M) or high glucose (25 mM) caused a significant elevation of cellular MG that was attenuated by co-incubation with the MG scavenger ALA (Fig. 4-1A, B). Treatment of SD rats for 4 weeks with MG caused a significant increase in aortic and renal MG levels which was attenuated by co-treatment with ALA (Fig. 4-1C, D).

Chronic methylglyoxal treatment increases blood pressure and plasma catecholamines, renin, angiotensin and aldosterone levels

Treatment of SD rats for 4 weeks with MG caused a significant increase in the BP which was attenuated by the MG scavenger ALA (Fig. 4-2A). ALA alone did not affect the BP. Chronic MG treatment also caused a significant increase in plasma norepinephrine, epinephrine,

dopamine (Fig. 4-2B), angiotensin (Fig. 4-2C), renin (Fig. 4-2D) and aldosterone (Fig. 4-2E) levels. The MG scavenger, ALA, attenuated the increase in catecholamines, angiotensin, renin and aldosterone caused by MG (Fig. 4-2).

MG increases α_{1D} receptor, AT_1 receptor and angiotensin expression in vascular smooth muscle cells and the aorta.

Glucose is a major precursor of MG formation (Dhar *et al.*, 2008). Incubation of VSMCs with MG (30 μ M) or high glucose (25 mM) for 24 h (Fig. 4-3Ai, ii, B) or 5 days (supplemental Fig. 4-S1) caused a significant increase in adrenergic α_{1D} receptor, angiotensin AT_1 receptor and angiotensin protein and mRNA, which were attenuated by ALA (Figs. 4-3Ai, ii, B, supplemental Fig. 4-S1).

Chronic treatment of SD rats with MG for 4 weeks significantly elevated aortic adrenergic α_{1D} receptor, angiotensin AT_1 receptor and angiotensin protein and mRNA, which were attenuated by co-treatment with ALA + MG (Fig. 4-3Aiii, C).

Chronic treatment of Sprague-Dawley rats with methylglyoxal increases renal AT_1 receptor, renin and angiotensin expression

Chronic treatment of Sprague-Dawley rats with MG for 4 weeks significantly increased renal AT_1 receptor, renin and angiotensin protein and mRNA, which were attenuated by co-treatment with ALA (Fig. 4-4A, B).

Acute or chronic treatment with MG increases phosphorylated extracellular signal related kinases 1/2 (p-Erk 1/2) and NFATc expression

Incubation of VSMCs with MG (30 μ M) for 24 h (Fig. 4-5A) caused an increase in phosphorylated Erk 1/2 (p-Erk 1/2) and NFATc protein expression which was attenuated by ALA (Fig. 4-5A). Chronic treatment of Sprague-Dawley rats with MG for 4 weeks increased aortic and renal protein expression of p-Erk 1/2 and NFATc, which was attenuated by ALA (Figs. 4-5B, C).

Effects of inhibition of NF- κ B or angiotensinogen siRNA and receptor for AGEs (RAGE) siRNA on MG induced increase in NF- κ B, angiotensin, AT₁ and α_{1D} receptors in VSMCs

Incubation of VSMCs with MG (30 μ M) for 24 h caused a significant increase in the protein and mRNA for NF- κ B, angiotensin, AT₁ and adrenergic α_{1D} receptors, which were attenuated by co-incubation with the NF- κ B inhibitor CAY 10512 (0.1 μ M) or the MG scavenger, ALA (100 μ M) (Fig. 4-6A-D). RAGE siRNA attenuated the increase in RAGE and NF- κ B p65 protein expression induced by MG (30 μ M) in cultured VSMCs (Fig. 4-6E). Angiotensinogen siRNA attenuated the increase in NF- κ B p65, angiotensin, AT₁ and adrenergic α_{1D} receptor protein expression induced by MG (30 μ M) in cultured VSMCs (Fig. 4-6F).

4.5 Discussion

We report the novel finding that MG, a reactive metabolite produced from glucose and fructose, significantly increases renin angiotensin aldosterone levels associated with an increase in the BP in Sprague-Dawley rats. Molecular studies in cultured VSMCs implicate RAGE and

NF- κ B in the signaling pathway of MG to cause these effects. Alagebrium attenuates the pathological effects of MG on the RAAS.

The association of hypertension, and increased cardiovascular fatalities such as myocardial infarction and stroke, with diabetes is well established (Ferrannini *et al.*, 2012; Gee *et al.*, 2012). High glucose (Kaufman *et al.*, 1991) and high fructose diets (Hwang *et al.*, 1987; Madero *et al.*, 2011) have been shown to significantly increase the BP in animals and humans but the molecular mechanisms are not very clear. In the case of high glucose diet activation of protein kinase C, increased oxidative stress and reduced bioavailability of NO have been reported (Brownlee, 2001). In the case of high fructose diet one proposed mechanism implicates unregulated metabolism of fructose causing ATP depletion and an increase in uric acid, oxidative stress and decrease in NO production (Perez-Pozo *et al.*, 2010; Sanchez-Lozada *et al.*, 2007). Glucose and fructose are both precursors of MG formation (Dhar *et al.*, 2008) and MG in turn is a well-established trigger for increased oxidative stress through multiple pathways (Desai *et al.*, 2008). MG is also a major precursor of the formation of AGEs (Ahmed *et al.*, 1997; Vander Jagt *et al.*, 2003).

To the best of our knowledge our study is the first one to report a direct effect of MG on the development of hypertension and the probable molecular mechanisms. One study had reported administration of MG in drinking water for 18 weeks, and an increase in BP (Vasdev *et al.*, 1998; Vasdev *et al.*, 2010) but a direct link between MG and increased BP was not established. For example, plasma or aortic or renal MG levels were not measured. Instead renal levels of aldehyde conjugates (AGEs) were measured which likely represent modification of several proteins/enzymes, and do not identify individual proteins/enzymes and molecular

mechanisms. Moreover, in their study (Vasdev *et al.*, 1998; Vasdev *et al.*, 2010) the authors did not address the role of the RAAS. Moreover, MG reacts with epithelial cells of the intestines and the colon (Baskaran *et al.*, 1990) and its oral bioavailability is questionable. An association of elevated MG with hypertension in rats has been shown but the cause effect relation was not established (Wang *et al.*, 2007b; Wang *et al.*, 2008).

Increased activity of the RAAS is seen in type 2 diabetics with hypertension (Hsueh *et al.*, 2011). Hyperglycemia and diabetes are known to increase renin angiotensin activity but the mechanism has not been clearly defined. In one study high levels of glucose were shown to cause the release of renin through local accumulation of succinate and activation of the kidney-specific G protein-coupled receptor, GPR91, in the glomerular endothelium in rat, mouse, and rabbit (Toma *et al.*, 2008). In another study 25 mM D-glucose increased the expression of angiotensinogen gene in cultured immortalized rat proximal tubular cells through synthesis of diacylglycerol and activation of protein kinase C (Toma *et al.*, 2008; Zhang *et al.*, 1999). We decided to examine the effect of MG on the RAAS.

The oral absorption of MG is doubtful as determined in our preliminary studies, which failed to cause any significant increase in plasma MG levels. Therefore, we administered MG by continuous infusion of very small amounts with a minipump (Dhar *et al.*, 2011), hoping to simulate the almost continuous production of MG in the body through metabolism. Administration of the dose of MG used in this study by minipump results in significant elevation of plasma MG levels to values reported in pathological conditions (Wang *et al.*, 2005; Wang *et al.*, 2008) and in our previous study (Dhar *et al.*, 2011). This mode of MG administration significantly elevated MG levels in the kidney and the aorta (Fig. 4-1), which implicates MG in

the pathological effects on the RAAS. MG treatment increased plasma levels of renin, angiotensin, aldosterone and catecholamines (Fig. 4-2) signifying increased RAAS and adrenergic activity, which most likely increased the blood pressure. The kidney is the main source of renin and the major regulator of BP. The kidney had increased protein expression of renin, which would explain increased plasma renin. The renin in turn would produce more angiotensin II and its multiple effects (Fyhrquist *et al.*, 2008; Hitomi *et al.*, 2007; Zaman *et al.*, 2002).

The aorta had significantly elevated MG. Previously we have shown that the aorta has significantly elevated levels of MG compared to other organs and tissues in normal SD rats, the reason being unknown, and it increases further after an acute infusion of MG (Dhar *et al.*, 2010a). The increased MG most likely increased aortic AT₁ receptor, α_{1D} receptor and angiotensin, because these effects were attenuated by the MG scavenger ALA. Both AT₁ and α_{1D} receptor activation can increase p-Erk 1/2 and NFATc, as seen here, which can contribute to increased vascular tone, inflammation and hypertension development (Hitomi *et al.*, 2007). Increased AT₁ and α_{1D} receptors can increase vascular tone and BP (Fyhrquist *et al.*, 2008; Zaman *et al.*, 2002). We have previously shown (Dhar *et al.*, 2010b) that MG can reduce endothelial nitric oxide synthase activity which can contribute to increased BP, although it was not investigated in this study.

Treatment of VSMCs with MG or high glucose caused a similar significant increase in cellular MG levels, along with increased expression of AT₁ receptor, α_{1D} receptor and angiotensin, which were all attenuated by ALA (Fig. 4-1), which is a MG scavenger and an AGEs breaker (Dhar *et al.*, 2010a; Wolffenbuttel *et al.*, 1998). Since the attenuation by ALA of

increased MG levels, AT₁ receptor, α_{1D} receptor and angiotensin occurred within 24 h in cultured VSMCs (Fig. 4-3), this indicates a direct effect of MG on AT₁ receptor, α_{1D} receptor and angiotensin, rather than an indirect effect through MG-induced AGEs, which normally take 5-7 days to form. High glucose similarly increased AT₁ receptor, α_{1D} receptor and angiotensin expression in VSMCs, which was attenuated by a MG scavenger, implicating MG as a mediator of the deleterious effects of hyperglycemia (Fig. 4-3). Since MG is a major precursor of AGEs formation, part of alagebrium's preventive effects against MG could be due to prevention of AGEs formation *in vivo*, but not in 24 h cell culture studies.

We investigated whether the effect of MG on angiotensin, AT₁ and α_{1D} receptors was direct, or indirect through some other mediators. RAGE is currently under intense investigation as a target to prevent diabetic complications (Ramasamy *et al.*, 2011). The activation of RAGE by AGEs has been reported to increase two key transcription factors, NF- κ B and early growth response-1 (Egr-1), and cause oxidative stress (Ramasamy *et al.*, 2011; Wendt *et al.*, 2002). We report the novel finding that MG can activate RAGE and up regulate it (Fig. 4-6E). RAGE is a receptor which is up regulated by its other known ligands (Ramasamy *et al.*, 2011). Thus, when we silenced RAGE we were able to prevent the increase in NF- κ B induced by MG (Fig. 4-6E). When we inhibited NF- κ B we were able to prevent the increase in angiotensin, AT₁ and α_{1D} receptors in VSMCs (Fig. 4-6B-D), indicating an indirect effect of MG on these parameters. When we silenced angiotensin mRNA we were able to prevent the increase in angiotensin, AT₁ and α_{1D} receptors and also the increase in NF- κ B induced by MG (Fig. 4-6F). These results indicate that MG activates RAGE which then increases NF- κ B followed by angiotensin, AT₁ and α_{1D} receptors. The increased angiotensin II in turn increases NF- κ B and sets up a vicious cycle.

Our results establish a probable sequence of molecular events resulting from elevated MG levels which have been reported in high carbohydrate diet fed animals and in diabetic patients (Wang *et al.*, 2007a; Wang *et al.*, 2008).

4.6 Conclusions

We have shown a direct effect of MG in the development of hypertension and identified the probable involvement of the RAAS and RAGE in molecular mechanisms. Since diabetic patients are known to have significantly elevated MG levels (Wang *et al.*, 2007a) and many people with diabetes develop hypertension, MG could possibly be a mediator of hyperglycemia-induced and diabetes associated hypertension. High carbohydrate diets are known to induce hypertension in animal models and since glucose and fructose are both precursors for MG formation, MG could very likely be causing high dietary carbohydrate-induced hypertension, which is currently being investigated by us. The involvement of MG as one of the causative factors of hypertension and activator of RAGE opens up the possibility of using effective and safe MG scavengers in preventing diabetes associated hypertension and vascular complications. While the dietary habits of millions of people worldwide and the use of high fructose containing processed foods is not likely to change overnight, preventive strategies will play a major role in attenuating the adverse health effects of high carbohydrate diets.

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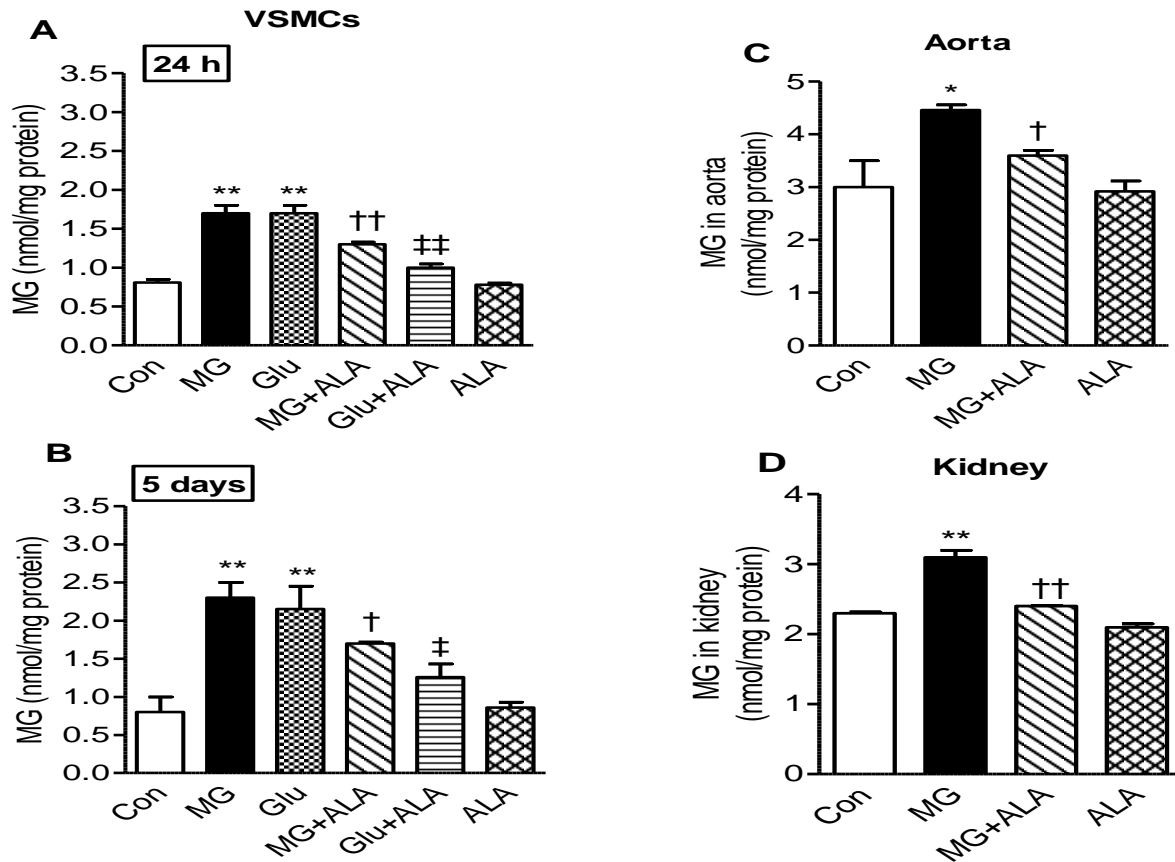


Figure 4-1. Methylglyoxal levels in cultured vascular smooth muscle cells and in the aorta and kidney of chronic methylglyoxal treated Sprague-Dawley rats. Vascular smooth muscle cells (A10 cell line) were incubated with MG (30 μ M) or high glucose (Glu, 25 mM) for 24 h (A) or 5 days (B). Alagebrium (30 μ M) was used as a MG scavenger. Groups ($n = 7$ each) of 12 week old male Sprague-Dawley rats were treated for 4 weeks with a continuous infusion of methylglyoxal (MG, 24 mg/day) delivered by a subcutaneous minipump (C, D). Control rats received 0.9% saline by pump. Alagebrium (ALA, 100 mg/kg/day in drinking water) was used as a MG scavenger. MG levels were determined with HPLC. * $P < 0.05$, ** $P < 0.01$ vs. respective control (Con). † $P < 0.05$, †† $P < 0.01$ vs. respective MG. ‡ $P < 0.05$, ‡‡ $P < 0.01$ vs. respective Glu group.

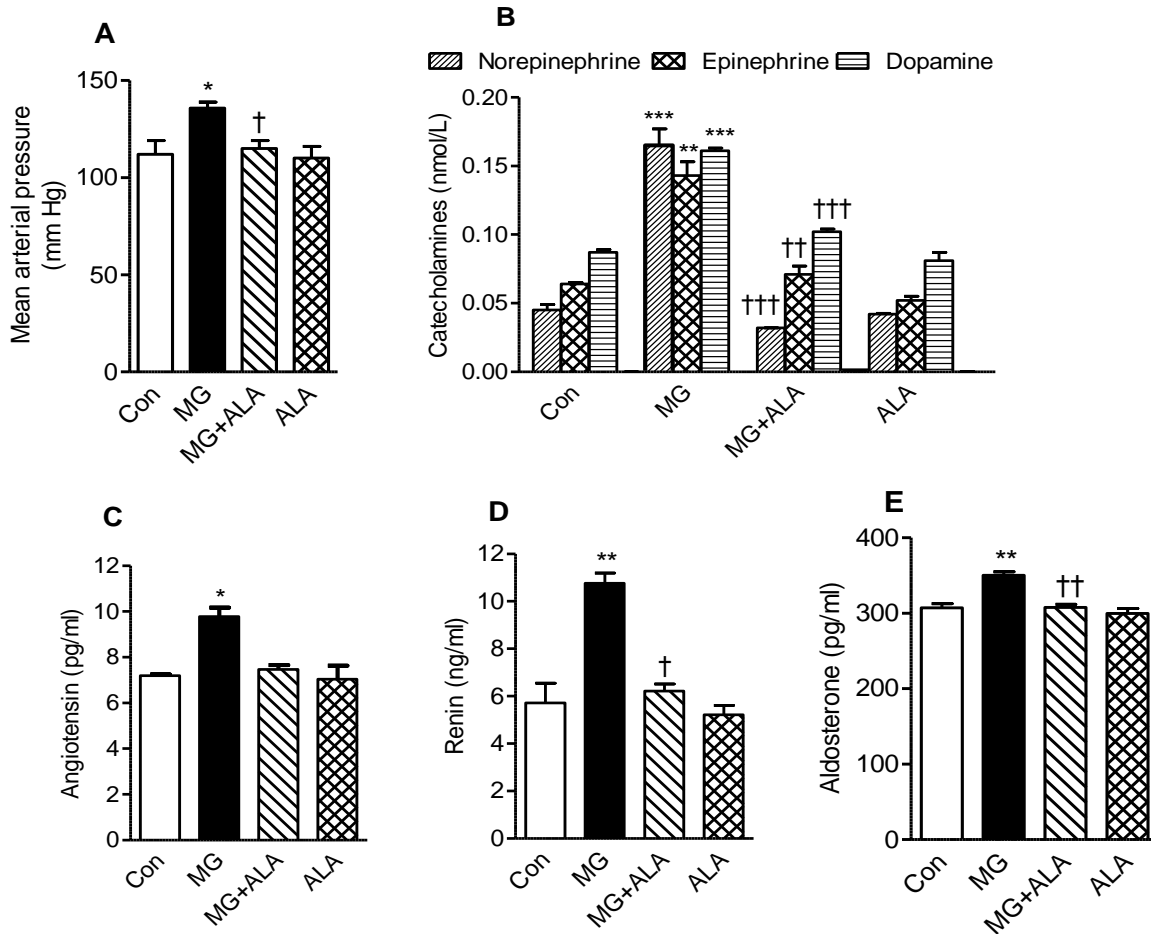


Figure 4-2. Mean arterial blood pressure, plasma catecholamines, angiotensin, renin, and aldosterone levels in chronic methylglyoxal treated Sprague-Dawley rats. Groups ($n = 7$ each) of 12 week old male Sprague-Dawley rats were treated for 4 weeks with a continuous infusion of methylglyoxal (MG, 24 mg/day) delivered by a subcutaneous minipump. Control rats received 0.9% saline by pump. Alagebrium (ALA, 100 mg/kg/day in drinking water) was used as a MG scavenger. Blood was collected and plasma was separated for analysis of MG by HPLC and catecholamines, renin, angiotensin and aldosterone by assay kits. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. respective control. † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ vs. respective MG group.

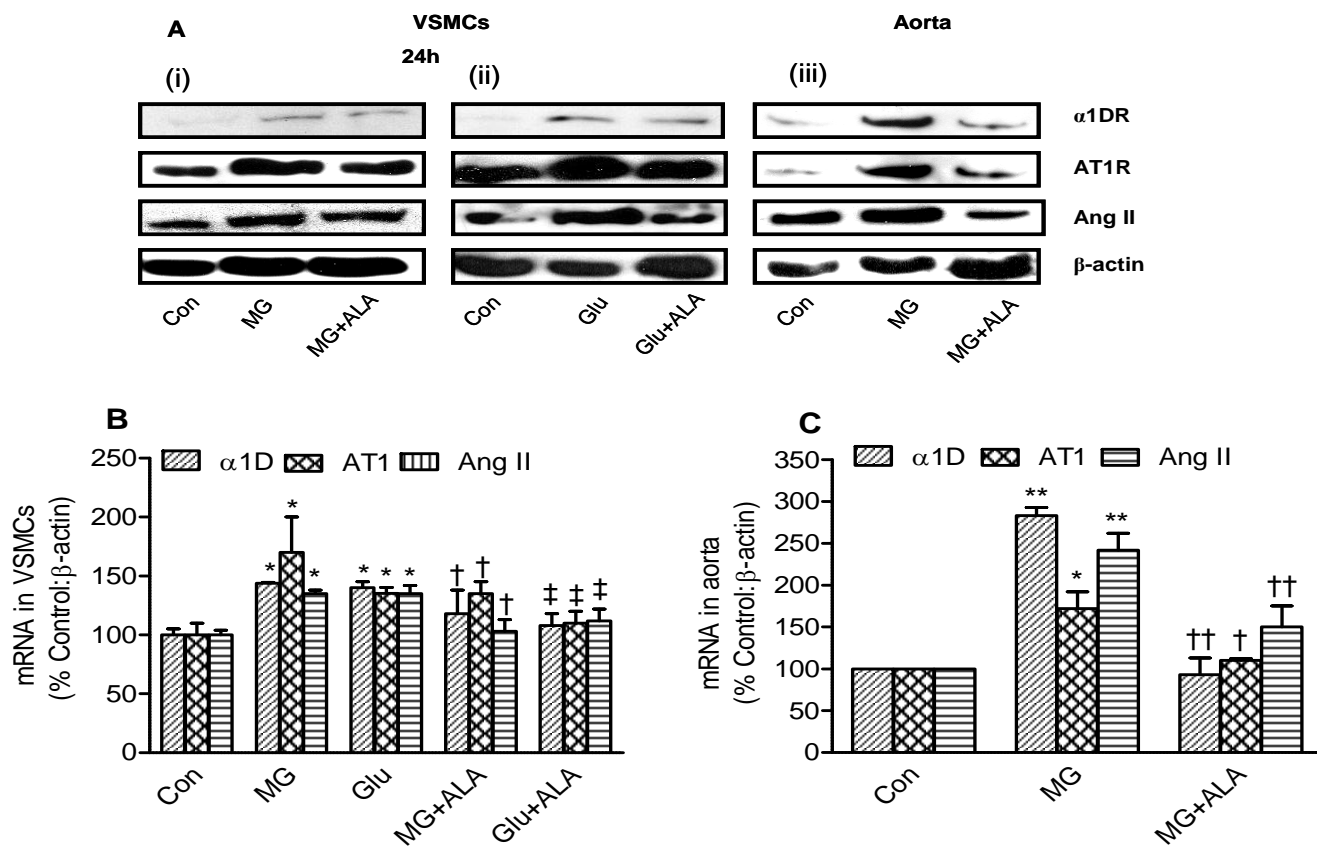


Figure 4-3. α_{1D} receptor, AT_1 receptor and angiotensin protein expression in cultured vascular smooth muscle cells and in the aorta and mRNA in VSMCs and aorta of chronic methylglyoxal treated Sprague-Dawley rats. Rat thoracic aorta smooth muscle cells (VSMCs, A10 cell line) were cultured and incubated with MG (30 μ M) (Ai, B) or high glucose (Glu, 25 mM) (Aii, B) for 24 h. Alagebrium (ALA, 30 μ M) was used as a MG scavenger. Groups ($n = 7$ each) of 12 week old male Sprague-Dawley rats were treated for 4 weeks with a continuous infusion of methylglyoxal (MG, 24 mg/day) delivered by a subcutaneous minipump (Aiii, C). Control rats received 0.9% saline by pump. ALA (100 mg/kg/day in drinking water) was used as a MG scavenger. Protein expression was determined by Western blotting using appropriate

primary antibodies, and mRNA with RT-PCR. * $P < 0.05$, ** $P < 0.01$ vs. respective control (Con).

† $P < 0.05$, †† $P < 0.01$ vs. respective MG group. ‡ $P < 0.05$ vs. respective Glu group.

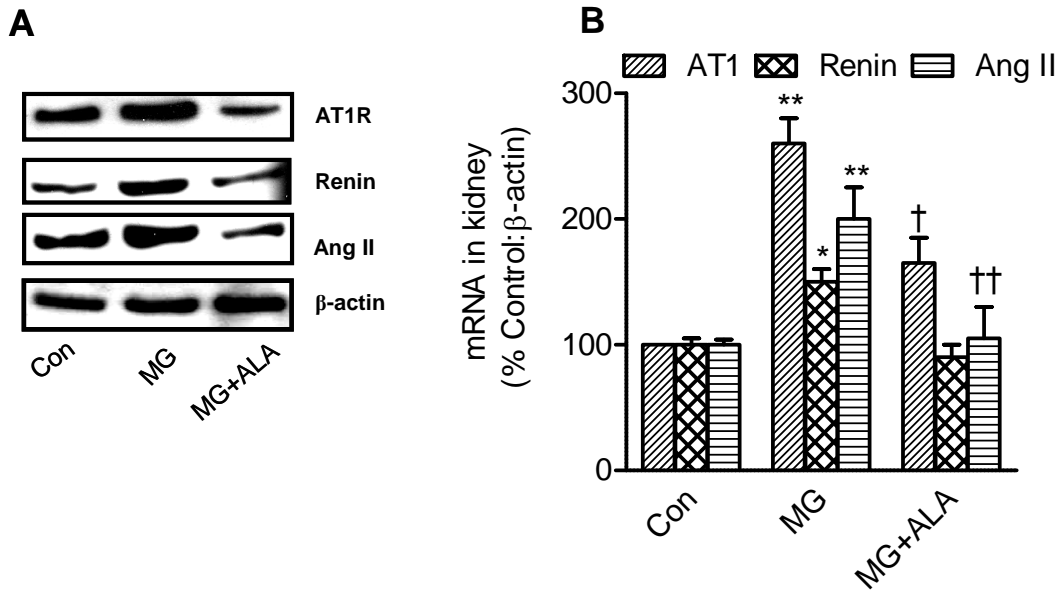


Figure 4-4. AT_1 receptor, renin and angiotensin protein expression and mRNA in the kidney of chronic methylglyoxal treated Sprague-Dawley rats. Groups ($n = 7$ each) of 12 week old male Sprague-Dawley rats were treated for 4 weeks with a continuous infusion of methylglyoxal (MG, 24 mg/day) delivered by a subcutaneous minipump. Control rats received 0.9% saline by pump. Alagebrium (ALA, 100 mg/kg/day in drinking water) was used as a MG scavenger. Protein expression was determined by Western blotting using appropriate primary antibodies, and mRNA with RT-PCR. * $P < 0.05$, ** $P < 0.01$ vs. respective control (Con). $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ vs. respective MG group.

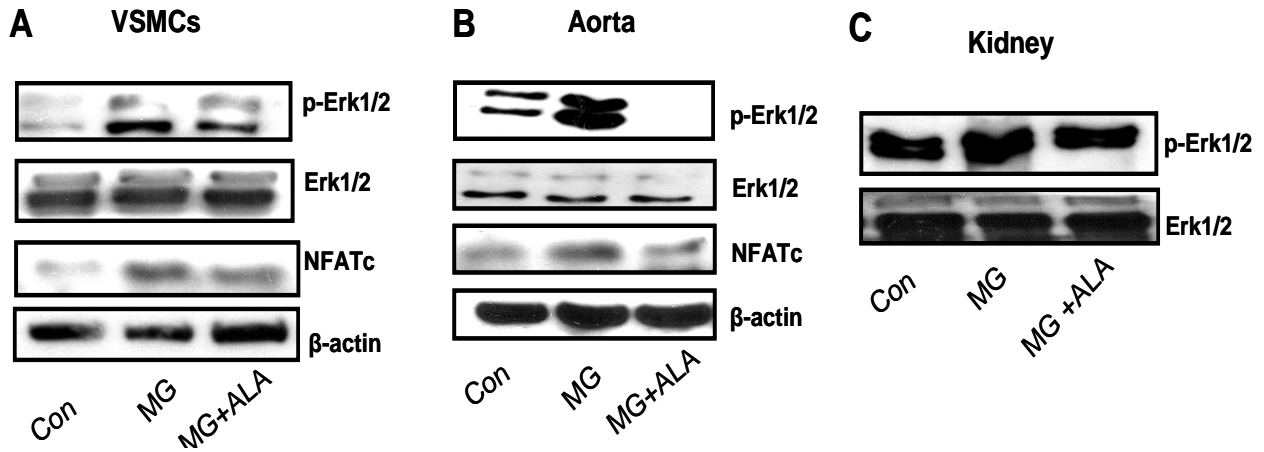


Figure 4-5. Phosphorylated extracellular signal-related kinases 1/2 (p-Erk 1/2), Erk 1/2 and NFATc expression in cultured vascular smooth muscle cells, and in the aorta and kidney of chronic methylglyoxal treated Sprague-Dawley rats. Rat thoracic aorta smooth muscle cells (VSMCs, A10 cell line) were cultured and incubated with MG (30 μ M) (A) for 24 h. Alagebrium (ALA, 30 μ M) was used as a MG scavenger. Groups ($n = 7$ each) of 12 week old male Sprague-Dawley rats were treated for 4 weeks with a continuous infusion of methylglyoxal (MG, 24 mg/day) delivered by a subcutaneous minipump (B, C). Control rats received 0.9% saline by pump. ALA (100 mg/kg/day in drinking water) was used as a MG scavenger. Protein expression was determined by Western blotting using appropriate primary antibodies, and mRNA with RT-PCR.

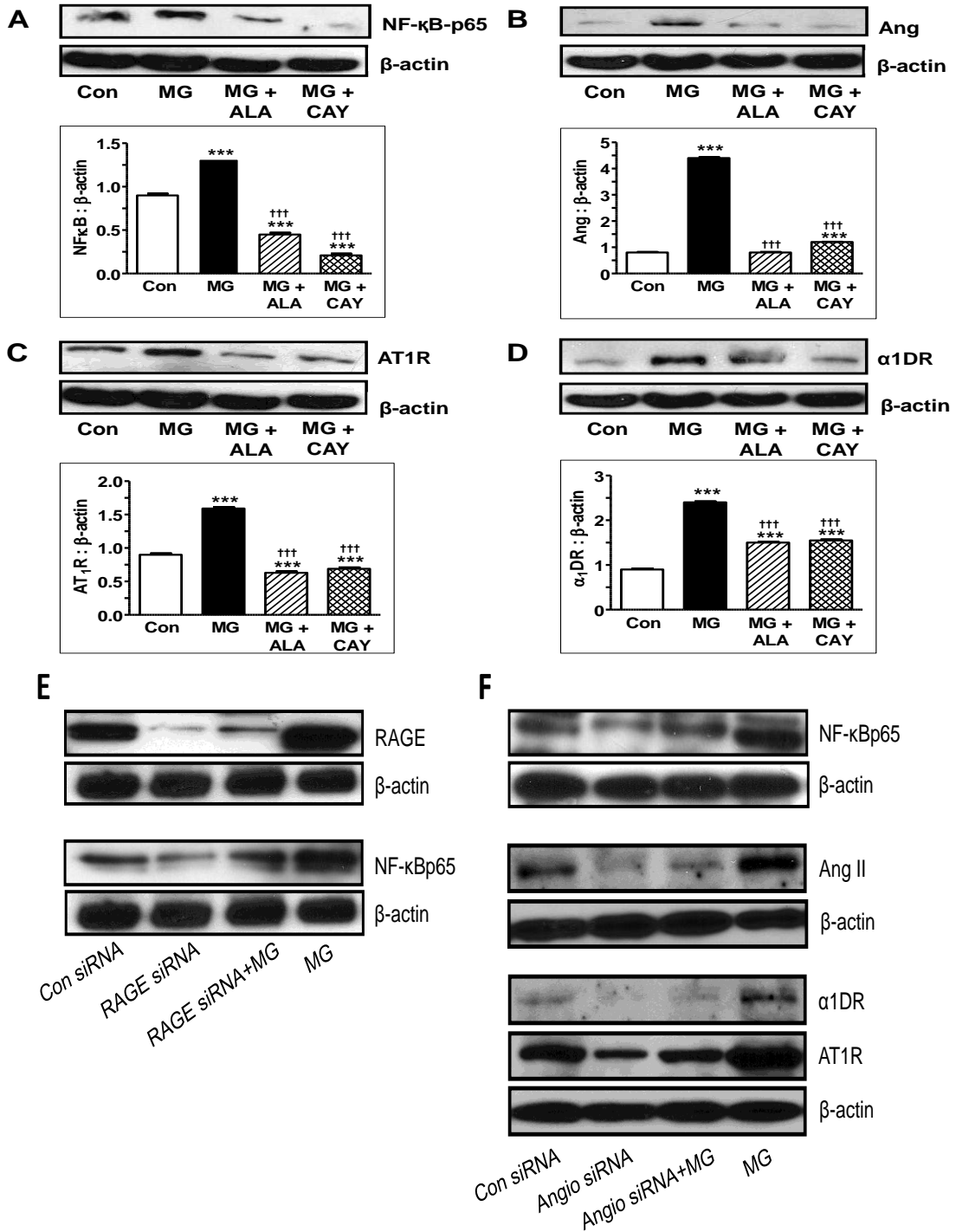


Figure 4-6. Nuclear factor κ B (NF- κ B), angiotensin, AT₁ receptor, α_{1D} receptor and receptor for advanced glycation endproducts (RAGE) expression in cultured vascular

smooth muscle cells treated with a NF- κ B inhibitor, or after RAGE siRNA or angiotensinogen siRNA treatment. Vascular smooth muscle cells (A10 cell line) were cultured and incubated with MG (30 μ M) for 24 h. CAY 10512 (0.1 μ M) was used a NF- κ B inhibitor. Alagebrium (ALA, 30 μ M) was used as a MG scavenger (A-D). RAGE siRNA (E) or angiotensinogen siRNA (F) was carried out as explained in methods. Protein expression was determined by Western blotting using appropriate primary antibodies, and mRNA with RT-PCR. $n = 4$ for each group. *** $P < 0.001$ vs. respective control. ††† $P < 0.001$ vs. respective MG group.

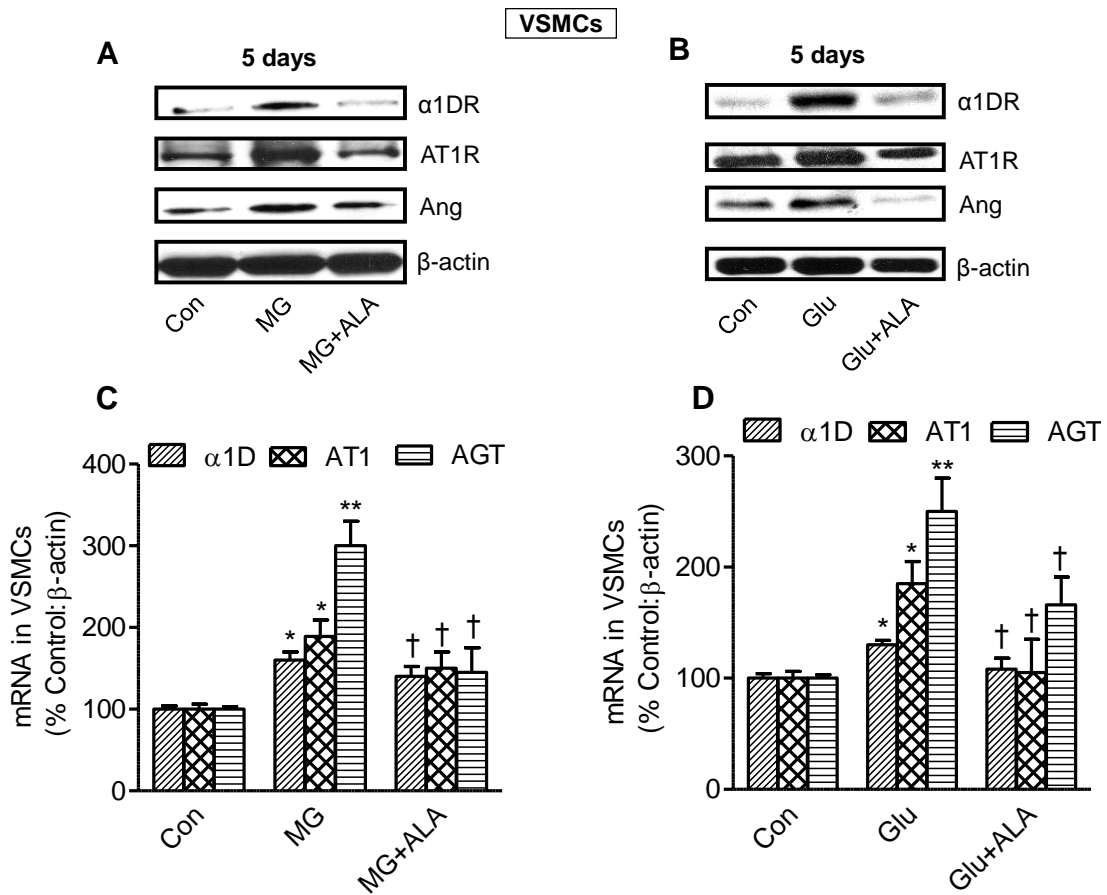


Figure 4-S1. $\alpha 1D$ receptor, AT1 receptor and angiotensin expression in cultured vascular smooth muscle cells treated with methylglyoxal or high glucose. Rat thoracic aorta smooth muscle cells (A10 cell line) were cultured and incubated with MG (30 μM) (A, C) or high glucose (Glu, 25 mM) (B, D) for 5 days. Alagebrium (ALA, 30 μM) was used as a MG scavenger. Protein expression was determined by Western blotting using appropriate primary antibodies, and mRNA with RT-PCR. * $P < 0.05$, ** $P < 0.01$ vs. respective control (Con).

CHAPTER 5

Increased methylglyoxal formation with upregulation of renin angiotensin system in fructose fed Sprague Dawley rats.

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5.1 Abstract

The current epidemic of obesity and type 2 diabetes is attributed to a high carbohydrate diet, containing mainly high fructose corn syrup and sucrose. More than two thirds of diabetic patients have hypertension. Methylglyoxal is a highly reactive dicarbonyl generated during glucose and fructose metabolism, and a major precursor of advanced glycation end products (AGEs). Plasma methylglyoxal levels are increased in hypertensive rats and diabetic patients. Our aim was to examine the levels of methylglyoxal, mediators of the renin angiotensin system and blood pressure in male Sprague-Dawley rats treated with a high fructose diet (60% of total calories) for 4 months. The thoracic aorta and kidney were used for molecular studies, along with cultured vascular smooth muscle cells (VSMCs). HPLC, Western blotting and Q-PCR were used to measure methylglyoxal and reduced glutathione (GSH), proteins and mRNA, respectively. Fructose treated rats developed a significant increase in blood pressure. Methylglyoxal level and protein and mRNA for angiotensin II, AT₁ receptor, adrenergic α_{1D} receptor and renin were significantly increased, whereas GSH levels were decreased, in the aorta and/or kidney of fructose fed rats. The protein expression of the receptor for AGEs (RAGE) and NF- κ B were also significantly increased in the aorta of fructose fed rats. MG treated VSMCs showed increased protein for angiotensin II, AT₁ receptor, and α_{1D} receptor. The effects of methylglyoxal were attenuated by metformin, a methylglyoxal scavenger and AGEs inhibitor. In conclusion, we report a strong association between elevated levels of methylglyoxal, RAGE, NF- κ B, mediators of the renin angiotensin system and blood pressure in high fructose diet fed rats.

Key words: Methylglyoxal, hypertension, renin angiotensin system, diabetes, fructose, receptor for advanced glycation endproducts.

5.2 Introduction

High dietary carbohydrates, increasing type 2 diabetes and obesity, and the associated hypertension and cardiovascular diseases are major health issues globally (Johnson *et al.*, 2007; Selvaraju *et al.*, 2012; Stanhope, 2012). The explosive increase in type 2 diabetes in the past 2-3 decades has been attributed to high dietary carbohydrates, especially fructose, combined with a sedentary lifestyle. Compared to the general population the risk of high blood pressure (BP, systolic BP ≥ 140 mmHg or diastolic BP ≥ 90 mmHg) is four times higher for people with diabetes (Diabetes, 2000). The pathogenesis of diabetes-associated hypertension is not clearly known. While it is believed to be initiated by hyperglycemia, the molecular mechanisms are far from clear.

The Western diet has high fructose content, mainly in the form of high fructose corn syrup, which has been proposed to induce hypertension (Johnson *et al.*, 2007; Stanhope, 2012). High fructose diet-fed Sprague-Dawley rats have been widely used as a model of insulin resistance and these rats also develop hypertension (Hwang *et al.*, 1987; Wang *et al.*, 2008).

We have shown that fructose-fed Sprague-Dawley rats have elevated levels of methylglyoxal (MG), a reactive metabolite of glucose and fructose (Wang *et al.*, 2008; Dhar *et al.*, 2008). Elevated plasma levels of MG have been reported in spontaneously hypertensive rats which correlate with the degree of hypertension (Wang *et al.*, 2005; Wang *et al.*, 2007), but the cause-effect relationship and the underlying molecular mechanisms are not known. People with diabetes have significantly elevated levels of MG (Wang *et al.*, 2007; McLellan *et al.*, 1994). We have recently reported that chronic MG induces features of type 2 diabetes in SD rats (Dhar *et al.*, 2011). MG is a major precursor for the formation of advanced glycation end products

(AGEs) (Desai & Wu, 2007; Vander & Husankar, 2003). MG reduces activity of antioxidant enzymes like glutathione reductase and glutathione peroxidase (Wu & Juurlink, 2002); leading to increased oxidative stress, which in turn is believed to cause the pathophysiological changes in diabetes, hypertension, and aging (Ceriello & Motz, 2004; Harman D, 1998). The pathogenesis of hypertension is multifactorial. Some of the factors include an increase in renin angiotensin aldosterone system (RAAS) activity, insulin resistance, renal disease and oxidative stress (Ceriello A, 2008; Cowley & Roman, 1996; Hall *et al.*, 1990; Manrique *et al.*, 2009). The RAAS plays an important role in maintaining fluid balance, vascular tone and blood pressure (Fyhrquist & Saijonmaa 2008; Zaman *et al.*, 2002). MG and angiotensin II (Ang II) both lead to an increase in oxidative stress (Manrique *et al.*, 2009; Hitomi *et al.*, 2007; Desai & Wu, 2008). Ang II stimulates NADPH oxidase by acting through the AT₁ receptor and increases superoxide, hydrogen peroxide, and peroxynitrite (Hitomi *et al.*, 2007). However, the cause and effect relationship between increased oxidative stress, RAAS activity and increased blood pressure has remained unclear.

Therefore, the aim of this study was to examine and correlate the levels of MG, mediators of the renin angiotensin system and blood pressure in high fructose diet fed Sprague-Dawley rats.

5.3 Materials and methods

Animals

Male Sprague-Dawley rats from Charles River Laboratories (Quebec, Canada) were used according to guidelines of the Canadian Council on Animal Care. All animal protocols were

approved by the University of Saskatchewan's Animal Research Ethics Board. 32 male 5 week old Sprague-Dawley rats were randomly divided into the following treatment groups ($n = 8$ in each group): 1. Control (normal rat chow), 2. High fructose diet (60% of total calories), 3. High fructose diet + metformin (500 mg/kg/day in drinking water, MG scavenger (Beisswenger *et al.*, 1999; Ruggiero-Lopez *et al.*, 1999), 4. Metformin (normal chow). The treatments were for 16 weeks.

At the end of the treatment period, the rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight, i.p.). The trachea was cannulated to allow spontaneous respiration. The carotid artery was cannulated and connected to a pressure transducer and a Powerlab system (AD Instruments Inc., Colorado Springs, CO, USA) using LabChart 7 software. After 15 min stabilization the blood pressure was recorded for 30 min (Desai *et al.*, 2006). Blood was collected from the carotid artery and plasma was separated and stored at -80°C . The anesthetized rat was euthanized by cutting the thorax and the heart, and causing exsanguination, as per the guidelines of the Canadian Council on Animal Care. Organs and tissues cleaned in ice-cold phosphate buffer saline were immediately frozen in liquid nitrogen and stored at -80°C until processing.

Cell culture

Rat thoracic aortic smooth muscle cells (A-10 cells, CRL-1476, American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2 , as described in our previous study (Dhar *et al.*,

2008). The cells were seeded in 100 mm dishes, with an equal amount of cells (10^6 /mL) in each dish, and cultured to confluence. Cells were starved in FBS-free DMEM medium for 24 h prior to exposure to different treatments alone or in combination: MG (30 μ M based on preliminary results), and metformin (100 μ M).

Methylglyoxal measurement

MG was measured by a specific and sensitive high-performance liquid chromatography (HPLC) method as described earlier (Dhar *et al.*, 2009). MG was derivatized with *o*-phenylenediamine to form the quinoxaline product, 2-methylquinoxaline, which is very specific for MG. The 2-methylquinoxaline and the internal standard 5-methylquinoxaline were quantified on a Hitachi D-7000 HPLC system (Hitachi, Ltd., Mississauga, ON, Canada) *via* a Symmetry C18 column (3.9 x 150 mm, and 4 μ m particle diameter; Waters Corp., Milford, MA, USA).

Western blotting

Total proteins from cultured cells, aorta and kidney, were isolated with RIPA (lysis) buffer using a polytron homogenizer. The supernatants (40 μ g of protein each) were separated by using 6-10% SDS-PAGE gel and subjected to Western blot analysis as previously described (Dhar *et al.*, 2011) with overnight incubation with primary antibodies to AT₁ receptor for angiotensin (AT₁R), adrenergic α_{1D} receptor (α_{1D} R), renin, Ang II, (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), receptor for AGEs (RAGE), nuclear factor κ B (NF- κ B) (Abcam, Cambridge, MA, USA), and anti- β -actin (diluted 1:1000) (Sigma-Aldrich Canada Ltd., Mississauga, ON, Canada). This was followed by incubation with horse radish peroxidase

conjugated secondary antibodies (Life Science, Hercules, CA, USA) (diluted 1:3000) for 2 h at room temperature. The proteins were then visualized with ECL chemiluminescence reagent (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and exposed to X-ray film (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Real time quantitative PCR (RT-PCR)

Total RNA from the kidney and the aorta was isolated using RNA isolation kit (Qiagen, Germantown, MD, USA). The pre-designed primers for Ang II, AT₁R, α_{1D} R, renin and RAGE were purchased from Qiagen, (Germantown, MD, USA). The real-time PCR was performed in an iCycler iQ apparatus (Life Science, Hercules, CA, USA) associated with the ICYCLER OPTICAL SYSTEM software (version 3.1) using SYBR Green PCR Master Mix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada).

Measurement of reduced glutathione (GSH)

Reduced glutathione was measured by derivatization with 5,5'-dithio-bis(2-nitrobenzoic acid), and reverse-phase HPLC using ultraviolet detection ([Wang *et al.*, 2008](#)).

Statistical Analysis

Data obtained from separate experiments are expressed as mean \pm SEM. Statistical analysis was performed using ANOVA with post hoc Bonferroni's test. A *P* value of less than 0.05 was considered to be statistically significant.

5.4 Results

Chronic fructose treatment increases blood pressure, aortic and renal methylglyoxal levels

Treatment of Sprague-Dawley rats with a high fructose diet for 16 weeks caused a significant increase in the blood pressure, which was attenuated by the MG scavenger metformin (Fig. 5-1A). Metformin alone did not affect the blood pressure. The high fructose diet also caused a significant elevation of aortic and renal MG levels, which was attenuated by co-treatment with metformin (Fig. 5-1B, C).

Chronic treatment with fructose increases aortic α_{1D} receptor, AT₁ receptor and angiotensin II expression

Chronic treatment of Sprague-Dawley rats with fructose for 16 weeks significantly elevated aortic adrenergic α_{1D} receptor, angiotensin AT₁ receptor and Ang II protein and mRNA, which were attenuated by co-treatment with metformin (Fig. 5-2). Metformin alone was without any effect.

Chronic treatment of Sprague-Dawley rats with fructose increases renal AT₁ receptor, renin and angiotensin II expression

Chronic treatment of Sprague-Dawley rats with fructose for 16 weeks significantly increased renal AT₁ receptor, renin and Ang II protein and mRNA, which were attenuated by co-treatment with metformin (Fig. 5-3). Metformin alone was without any effect.

Chronic treatment with fructose increases phosphorylated extracellular signal related

kinases 1/2 (p-Erk 1/2), and NFATc expression

Chronic treatment of Sprague-Dawley rats with fructose for 16 weeks increased aortic and renal protein expression of phosphorylated Erk 1/2 (p-Erk 1/2) and NFATc in the aorta, which was attenuated by metformin (Fig. 5-4A, B).

Chronic treatment with fructose increases NF- κ B and RAGE protein expression

Chronic treatment of SD rats with fructose for 16 weeks significantly increased aortic and renal NF- κ B and RAGE protein expression, which was attenuated by co-treatment with metformin (Fig. 5-5A, B).

Chronic treatment with fructose decreases levels of reduced glutathione in the aorta and kidney

Chronic treatment of Sprague-Dawley rats with fructose for 16 weeks significantly decreased aortic and renal levels of reduced glutathione, which were attenuated by co-treatment with metformin (Fig. 5-6A, B).

Methylglyoxal increases α_{1D} receptor, AT₁ receptor and angiotensin II expression in cultured vascular smooth muscle cells

Treatment of rat thoracic aortic cultured vascular smooth muscle cells with MG (30 μ M) for 24 h significantly elevated adrenergic α_{1D} receptor, angiotensin AT₁ receptor and Ang II protein, which were attenuated by co-treatment with metformin (Fig. 5-7). Metformin alone was without any effect.

Metformin has MG scavenging ability

As shown in table 1, 100 μ M metformin significantly reduced the detectable free MG in solution after 3 h incubation, and about 30% of MG was detectable after 24 h incubation. A higher concentration of metformin (1 mM) was more effective in scavenging MG and it significantly reduced detectable free MG after 1 h of incubation and only about 10% of MG was detectable after 24 h.

5.5 Discussion

Here we show that 12 week old male Sprague-Dawley rats treated for 16 weeks with a high fructose diet, a precursor of MG, develop a significant increase in blood pressure. The kidney and aorta from fructose treated rats had significantly increased MG levels, and protein and mRNA for renin, Ang II, AT₁ and adrenergic α_{1D} receptors, which were attenuated by the MG scavenger metformin. MG treated cultured vascular smooth muscle cells had increased expression of Ang II, AT₁ and α_{1D} receptors, which was attenuated by metformin.

Although high glucose (Kaufman *et al.*, 1991) and high fructose diets (Hwang *et al.*, 1987; Madero *et al.*, 2011) have been shown to significantly increase the blood pressure in animals and humans, the molecular mechanisms are not very clear. A high glucose diet-induced increase in blood pressure has been attributed to activation of protein kinase C, increased oxidative stress and reduced bioavailability of nitric oxide (Brownlee M, 2001). Fructose is metabolized differently than glucose with up to 75% of orally absorbed fructose being metabolized by the liver (Johnson *et al.*, 2009). Moreover, the key enzyme of fructose metabolism, fructokinase is not feedback regulated by fructose metabolites (Johnson *et al.*,

2009). Thus, all of the orally absorbed fructose is phosphorylated by fructokinase (Johnson *et al.*, 2009; Le *et al.*, 2012). Unregulated metabolism of fructose causing ATP depletion, oxidative stress and decrease in nitric oxide production has been proposed as one of the mechanisms of fructose-induced increase in blood pressure (Perez-Pozo *et al.*, 2010; Sanchez-Lozada *et al.*, 2007). Both glucose and fructose are precursors of MG formation (Dhar *et al.*, 2008) and MG is also a well-established trigger for increased oxidative stress through multiple pathways (Desai & Wu, 2008). MG is also a major precursor of the formation of AGEs. (Desai & Wu, 2007; Vander & Husankar, 2003).

The aorta and the kidney of fructose treated rats had significantly elevated MG levels (Fig. 5-1) which implicates MG as a factor in hypertension development. The kidney had increased protein expression of renin, which in turn would produce more Ang II and its multiple effects (Hitomi *et al.*, 2007). The increased aortic MG, as observed in the present study, most likely increased aortic AT₁ receptor, α_{1D} receptor and Ang II. This effect of MG was confirmed by increased expression of AT₁ receptor, α_{1D} receptor and Ang II in MG treated cultured vascular smooth muscle cells (Fig. 5-7). Both AT₁ and α_{1D} receptor activation can increase p-Erk 1/2, and NFATc, as seen here, which can contribute to increased vascular contractility, inflammation and hypertension development (Hitomi *et al.*, 2007; Michelotti *et al.*, 2000). The increased Ang II may be responsible for the increased α_{1D} receptor expression (Fyhrquist & Saijonmaa, 2008; Zaman *et al.*, 2000). The increased Ang II, AT₁ and α_{1D} receptors in the aorta signify increased vascular tone. We were not able to harvest enough tissue from the mesenteric artery for western blot and PCR analysis. GSH levels were reduced in the aorta and the kidney of fructose treated rats (Fig. 5-6). GSH plays a central role in the degradation of MG by binding MG and making it

available to the glyoxalase enzymes (Vander & Husankar, 2003). A reduction in GSH would decrease MG degradation, increase its levels, and set up a vicious cycle.

We addressed the possibility that MG was increasing oxidative stress, which has been implicated in the pathogenesis of hypertension (Ceriello A, 2008). The effect of MG on RAGE has not been reported before. RAGE is currently under intense investigation as a target to prevent diabetic complications. The activation of RAGE by AGEs has been reported to increase two key transcription factors, NF- κ B and early growth response-1 (Egr-1), and cause oxidative stress (Ramasamy *et al.*, 2011; Wendt *et al.*, 2002). We observed increased RAGE and NF- κ B in the aorta and the kidney of fructose treated rats. These results suggest that fructose treatment increases MG levels, which in turn can activate RAGE, which then increases NF- κ B and oxidative stress. The increased oxidative stress increases expression of Ang II, AT₁ and α_{1D} receptors. The increased Ang II in turn can increase NF- κ B and oxidative stress (Hitomi *et al.*, 2007) and set up a vicious cycle. Our results suggest a possible sequence of molecular events resulting from elevated endogenous MG levels which have been reported in high carbohydrate diet fed animals and in diabetic patients (Hwang *et al.*, 1987; Wang *et al.*, 2008; Wang *et al.*, 2007; McLellan *et al.*, 1994).

Metformin is used clinically as an oral anti-diabetic drug in patients with type 2 diabetes. Metformin has multiple effects *in vivo*. For example, it inhibits mitochondrial respiration and gluconeogenesis in the liver, activates AMP-activated protein kinase, increases insulin sensitivity, antagonizes the action of glucagon and increases fatty acid oxidation (Rena *et al.*, 2013). These *in vivo* antidiabetic actions of metformin can make interpretation of results difficult, making it a less than ideal experimental MG scavenger. Even though the MG

scavenging ability of metformin has been studied in specific experiments and reported before (Beisswenger *et al.*, 1999; Ruggiero-Lopez *et al.*, 1999), we tested the MG scavenging ability of metformin in cultured vascular smooth muscle cells treated with MG, where multiple *in vivo*, especially hepatic, actions of metformin do not come into play. We also performed an *in vitro* MG scavenging assay for metformin. In cultured vascular smooth muscle cells metformin attenuated the increased expression of α_{1D} and AT₁ receptors, and Ang II (Fig. 5-7). These results support a direct effect of MG on these RAAS mediators and the MG scavenging action of metformin. As shown in table 1 metformin displayed significant MG scavenging ability when the two were mixed in solution.

5.6 Conclusions

In conclusion, high fructose diet fed rats had significantly elevated blood pressure, MG levels in the aorta, and the kidney, and increased expression of Ang II, AT₁ receptor, α_{1D} receptor, renin, RAGE and NF- κ B and decreased levels of reduced glutathione in the aorta and/or the kidney. The MG scavenger metformin attenuated these effects. Our results show a strong association between elevated levels of methylglyoxal, RAGE, NF- κ B, mediators of the renin angiotensin system and blood pressure in high fructose diet fed rats. Therefore, it is possible that MG could be a mediator of high fructose diet-induced hypertension, probably acting through RAGE and NF- κ B, and up regulating the renin angiotensin system.

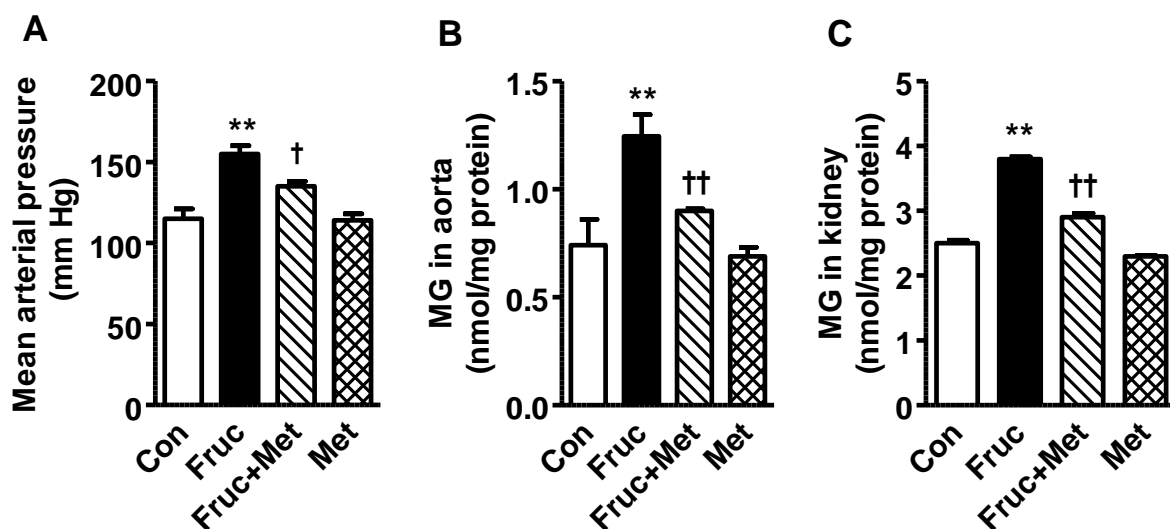


Figure 5-1. Mean arterial blood pressure and methylglyoxal (MG) levels in the aorta and kidney of high fructose diet treated Sprague-Dawley rats. Groups ($n = 8$ each) of 5 week old male Sprague-Dawley rats were treated for 16 weeks with a high fructose diet (Fruc, 60% of total calories). Control rats received normal chow. Metformin (Met, 500 mg/kg/day in drinking water) was used as a MG scavenger. Mean arterial pressure was measured with an intra-carotid artery catheter in anesthetized rats. MG levels were determined with HPLC. ** $P < 0.01$ vs. respective control (Con). † $P < 0.05$, †† $P < 0.01$ vs. respective Fruc group.

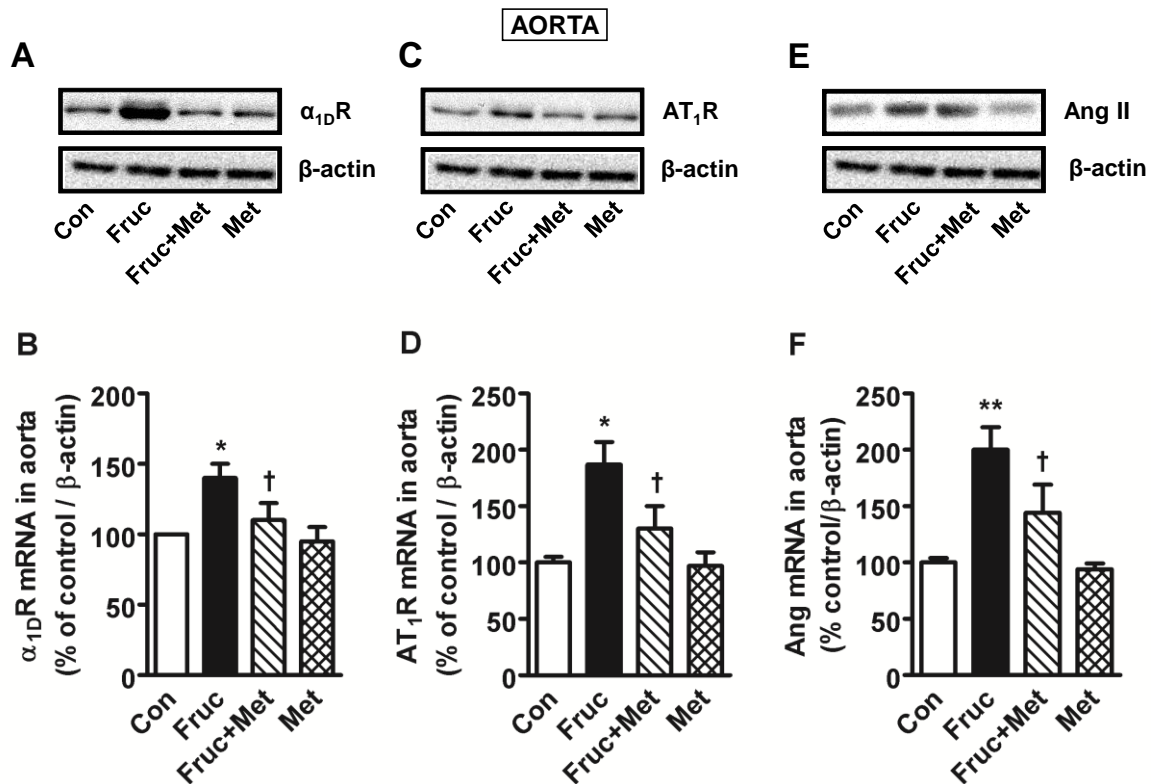


Figure 5-2. Adrenergic α_{1D} receptor ($\alpha_{1D}R$), AT₁ angiotensin receptor (AT₁R) and angiotensin II (Ang II) expression and mRNA in the aorta of high fructose diet treated Sprague-Dawley rats. Groups ($n = 8$ each) of 5 week old male Sprague-Dawley rats were treated for 16 weeks with a high fructose diet (Fruc, 60% of total calories). Control rats received normal chow. Metformin (Met, 500 mg/kg/day in drinking water) was used as a MG scavenger. Protein expression was determined by Western blotting using appropriate primary antibodies, and mRNA with RT-PCR. * $P < 0.05$, ** $P < 0.01$ vs. respective control (Con). † $P < 0.05$ vs. respective Fruc group.

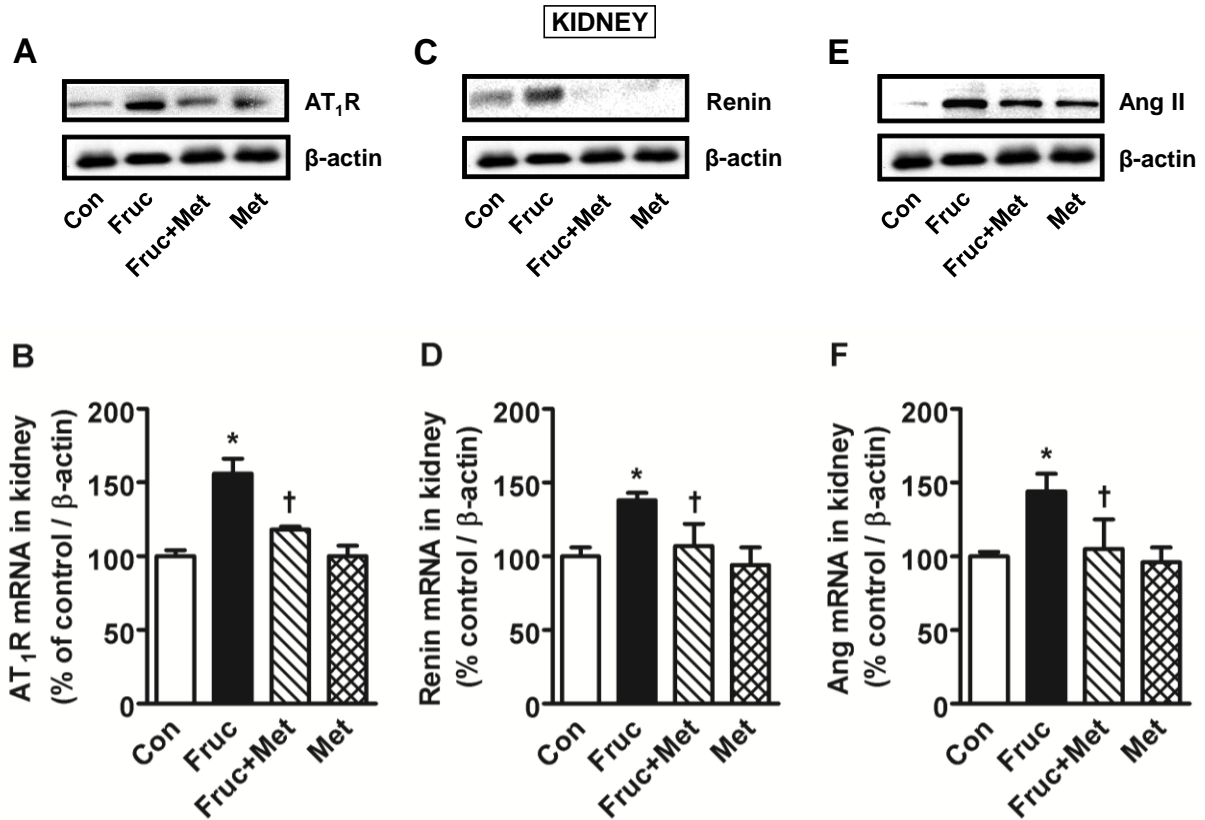


Figure 5-3. AT₁ angiotensin receptor (AT₁R), renin and angiotensin II (Ang II) expression and mRNA in the kidney of high fructose diet treated Sprague-Dawley rats. Groups ($n = 8$ each) of 5 week old male Sprague-Dawley rats were treated for 16 weeks with a high fructose diet (Fruc, 60% of total calories). Control rats received normal chow. Metformin (Met, 500 mg/kg/day in drinking water) was used as a MG scavenger. Protein expression was determined by Western blotting using appropriate primary antibodies, and mRNA with RT-PCR. * $P < 0.05$ vs. respective control (Con). † $P < 0.05$ vs. respective Fruc group.

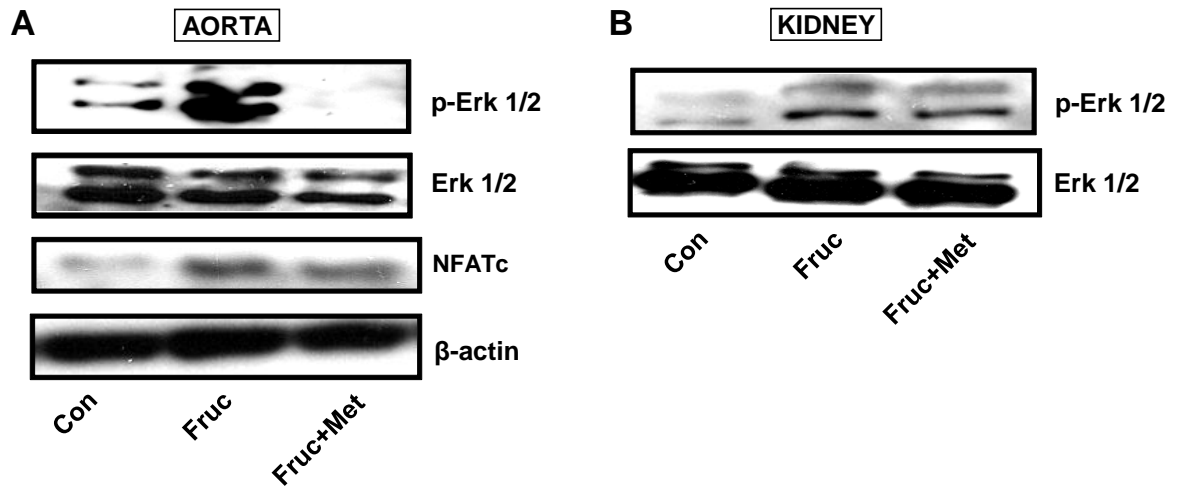


Figure 5-4. Phosphorylated extracellular signal-related kinases 1/2 (p-Erk 1/2), Erk 1/2 and NFATc expression in the aorta and kidney of high fructose diet treated Sprague-Dawley rats. Groups ($n = 8$ each) of 5 week old male Sprague-Dawley rats were treated for 16 weeks with a high fructose diet (Fruc, 60% of total calories). Control rats received normal chow. etformin (Met, 500 mg/kg/day in drinking water) was used as a MG scavenger. Protein expression was determined by Western blotting using appropriate primary antibodies.

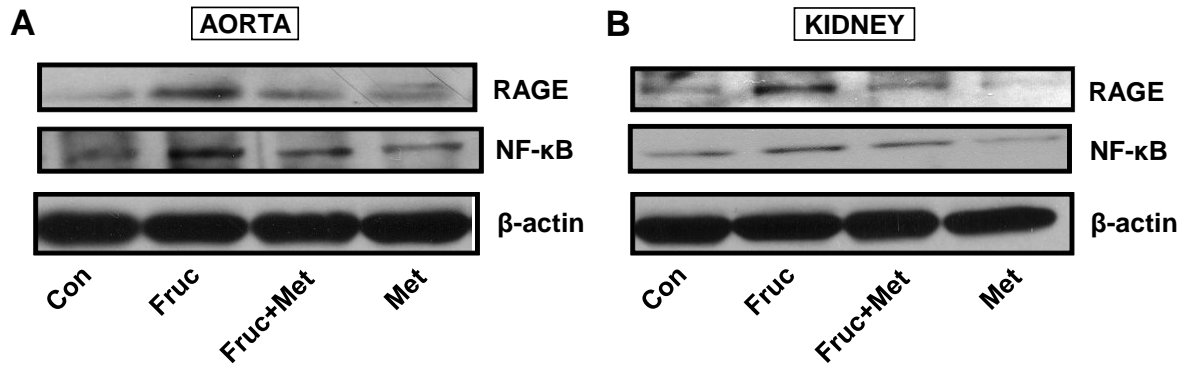


Figure 5-5. Receptor for advanced glycation endproducts (RAGE) and nuclear factor κ B (NF- κ B) expression in the aorta and kidney of high fructose diet treated Sprague-Dawley rats. Groups ($n = 8$ each) of 5 week old male Sprague-Dawley rats were treated for 16 weeks with a high fructose diet (Fruc, 60% of total calories). Control rats received normal chow. Metformin (Met, 500 mg/kg/day in drinking water) was used as a MG scavenger. Protein expression was determined by Western blotting using appropriate primary antibodies.

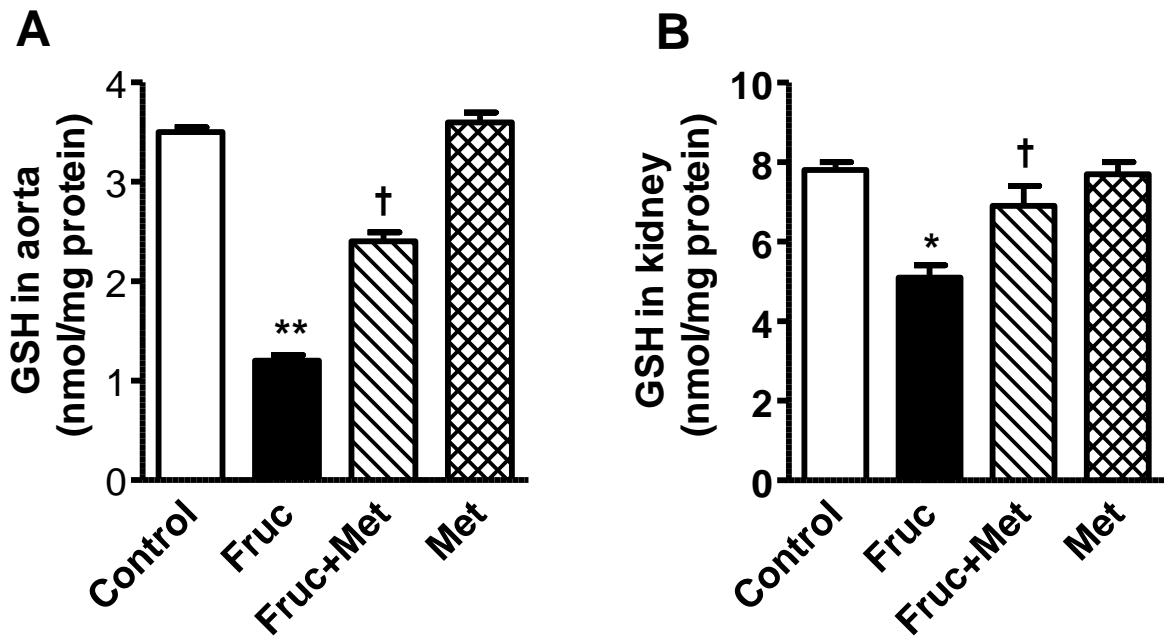


Figure 5-6. Reduced glutathione (GSH) levels in the aorta and kidney of high fructose diet treated Sprague-Dawley rats. Groups ($n = 8$ each) of 5 week old male Sprague-Dawley rats were treated for 16 weeks with a high fructose diet (Fruc, 60% of total calories). Control rats received normal chow. Metformin (Met, 500 mg/kg/day in drinking water) was used as a MG scavenger. Glutathione levels were determined with HPLC. * $P < 0.05$, ** $P < 0.01$ vs. respective control. † $P < 0.05$, vs. respective Fruc group.

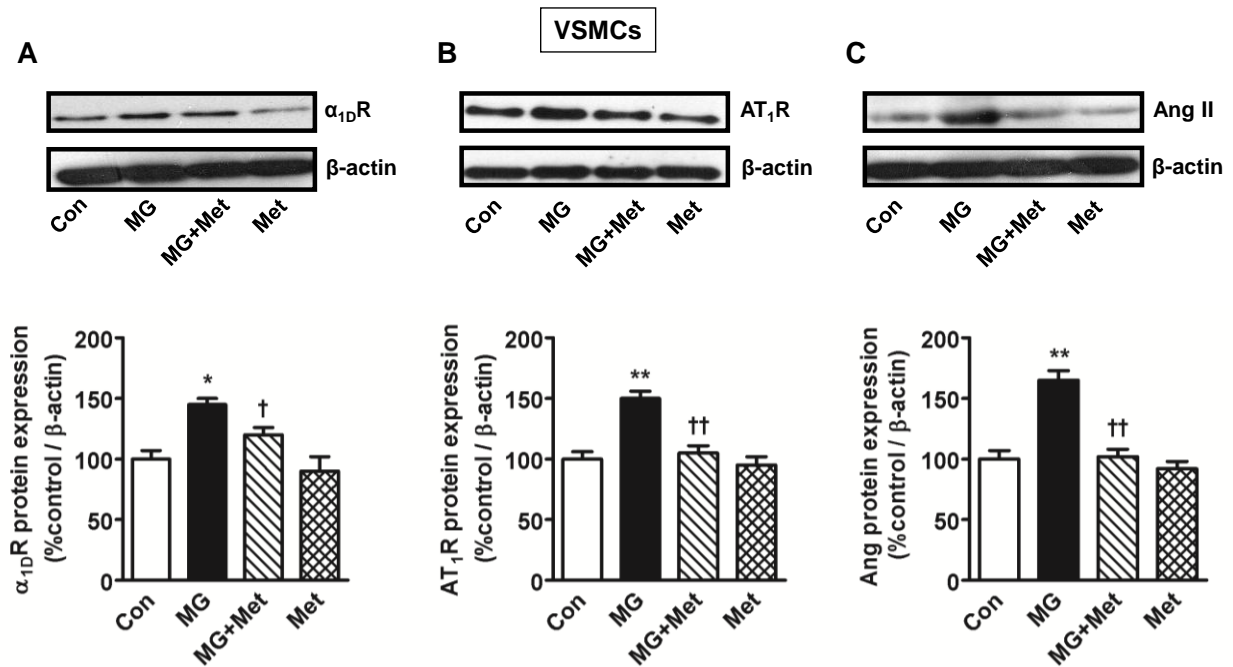


Figure 5-7. α_{1D} receptor ($\alpha_{1D}R$), AT1 receptor (AT_1R) and Angiotensin II (Ang II) expression in cultured vascular smooth muscle cells (VSMCs) treated with methylglyoxal.

Rat thoracic aorta smooth muscle cells (A10 cell line) were cultured and incubated with MG (30 μ M) for 24 h. Metformin (100 μ M) was used as a MG scavenger. Protein expression was determined by Western blotting using appropriate primary antibodies. $n = 4$ for each group.

* $P < 0.05$, ** $P < 0.01$ vs. respective control (Con). † $P < 0.05$, †† $P < 0.01$ vs. respective MG group.

Incubation time	MG (30 μM)	MG (30 μM) + metformin (100 μM)	MG (30 μM) + metformin (1 mM)	Metformin (100 μM)	Metformin (1 mM)
15 min	24.89 \pm 0.15	21.69 \pm 0.17	18.79 \pm 0.21	0	0
30 min	25.28 \pm 0.21	19.29 \pm 0.06	15.28 \pm 0.32	0	0
60 min	26.82 \pm 0.15	17.87 \pm 0.31	10.38 \pm 0.51*	0	0
3 h	25.77 \pm 0.14	12.52 \pm 0.08*	7.36 \pm 0.27**	0	0
24 h	25.23 \pm 0.31	9.43 \pm 0.41**	3.39 \pm 0.32***	0	0

Table 5-1. *In vitro* assay to determine the methylglyoxal (MG) scavenging ability of metformin. MG was incubated with metformin at different concentrations at 37°C for different times. The solution was analyzed for free MG by HPLC after the given incubation period. The values (μ M) are expressed as mean \pm SEM ($n = 6$ in each).

CHAPTER 6

Arginine attenuates methylglyoxal- and high glucose-induced endothelial dysfunction by an eNOS-independent mechanism

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6.1 Abstract

Aims. Methylglyoxal (MG), a reactive metabolite of glucose, has high affinity for arginine and is a major precursor of advanced glycation endproducts. Specific and safe MG scavengers are not available. We investigated whether L-arginine and D-arginine can attenuate endothelial dysfunction and oxidative stress induced by MG and high glucose, and implications for arginase for which L-arginine is a substrate.

Methods and results. We used isolated aortic rings from 12 week old male Sprague-Dawley rats and cultured human umbilical vein endothelial cells (HUVECs) and vascular smooth muscle cells (VSMCs). L- and D-arginine both attenuated MG and high glucose induced reduced endothelium-dependent relaxation by acetylcholine, but only L-arginine attenuated inhibition of relaxation by the nitric oxide synthase inhibitor, N ω -nitro-L-arginine methyl ester. MG and high glucose increased protein expression of arginase, NADPH oxidase 4, nuclear factor kappa B, and reactive oxygen species production in HUVECs and VSMCs, which were attenuated by L- and D-arginine. However, L- and D-arginine did not attenuate the MG and high glucose-induced increased arginase activity in VSMCs. MG and high glucose-induced increased formation of the MG-specific advanced glycation endproduct, N ϵ -carboxyethyl lysine, in VSMCs was attenuated by L- and D-arginine.

Conclusions. MG and high glucose increase arginase protein and activity which may contribute to endothelial dysfunction. L-arginine and D-arginine attenuate the endothelial dysfunction and increased oxidative stress induced by MG and high glucose by an endothelial nitric oxide synthase independent mechanism, despite increased arginase activity. The potential of arginine as a safe MG scavenger needs to be followed up.

6.2 Introduction

L-arginine (L-Arg), but not D-arginine (D-Arg), is a substrate for nitric oxide synthase (NOS), which catalyzes the formation of nitric oxide (NO) and L-citrulline (Palmer *et al.*, 1988). There are three isoforms of NOS, endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) (Moncada *et al.*, 1991). eNOS is mainly found in the endothelial cells and mediates endothelium-dependent agonist induced vessel relaxation (Moncada *et al.*, 1991). Reduced production or availability of NO is a common feature of endothelial dysfunction (De Vriese *et al.*, 2000; Potenza *et al.*, 2009), which is commonly defined as reduced endothelium-dependent vascular relaxation, and is a feature of diabetes, atherosclerosis, hypertension and several other conditions.

L-Arg, but not D-Arg, is also a substrate for arginase, which is an enzyme of the urea cycle that catalyzes the formation of urea and ornithine. There are two isoforms, arginase I and arginase II (Haraguchi *et al.*, 1987; Morris *et al.*, 1997). In blood vessels, arginases are mainly expressed in the endothelium and at low levels in vascular smooth muscle cells (VSMCs) (Wei *et al.*, 2000). Increased expression of VSMC arginase may contribute to intimal hyperplasia and vascular stiffness (Marinova *et al.*, 2008; Wei *et al.*, 2001). Endothelial arginase may be affecting eNOS function and can be responsible for endothelial dysfunction (Berkowitz *et al.*, 2003; Zhang *et al.*, 2001). High glucose (25 mM) has been shown to increase arginase I activity, but not protein expression, in bovine coronary endothelial cells, and arginase I is increased in the aorta of streptozotocin diabetic rats (Romero *et al.*, 2008), causing endothelial dysfunction (Ishizaka *et al.*, 2007; Romero *et al.*, 2008).

Methylglyoxal (MG) is produced during glucose, fatty acid and amino acid metabolism to varying degrees (Thornalley, 1996). MG is a reactive dicarbonyl molecule which can react with arginine (Shipanova *et al.*, 1997; Takahashi, 1977), lysine and cysteine residues (Ahmed *et al.*, 1997) of exposed functional groups of proteins and enzymes and cause their structural and functional disruption (Dhar *et al.*, 2010b; Jia *et al.*, 2006). MG is a major precursor for the formation of advanced glycation end products (AGEs) and is clearly of great pathological significance (Kilhovd *et al.*, 2003). Under physiological conditions the MG produced in the body is efficiently degraded by the glyoxalase enzymes and reduced glutathione (GSH) into D-lactate (Thornalley, 1993; Vander Jagt *et al.*, 2003) to maintain plasma MG levels at around 1 μ M or less. MG formation increases in diabetes (Thornalley, 1988) and diabetic patients have 3-4 fold elevated plasma MG levels (McLellan *et al.*, 1994; Wang *et al.*, 2007).

Incubation of vascular smooth muscle cells with 25 mM glucose or fructose for 3 h increases MG production 3.5 or 3.9 fold, respectively, and increases oxidative stress (Dhar *et al.*, 2008). We have recently shown that in cultured rat aortic and human umbilical vein endothelial cells MG and high glucose reduced basal and bradykinin-stimulated nitric oxide (NO) production, cyclic guanosine monophosphate levels, and serine-1177 phosphorylation and activity of endothelial nitric oxide synthase (eNOS) without affecting threonine-495 and Akt phosphorylation, and total eNOS protein (Dhar *et al.*, 2010b). Chronic treatment of Sprague-Dawley rats with MG for 4 weeks induces features characteristic of type 2 diabetes mellitus.

Evidently, safe and specific MG scavengers have the potential to prevent several different pathological conditions such as endothelial dysfunction, type 2 diabetes and AGEs formation. Currently, there is a lack of safe and specific MG scavengers. Several investigators have used

different compounds as MG scavengers, which have other actions and thus are non-specific and can even produce toxicity. These compounds include aminoguanidine (Brownlee *et al.*, 1986; Edelstein *et al.*, 1992), alagebrium (Dhar *et al.*, 2010a; Little *et al.*, 2005; Wolffenbuttel *et al.*, 1998), N-acetyl cysteine (Jia *et al.*, 2007; Vasdev *et al.*, 1998) and metformin (Beisswenger *et al.*, 2003; Ruggiero-Lopez *et al.*, 1999; Wang *et al.*, 2008). Since MG has been reported to have high affinity for arginine (Takahashi, 1977), our aim was to investigate and compare the effects of L-Arg and D-Arg on MG and high glucose-induced endothelial dysfunction and oxidative stress. We also examined the effects of MG on arginase expression and activity, which has not been reported previously.

6.3 Methods and materials

Animals

Male 11-week old Sprague-Dawley rats from Charles River Laboratories (Quebec, Canada) were used according to a protocol approved by the Animal Care Committee at The University of Saskatchewan, following guidelines of the Canadian Council on Animal Care. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). After one week of acclimatization, the rats were anesthetized with an inhalation of isoflurane (Forane, 4% in oxygen), delivered through a precision vaporizer and circle absorption breathing system (Ohio, 30/70 Proportioner Anesthesia Machine, Madison, Wisconsin). When anesthesia reached sufficient depth as determined by the absence of the leg flexor response and the eyelid reflex, the thorax was quickly opened by a midline incision and the rat was killed by cutting the heart open

and causing exsanguination. The aorta was quickly removed without damaging the endothelium and placed in a beaker filled with Krebs's solution and bubbled with 95% O₂ + 5% CO₂.

Isometric tension studies on aortic rings

A group of 24 Sprague-Dawley rats was used. Isometric tension studies were carried out on rat aortic rings as described (Dhar *et al.*, 2010b). Briefly, 3-4 mm thoracic aortic rings from Sprague-Dawley rat were mounted under a 2 g load in four separate 10 mL organ baths containing Krebs solution with 5 mM glucose and maintained at 37° C and bubbled with 95% O₂ + 5% CO₂. After a 90 min equilibration period the rings were pre-contracted with phenylephrine (1 μM) and cumulative concentration-dependent relaxation in response to acetylcholine (ACh) was obtained before (Control) and 2 h after incubation with either glucose (25 mM) or MG (100 μM) (Dhar *et al.*, 2010b), or the nitric oxide synthase inhibitor, N^ω-nitro-L-arginine methyl ester (L-NAME, 10 μM). In initial experiments the responses to ACh were repeated before and 2 h after incubation with normal Krebs solution to confirm reproducibility of responses to ACh. Some sets of rings were co-incubated with either L-Arg (300 μM) or D-Arg (300 μM) for 2 h. Treatment with each compound was tested in rings from at least 5 different rats. Isometric tension was measured with isometric force transducers with the 'Chart' software and Powerlab equipment (AD Instruments Inc., Colorado Springs, CO, USA).

Methlyglyoxal assay

MG was measured by a specific and sensitive HPLC method as described previously (Dhar *et al.*, 2009). MG was derivatized with *o*-phenylenediamine (*o*-PD) to specifically form 2-

methylquinoxaline. The samples were incubated in the dark for 24 h with 0.45 N perchloric acid (PCA) and 10 mM *o*-PD at room temperature. Samples were centrifuged at 12000 rpm for 10 min. 2-methylquinoxaline and quinoxaline internal standard (5-methylquinoxaline) were quantified on a Hitachi D-7000 HPLC system (Hitachi, Ltd., Mississauga, ON, Canada) *via* Nova-Pak® C18 column (3.9×150 mm, and 4 µm particle diameter, Waters Corporation, Milford, MA, USA).

Cell culture

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% fetal bovine serum (FBS) at 37° C in a humidified atmosphere of 95% air and 5% CO₂, as described in our previous studies (Dhar *et al.*, 2008). A-10 cells were seeded either in 100 mm dishes for MG measurement or in 96-well plates for other assays, with an equal amount of cells (10⁶/mL) in each well, and cultured to confluence. For immunocytochemistry staining, cells were seeded on cover glass slides (2×10⁶/mL). Cells were starved in FBS-free DMEM for 24 h before exposure to different metabolic precursors.

Human umbilical vein endothelial cells (HUVECs) from American Type Culture Collection were cultured in Kaighn's F12K medium containing 10% fetal bovine serum (FBS), 0.1 mg/mL heparin and 0.03-0.05 mg/mL endothelial cell growth supplement (Dhar *et al.*, 2010b).

Western blotting

Cell lysates were prepared as described earlier (Dhar *et al.*, 2010b; Dhar *et al.*, 2011) and the protein concentration in the supernatant was determined by the BCA Protein assay (Bio-Rad, Hercules, CA, USA). Aliquots of cell lysates (50 µg of protein each) were separated on 7.5-10% SDS-PAGE, electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), blocked with 5% nonfat milk in TBS-Tween buffer for 2 h at room temperature, and incubated overnight at 4° C with the primary antibodies to arginase I, arginase II, nuclear factor kappa B (NF-κB) or NADPH oxidase 4 (NOX4) (Santa Cruz, CA, USA), and then with horseradish peroxidase conjugated secondary antibody (Santa Cruz, CA, USA) for 1 h at room temperature. After extensive washing, the immunoreactive proteins were detected with an Enhanced Chemiluminescence Detection System (ECL; Amersham Biosciences Corp., Piscataway, NJ, USA) (Dhar *et al.*, 2010b; Dhar *et al.*, 2011).

Arginase activity assay

Arginase activity was measured using an arginase assay kit (BioAssays Systems, CA, USA). Arginase catalyses the conversion of arginine to ornithine and urea. Briefly, cell lysates were mixed with substrate arginine buffer and incubated for 2 h. Urea reagent was added to stop the reaction and the optical density measured at 430 nm.

Measurement of reactive oxygen species

Confluent cells were loaded with a membrane-permeable, nonfluorescent probe 2',7'-dichlorofluorescein diacetate (CM-H2DCFDA, 5 µM) for 2 h at 37° C in FBS-free medium in the

dark. The cells treated with MG (30 μ M) or glucose (25 mM) for 24 h were assayed for fluorescent oxidized dichlorofluorescein (DCF) as an indicator of production of reactive oxygen species (ROS) as described earlier (Dhar *et al.*, 2008; Dhar *et al.*, 2010b). The protein content of the homogenate was measured by BCA Protein assay (Bio-Rad, Hercules, CA, USA).

Immunocytochemistry

A-10 cells were seeded on glass cover slips followed by incubation with different test compounds for 24 h, and subjected to staining for the MG-induced AGE, N ϵ -carboxyethyl lysine (CEL). As described previously (Dhar *et al.*, 2008; Dhar *et al.*, 2011), the treated cells were fixed in 4% paraformaldehyde for 30 min at room temperature and washed twice with 0.01 N phosphate buffered saline (PBS). After permeation with 0.1% Triton X-100 for 5 min and two washes with PBS, the cells were incubated with normal goat serum (diluted 1:30 in 0.1 N PBS) for 1 h to block non-specific binding sites. After shaking off the goat serum the slides were incubated with the CEL antibody (1:100; a generous gift from Novo Nordisk, A/S, Denmark) overnight at room temperature. Cells were washed twice in PBS (0.01 N) for 5 min and incubated with secondary fluorescein isothiocyanate (FITC) conjugated anti-CEL antibody (Molecular Probes) for 2 h. After washing thrice with PBS the slides were mounted in glycerol:PBS (3:7), coverslipped and observed under a fluorescence microscope. Staining intensity was quantified using the Metamorph image analysis software (v. 7, Molecular Devices). Slides from four different experiments were analyzed with 5 fields per slide observed and averaged.

Chemicals and statistical analysis

All chemicals were of analytical grade. Methylglyoxal, D-glucose, L-arginine and D-arginine were purchased from Sigma Aldrich, Oakville, ON, Canada. Cell culture media and reagents were purchased from Invitrogen Canada Inc., Burlington, ON, Canada. Data are expressed as mean \pm SEM and analyzed using one way ANOVA and *post hoc* Bonferroni's test. The *P* value was considered significant when it was less than 0.05.

6.4 Results

L-arginine and D-arginine prevent methylglyoxal and high glucose induced reduction of acetylcholine-induced relaxation of aortic rings

In rat aortic rings precontracted with phenylephrine (1 μ M), MG (100 μ M, Fig. 6-1A, B) as well as high glucose (25 mM, Fig. 6-1C, D) incubated for 2 h in the bath caused significant inhibition of ACh-induced endothelium-dependent relaxation, which was prevented by coincubation of L-Arg (300 μ M, Fig. 6-1A, C) or D-Arg (300 μ M, Fig. 6-1B, D) with MG (Fig. 6-1A, B) or high glucose (Fig. 6-1C, D). Incubation of the eNOS inhibitor, N ω -nitro-L-arginine methyl ester (L-NAME 10 μ M, Fig. 6-1E, F) prevented ACh-induced relaxation, which was restored by co-incubation of L-Arg (300 μ M, Fig. 6-1E), but not D-Arg (300 μ M, Fig. 6-1F), with L-NAME.

L-Arg and D-Arg (300 μ M) did not affect ACh-induced relaxation of the rings (data not shown). MG and glucose did not affect endothelium-independent relaxation of aortic rings induced by sodium nitroprusside (data not shown).

L-arginine and D-arginine prevent high glucose-induced elevation of methylglyoxal levels in vascular smooth muscle cells

Incubation of VSMCs with MG (30 μ M) (Fig. 6-2A) or high glucose (25 mM) (Fig. 6-2B) for 24 h significantly increased the level of cellular MG to a similar extent, which was prevented by cocubation with L-Arg or D-Arg (300 μ M) (Fig. 6-2). L-Arg or D-Arg alone did not affect basal MG levels in VSMCs (Fig. 6-2).

Methylglyoxal and high glucose increase arginase I and II expression and activity

Incubation of HUVECs (Fig. 6-3A, C) or VSMCs (Fig. 6-3B, D) with MG (30 μ M) (Fig. 6-3A, B) or high glucose (25 mM) (Fig. 6-3C, D) for 24 h significantly increased arginase I and II protein expression in both cell types, which was attenuated by co-incubation with L-Arg (300 μ M) or D-Arg (300 μ M).

Incubation of VSMCs with MG (30 μ M) or high glucose (25 mM) for 24 h significantly increased total arginase activity (Fig. 6-3E). L-Arg (300 μ M) or D-Arg (300 μ M) co-incubated with MG or high glucose further increased arginase activity (Fig. 6-3E). However, L-Arg (300 μ M) or D-Arg (300 μ M) alone did not increase arginase activity (Fig. 6-3E).

L-arginine and D-arginine prevent methylglyoxal and high glucose-induced increased expression of nuclear factor κ B and NADPH oxidase 4

Incubation of cultured HUVECs (Fig. 6-4A, C) or VSMCs (Fig. 6-4B, D) with MG (30 μ M) (Fig. 6-4A, B) or with high glucose (25 mM) (Fig. 6-4C, D) for 24 h significantly increased

the protein expression of NF- κ B and NOX4 in both cell types, which was attenuated by co-incubation with L-Arg (300 μ M) or D-Arg (300 μ M) (Fig. 6-4).

L-arginine and D-arginine prevent methylglyoxal and high glucose-induced oxidative stress

Incubation of cultured HUVECs (Fig. 6-5) or VSMCs (Fig. 6-6) with MG (30 μ M or 100 μ M) (Figs. 6-5A, B and 6-6A, B), or with high glucose (25 mM) (Figs. 6-5C and 6-6C) for 3 h or 24 h significantly increased oxidative stress as measured by oxidized dichlorofluorescein. Both L-Arg (300 μ M) and D-Arg (300 μ M) co-incubated with MG or glucose attenuated the increase in oxidative stress (Figs. 6-5 and 6-6). L-Arg and D-Arg alone did not affect basal DCF levels (data not shown).

L-arginine and D-arginine prevent methylglyoxal and high glucose-induced increased formation of N ϵ -carboxyethyl lysine (CEL)

Incubation of cultured VSMCs with MG (30 μ M) or with high glucose (25 mM) (Figs. 6-5C and 6-6C) for 24 h significantly increased the formation of the MG-specific AGE, CEL (bright green stain) (Fig. 6-7). Co-incubation with L-Arg (300 μ M) or D-Arg (300 μ M) attenuated the formation of CEL (Fig 6-7).

6.5 Discussion

We aimed to investigate the potential of arginine as a MG scavenger by focusing on endothelial dysfunction induced by MG and high glucose. We have reported the molecular

mechanisms of eNOS dysfunction caused by MG and high glucose in a recent paper (Dhar *et al.*, 2010b), so we investigated whether L-Arg and D-Arg can attenuate MG and high glucose-induced reduced ACh-induced relaxation in aortic rings. Additionally and importantly, we also considered effects on arginase since it has been recognized as a contributor to endothelial dysfunction (Berkowitz *et al.*, 2003; Ishizaka *et al.*, 2007) and also uses L-Arg as a substrate (Morris, 2009). We report the following novel findings: 1. MG up regulates arginase I and arginase II protein expression in cultured HUVECs and VSMCs, 2. MG increases arginase activity in VSMCs, 3. high glucose up regulates arginase I and II protein expression and activity in VSMCs, 4. L-Arg and D-Arg attenuate the effects of MG and high glucose in rat isolated aortic rings and cultured HUVECs and VSMCs for the parameters tested except for increased arginase activity, and the effect of L-Arg and D-Arg against MG and high glucose is most likely independent of eNOS.

We examined endothelial dysfunction, which is a hallmark of prediabetes (Su *et al.*, 2008), type 1 and type 2 diabetes (De Vriese *et al.*, 2000; Potenza *et al.*, 2009) and precedes the development of cardiovascular complications of diabetes (De Vriese *et al.*, 2000; Potenza *et al.*, 2009; Su *et al.*, 2008). It has been widely ascribed to reduced NO availability (De Vriese *et al.*, 2000; Dhar *et al.*, 2010b; Potenza *et al.*, 2009). We have recently shown that MG and high glucose cause reduced serine 1177 phosphorylation and activity of eNOS, NO production and ACh-induced endothelium-dependent relaxation, which was prevented by the MG scavenger aminoguanidine (Dhar *et al.*, 2010b).

L-Arg as well as D-Arg attenuated the reduced ACh-induced endothelium dependent relaxation of the aortic rings, but only L-Arg prevented the inhibition of ACh-induced relaxation

by the NOS inhibitor, L-NAME (Fig. 6-1). L-Arg, but not D-Arg, is a substrate of NOS (Palmer *et al.*, 1988) and since D-Arg also prevented MG and high glucose-induced reduced relaxation but not that caused by L-NAME, it strongly suggests that arginine prevents MG and high glucose-induced reduced relaxation by an eNOS independent mechanism.

One of the deleterious effects of MG and high glucose is an increase in oxidative stress (Dhar *et al.*, 2008; Dhar *et al.*, 2010b; Wu *et al.*, 2002), which can be attributed to increased activity of NADPH oxidase (Dhar *et al.*, 2010b) and NF- κ B (Wu *et al.*, 2002). We have recently shown that MG and high glucose cause reduced ACh-induced relaxation of aortic rings partly by increasing NADPH oxidase activity and oxidative stress (Dhar *et al.*, 2010b). In this study L-Arg and D-Arg attenuated the increased expression of NOX4 and NF- κ B (Fig.6-4) and increased production of free radicals caused by MG and high glucose (Figs. 6-5, 6-6). Oxidative stress can be increased by multiple mechanisms. MG is a key inducer of oxidative stress (Desai *et al.*, 2008) and scavenging MG would prevent activation of multiple pathways of increased free radical generation.

Arginase has also been implicated in causing endothelial dysfunction, mainly by competing with eNOS for the common substrate, L-Arg (Berkowitz *et al.*, 2003; Romero *et al.*, 2008). If L-Arg and D-Arg are to be used to attenuate the effects of MG or high glucose, then their effects on arginase activity have to be considered. High glucose has been reported to cause increased expression of arginase in endothelial cells (Romero *et al.*, 2008). The effect of MG on arginase has not been studied. We report for the first time that MG increases arginase I and II expression in endothelial cells and VSMCs. We also found that high glucose also increases arginase I and II expression in VSMCs (Fig. 6-3) besides endothelial cells. L-Arg and D-Arg

attenuated the increased arginase expression caused by MG and high glucose in both cell types (Fig. 6-3), possibly by scavenging MG. MG and high glucose also increased the activity of arginase, which seems to be independent of increased protein expression because, even when the protein expression of arginase was normalized by co-treatment with MG or high glucose and L-Arg or D-Arg, we still observed increased arginase activity (Fig.6-3). Moreover, L-Arg and D-Arg alone did not increase arginase activity. While, the increased arginase activity seen with MG or high glucose plus L-Arg can be explained by increased substrate availability, the increased arginase activity seen with MG or high glucose plus D-Arg is surprising and needs further evaluation. One very assuring finding is that the use of L-Arg or D-Arg to prevent the deleterious effects of MG and high glucose on endothelial function or possibly on other biological functions, will not be compromised by their effects on arginase or eNOS.

A major deleterious effect of high glucose and reactive dicarbonyl metabolic intermediates, such as MG, glyoxal and 3-deoxyglucosone, is the formation of AGEs ([Ahmed *et al.*, 1997](#); [Glomb *et al.*, 2001](#); [Kilhovd *et al.*, 2003](#); [Shipanova *et al.*, 1997](#)), which are strongly implicated in the pathogenesis of several conditions such as vascular complications of diabetes, neurodegenerative diseases, atherosclerosis and aging ([Vlassara *et al.*, 1994](#)). The attenuation of the formation of the MG-specific AGE, CEL, in VSMCs (Fig. 6-7) is very promising in this regard. Prevention of dicarbonyls-induced AGEs, which is a major source of AGEs formation ([Kilhovd *et al.*, 2003](#); [Mostafa *et al.*, 2007](#); [Vlassara *et al.*, 1994](#)), by safer and specific scavengers of reactive dicarbonyls is an attractive therapeutic option. The potential of L-Arg and D-Arg in this regard is discussed below.

Besides its effects on endothelial function, MG has other effects. Thus, acute and chronic MG cause pancreatic β cell dysfunction, reduced adipose tissue glucose uptake, reduced insulin secretion and type 2 diabetes in Sprague-Dawley rats (Dhar *et al.*, 2010a; Dhar *et al.*, 2011). MG has been shown to modify insulin making it dysfunctional (Jia *et al.*, 2006), and LDL making it more atherogenic (Rabbani *et al.*, 2011). Scavengers of MG can be very effective in preventive strategies against the deleterious effects of hyperglycemia, hyperfructosemia (another source of MG) (Dhar *et al.*, 2008) and elevated MG levels.

Unfortunately, specific and safe MG scavengers are not available. The compounds currently used for their MG scavenging ability are non-specific and have other effects, which can even be toxic. Aminoguanidine is the most widely used MG scavenger and AGEs inhibitor (Brownlee *et al.*, 1986; Edelstein *et al.*, 1992), which unfortunately proved toxic in clinical trials (Bolton *et al.*, 2004; Freedman *et al.*, 1999). Metformin, an orally used drug to reduce hyperglycemia in type 2 diabetes, and N-acetyl cysteine also have MG scavenging ability but have other effects (Beisswenger *et al.*, 2003; Jia *et al.*, 2007; Millea, 2009; Ruggiero-Lopez *et al.*, 1999; Vasdev *et al.*, 1998). MG has great affinity for L-Arg and is proposed to react rapidly with the guanidino group of L-Arg (Takahashi, 1977). Since arginine has strong affinity for MG, it is highly likely that arginine binds MG, administered from outside and produced from glucose and other sources in the body, and thus, attenuates the deleterious effects of MG and high glucose on endothelial function. While L-arginine is used as a nutritional supplement, it is a substrate for five different enzymes including eNOS and arginase (Morris, 2009). The metabolic consequences of orally administered L-Arg, which is known to enter multiple metabolic processes in the liver and the body, are difficult to predict. D-Arg, the inactive isomer of L-Arg,

may prove to have more attractive pharmacokinetic and pharmacodynamic properties, and therapeutic potential than L-Arg, if its MG scavenging ability can be demonstrated to be specific at multiple functional and biochemical levels. Thus, D-Arg needs to be evaluated more rigorously for its pharmacokinetic properties and metabolic fate.

In conclusion, L-Arg and D-Arg attenuate MG and high glucose induced endothelial dysfunction, by an eNOS independent mechanism, most probably by binding and inactivating MG, given from outside or produced from high glucose. MG increases arginase I and II expression and activity. L-Arg and D-Arg attenuate the increased protein expression but not the increased activity of arginase induced by MG and high glucose, which did not restrict the attenuation of endothelial dysfunction. The effects of L-Arg and D-Arg on the other deleterious effects of MG and high glucose, need to be evaluated by many more separate studies, for example on MG-induced pancreatic islet dysfunction and insulin resistance, before definitive statements on their therapeutic potential can be made.

Acknowledgements

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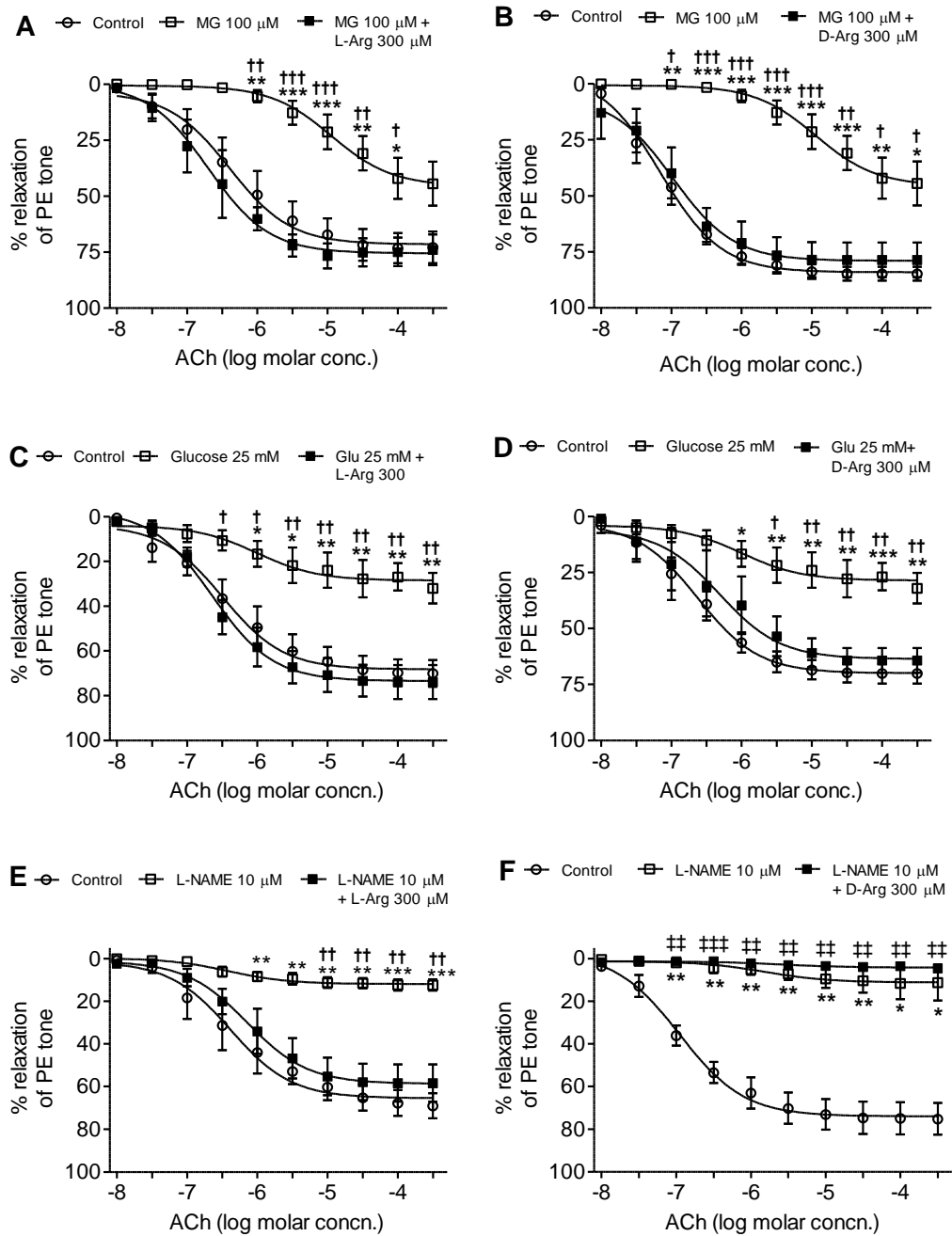


Figure 6-1. L-arginine and D-arginine attenuate MG and high glucose induced, but not inhibition of eNOS-induced, reduced endothelium-dependent relaxation in isolated aortic rings from Sprague-Dawley rats.

Concentration-related responses were obtained to ACh in phenylephrine (PE, 1 μ M) precontracted rings before (control) and 2 h after incubation with MG (100 μ M) (A and B), or glucose (Glu, 25 mM) (C and D) or 15 min after incubation with the eNOS inhibitor L-NAME (10 μ M) (E and F). In some sets of rings L-arginine (L-Arg, 300 μ M) or D-arginine (D-Arg, 300 μ M) was preincubated for 15 min followed by co-incubation for 2 h with MG (100 μ M) (A and B), or Glu (25 mM) (C and D) or for 15 min with L-NAME (10 μ M) (E and F). ($n = 6$ rings from different rats for each treatment group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. corresponding control values. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ vs. corresponding L-Arg or D-Arg co-treated values. $\#\dagger P < 0.01$, $\#\dagger\dagger P < 0.001$ vs. corresponding control values.

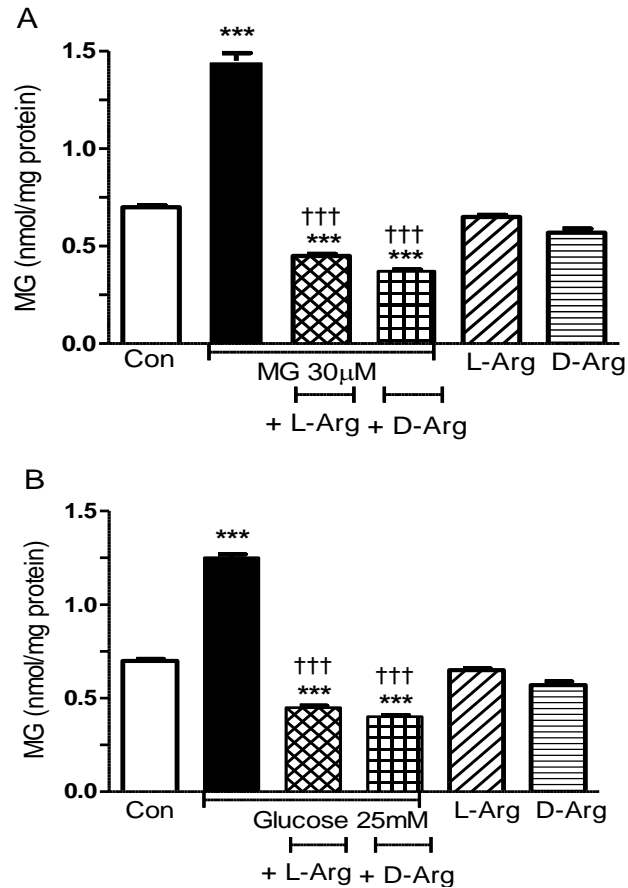


Figure 6-2. Exogenous methylglyoxal (MG) and high glucose and increase cellular MG levels in cultured vascular smooth muscle cells: attenuation by L-arginine and D-arginine.

Confluent rat aortic vascular smooth muscle cells were incubated with normal culture medium (Control, Con) or medium containing MG (30 μ M) (A), or glucose (25 mM) (B), for 24 h. L-arginine (L-Arg, 300 μ M) or D-arginine (D-Arg, 300 μ M) was incubated alone or with MG (30 μ M) (A) or glucose (25 mM) (B) for 24 h. Cellular MG was measured by HPLC. $n = 6$ for each treatment. *** $P < 0.001$ vs. corresponding control value, ††† $P < 0.001$ vs. corresponding MG (30 μ M) (A), or glucose (25 mM) value (B).

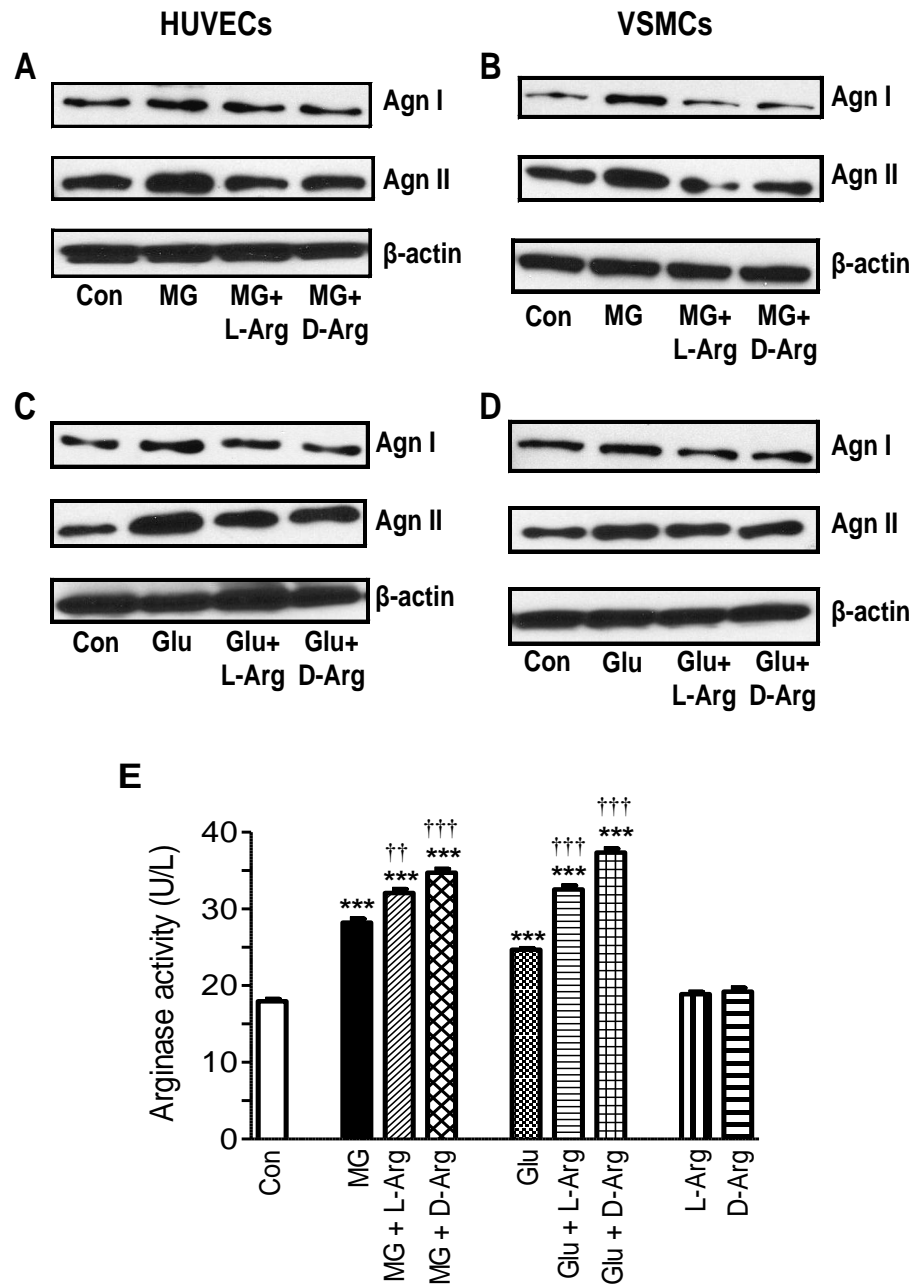


Figure 6-3. Attenuation by L-arginine and D-arginine of methylglyoxal (MG) and high glucose-induced increased protein expression, but not activity of arginase (Agn) I and II in

cultured human umbilical vein endothelial cells (HUVECs) and vascular smooth muscle cells (VSMCs).

Cultured confluent HUVECs (A, C) or VSMCs (A-10 cell line) (B, D, E) were incubated with MG (30 μ M) or glucose (Glu, 25 mM) for 24 h. In separate dishes L-arginine (L-Arg, 300 μ M) or D-arginine (D-Arg, 300 μ M) was incubated alone (E) or coincubated with MG (30 μ M) or glucose (25 mM) for 24 h following which the arginase I and arginase II proteins were determined by western blotting (A-D). In (E) an arginase activity assay was performed 24 h after the treatments, based on conversion of arginine to ornithine and urea, using an activity assay kit. $n = 6$ for each group. *** $P < 0.001$ vs. control, †† $P < 0.01$, ††† $P < 0.001$ vs. corresponding MG 30 μ M or glucose 25 mM value.

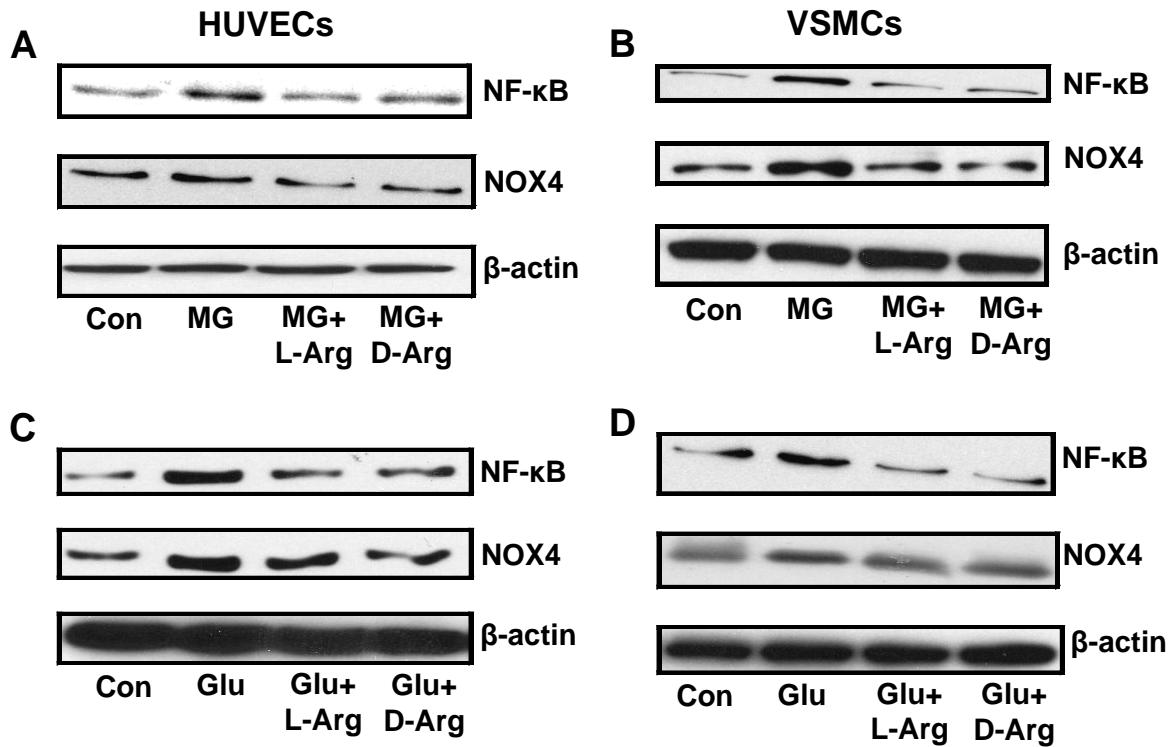


Figure 6-4. Attenuation by L-arginine and D-arginine of methylglyoxal (MG) and high glucose-induced increased protein expression of nuclear factor kappa B (NF-κB) and NADPH oxidase 4 (NOX-4) in cultured human umbilical vein endothelial cells (HUVECs) and vascular smooth muscle cells (VSMCs).

Cultured confluent HUVECs (A, C) or VSMCs (A-10 cell line) (B, D) were incubated with MG (30 μM) or glucose (Glu, 25 mM) for 24 h. In separate dishes L-arginine (L-Arg, 300 μM) or D-arginine (D-Arg, 300 μM) was coincubated with MG (30 μM) or glucose (25 mM) for 24 h following which the NF-κB and NOX-4 proteins were determined by western blotting.

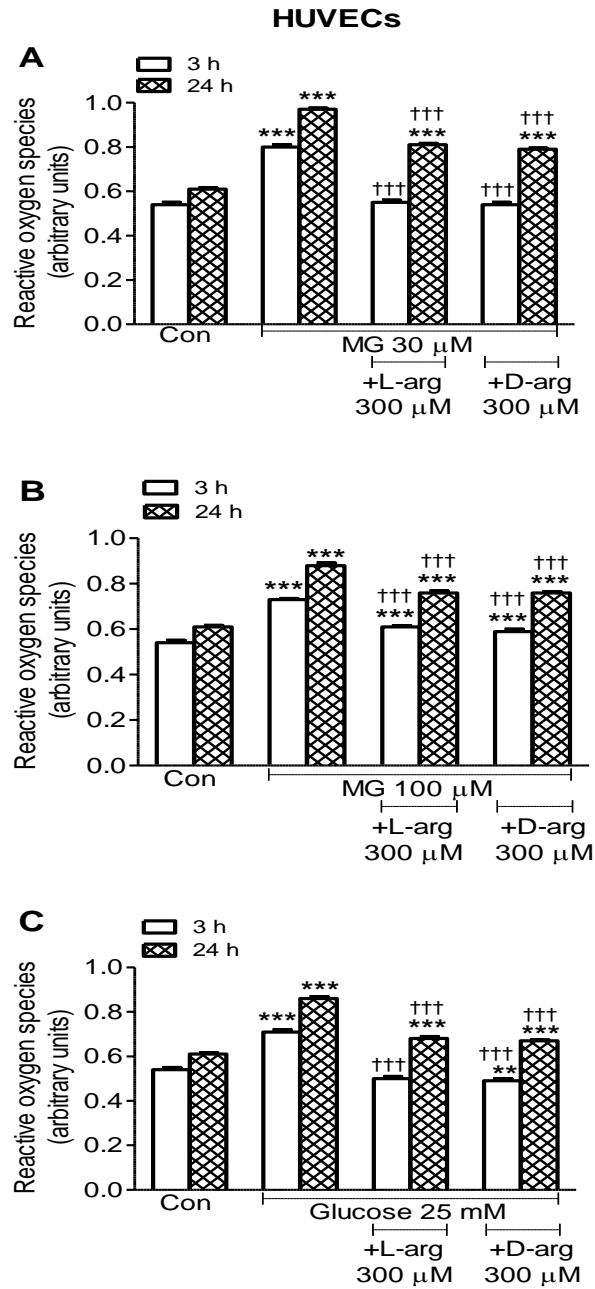


Figure 6-5. Attenuation by L-arginine and D-arginine of methylglyoxal (MG) and high glucose-induced increased reactive oxygen species production in cultured human umbilical vein endothelial cells (HUVECs).

Incubation of cultured HUVECs with MG (30 μ M) (A), or MG (100 μ M) (B) or glucose (25 mM) (C) for 3 h or 24 h increased reactive oxygen species (ROS) production, measured as oxidized dichlorofluorescein (DCF), which was attenuated by co-incubation with either L-arginine (L-Arg, 300 μ M) or D-arginine (D-Arg, 300 μ M). $n = 8$ for each group. $**P < 0.01$, $***P < 0.001$ vs. corresponding control, $^{\dagger\dagger}P < 0.001$ vs. corresponding MG (30 μ M), or MG (100 μ M), or glucose (25 mM) value.

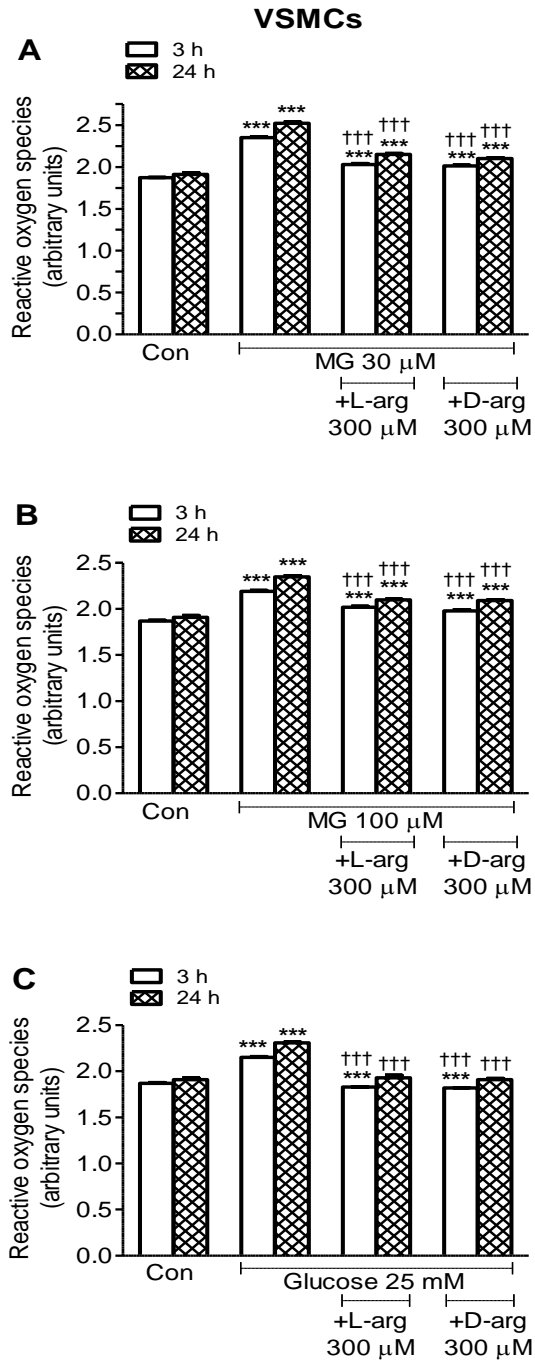


Figure 6-6. Attenuation by L-arginine and D-arginine of methylglyoxal (MG) and high glucose-induced increased reactive oxygen species production in cultured rat aortic

vascular smooth muscle cells (VSMCs).

Incubation of cultured VSMCs (A-10 cell line) with MG (30 μ M) (A), or MG (100 μ M) (B) or glucose (25 mM) (C) for 3 h or 24 h increased reactive oxygen species (ROS) production, measured as oxidized dichlorofluorescein (DCF), which was attenuated by co-incubation with either L-arginine (L-Arg, 300 μ M) or D-arginine (D-Arg, 300 μ M). $n = 8$ for each group. *** $P < 0.001$ vs. corresponding control, $\dagger\dagger P < 0.001$ vs. corresponding MG (30 μ M), or MG (100 μ M), or glucose (25 mM) value.

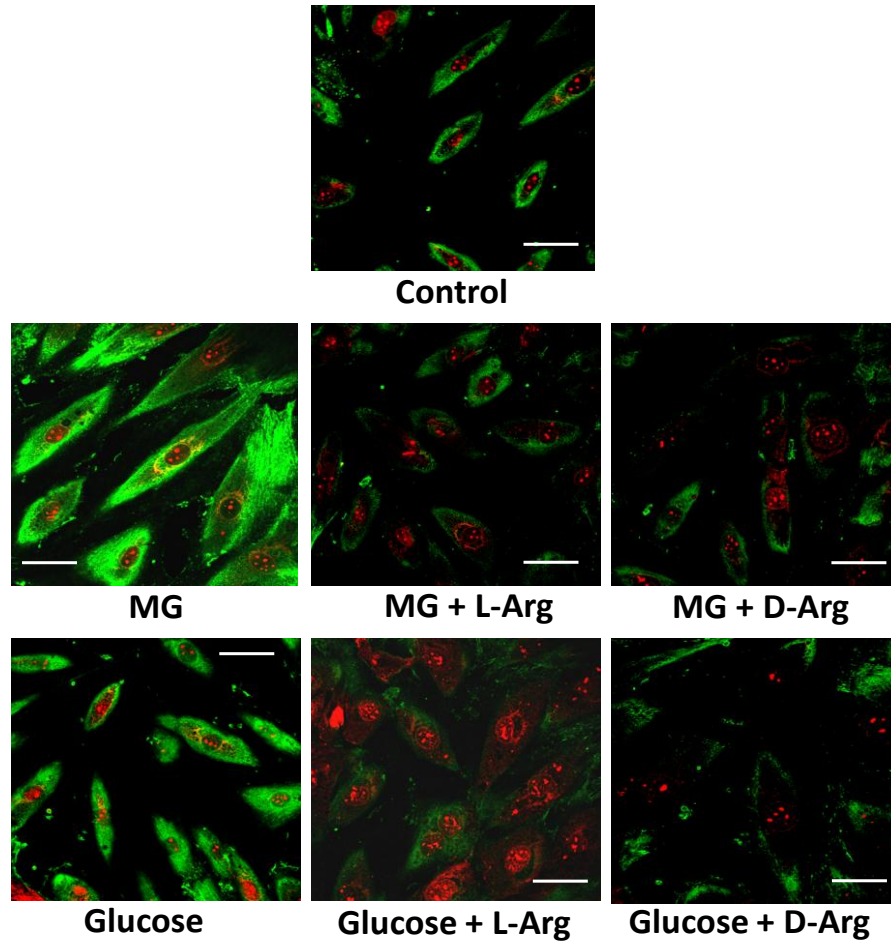


Figure 6-7. Detection of methylglyoxal-induced advanced glycation endproduct (AGEs). N^ε-carboxyethyl-lysine (CEL).

AGEs in cultured rat aortic vascular smooth muscle cells (A-10 cells) were detected after incubation with MG (30 μ M) or glucose (25 mM) alone, or coincubated with either L-arginine (L-Arg, 300 μ M) or D-arginine (D-Arg, 300 μ M) for 24 h. Immunocytochemistry was performed on fixed cells with specific CEL antibody and fluorescein isothiocyanate (FITC) conjugated secondary antibody. Scale bar 10 μ m.

Incubation Time	MG (30 μM) Alone	MG (30 μM) +L-Arg (300 μM)	MG (30 μM) +D-Arg (300 μM)	MG (30 μM) + NAC (600 μM)	NAC (600 μM)	L-Arg (300 μM)	D-Arg (300 μM)
15 min	26.7 \pm 0.7	12.6 \pm 0.7 ^{***}	12.2 \pm 0.6 ^{***}	15.2 \pm 0.7 ^{***}	0	0	0
30 min	26.2 \pm 0.7	7.4 \pm 0.5 ^{***}	7.00 \pm 0.3 ^{***}	8.8 \pm 0.6 ^{***}	0	0	0
60 min	27.2 \pm 0.6	5.7 \pm 0.2 ^{***}	4.3 \pm 0.12 ^{***}	7.00 \pm 0.3 ^{***}	0	0	0
3 h	27.6 \pm 0.6	3.5 \pm 0.09 ^{***}	2.1 \pm 0.05 ^{***}	5.3 \pm 0.06 ^{***}	0	0	0
24 h	26.3 \pm 0.6	1.2 \pm 0.07 ^{***}	0.8 \pm 0.08 ^{***}	2.7 \pm 0.08 ^{***}	0	0	0

Table 6-1. Methylglyoxal incubation assay

Methylglyoxal (MG) was incubated with L-arginine (L-Arg), D-arginine (D-Arg) or N-acetyl cysteine (NAC) in PBS at 37° C for different times. The solution was analyzed for free MG by HPLC after the given incubation period. The values are expressed as mean \pm SEM ($n = 6$ each). *** $P < 0.001$ vs. MG alone.

CHAPTER 7

DISCUSSION AND CONCLUSIONS

7.1 GENERAL DISCUSSION

The association of diabetes and hypertension is well known. It adds to the treatment challenges of diabetes. The pathogenesis of hypertension in diabetics is unclear. For example, it is not clear if it is due to hyperglycemia *per se* or due to insulin resistance or some other cause. It may even be due to obesity (Go *et al.*, 2013; Landsberg *et al.*, 2013) which is the highest risk factor for type 2 diabetes. Up regulation of the RAAS has been reported in diabetes (Hsueh and Wyne, 2011; Goossens, 2012). Our lab has reported an elevated level of plasma and tissue MG in SHR and fructose fed SD rats, an animal model of insulin resistance that also develops hypertension (Wang *et al.*, 2005; Wang *et al.*, 2008). High fructose-diet in SD rats (Wang *et al.*, 2008) had increased plasma and aortic MG levels, and significantly increased BP. However, the molecular mechanisms were not examined and it was not clear whether the elevated MG levels were the cause or effect of hypertension. For example, one popular theory proposes that metabolism of fructose by fructokinase, mainly in the liver, consumes ATP and raises tissue and plasma levels of uric acid (Johnson *et al.*, 2007; Stirpe *et al.*, 1970). Elevated uric acid reduces NO production in endothelial cells and the kidney, and causes endothelial dysfunction and increased renin secretion (Mazzali *et al.*, 2001). Thus, in the high fructose-diet study (Wang *et al.*, 2008) it is possible that elevated BP may be caused by increased MG levels. Similarly, in the SHR (Wang *et al.*, 2004; Wang *et al.*, 2005), it was not clear whether the hypertension was caused by elevated MG in the plasma, the aorta and the kidney. The molecular mechanisms were not addressed in these studies. To evaluate whether MG is directly involved in the pathogenesis of hypertension, the logical step was to administer MG to normal animals, such as Sprague-

Dawley rats and to investigate whether it induces hypertension and if so what is/are the possible mechanism/s.

MG was administered to SD rats for some preliminary data. It is a known fact that MG is a potent inducer of oxidative stress, and oxidative stress has been implicated as one of the causative factors of hypertension. Also, up regulation of the RAAS has been reported in hyperglycemia. Increased oxidative stress and RAAS are known to feed each other ([Hitomi *et al.*, 2007](#)). Since increased MG, oxidative stress, and RAAS have links it was hypothesized that MG up regulates RAAS, but this idea has not been tested before. Some common mediators of the RAAS are the AT₁ receptor for angiotensin, Ang II and adrenergic α_1 receptor. It was therefore decided to look at these parameters in cultured VSMCs treated with MG or high glucose. The initial experiments on VSMCs treated with MG were positive. Therefore, I made a protocol to treat SD rats with MG.

MG was administered to rats in drinking water in preliminary studies, and it did not significantly increase the plasma MG levels. One possible reason for this could be that MG being highly reactive is likely to react with proteins in the epithelial cells of the intestines, as reported in one study ([Baskaran *et al.*, 1990](#)). This might hinder the absorption of MG. MG is most likely generated continuously within the body from the ongoing metabolic processes. To simulate this situation as best as possible, MG was administered by continuous infusion with a minipump. Our lab has had great success with this mode of administration in a previous study ([Dhar *et al.*, 2011](#)) where the plasma concentration significantly increased to values reported in pathological conditions.

Treatment of SD rats with MG for 4 weeks resulted in a significant increase in the BP, and MG in the plasma, aorta and the kidney. The kidney plays a major role in BP regulation and RAAS activity, therefore, elevation of renal MG levels was of great interest. There was an elevation of plasma catecholamines, renin, angiotensin and aldosterone levels in MG treated rats, which was building up our evidence of up regulated RAAS. The effectors of RAAS were investigated and there was an increased expression of angiotensinogen, AT₁, α_{1D}, and renin in aorta and/or kidney of MG treated rats. One reason for choosing the aorta is that the expression of tissue RAAS has been well studied in the aorta (Campbell, 1987; Holycross *et al.*, 1992; Naftilan *et al.*, 1989). For example, the activity of VSMCs RAS is increased in conditions such as hypertension and atherosclerosis (Dzau, 1993). There were not enough of fat free mesenteric vessels to determine angiotensinogen, AT₁, α_{1D} in this tissue. To investigate the possible molecular mechanisms of how MG caused up regulation of the RAAS, cultured VSMCs were used, where interference from interacting factors found *in vivo* can be avoided. Normally, activation of a receptor by a ligand produces its effects through a simple or complicated signal transduction process. RAGE is a receptor for AGEs. It has two main functions. The membrane bound RAGE is believed to increase oxidative stress in response to stimulation by AGEs. However, MG has never been investigated as a ligand for RAGE. This idea was tested for the first time, and showed that silencing of RAGE prevented the increase in the expression of NF-κB, a key trigger of increased oxidative stress (Wu *et al.*, 2002; Desai and Wu, 2008). So it established a likely initiating point for the signal transduction of MG that up regulates the RAAS and increases BP. Next I tried inhibiting NF-κB with CAY10512. Surely enough, inhibition of NF-κB prevented the increase in AT₁ and α_{1D} receptors in VSMCs. Ang II is known to up

regulate its own receptor and also NF- κ B. To further establish the signal transduction I silenced angiotensinogen and was able to attenuate the increase in AT₁, α_{1D} , and also NF- κ B, caused by MG. NF- κ B can activate several oxidative stress pathways.

High dietary carbohydrates have been shown to increase BP in animal and human studies. High fructose levels in the diet in the form of sugar and high fructose corn syrup have created great controversy about their adverse health effects (Lustig *et al.*, 2012; Lustig, 2010). The main dietary carbohydrate in our diet is sucrose, in the form of table sugar and high fructose corn syrup (HFCS). Sucrose in the form of table sugar is broken down into one molecule of fructose and glucose each in the body. On the other hand high fructose corn syrup is prepared by mixing free glucose and fructose in different proportions. The two most commonly used form of high fructose corn syrup are HFCS 55 (55% fructose and 42% glucose) and HFCS 42 (42% fructose and 53% glucose) (Fulgoni, 2008). Glucose and fructose are metabolized differently in the body (Bruckdorfer *et al.*, 1973; Johnson *et al.*, 2007; Johnson *et al.*, 2009; Nakagawa *et al.*, 2006; Wolfe *et al.*, 1977), yet a high glucose, high fructose as well as a high sucrose diet have all been shown to increase BP in animal and human studies (Raben *et al.*, 2002; Israel *et al.*, 1983; Tom *et al.*, 2011; Kaufman *et al.*, 1991; Hwang *et al.*, 1987; Jia & Wu, 2007). Male SD rats fed a high glucose diet developed a 7% increase in BP compared to rats fed a normal diet (Kaufman *et al.*, 1991). SD rats fed with a diet containing 66% fructose as a percentage of total calories for approximately 2 weeks developed a significant increase in systolic BP from 124 ± 2 to 145 ± 2 (SEM) mmHg, whereas no change occurred in the control group (Hwang *et al.*, 1987). Even lower doses of fructose (10% in drinking water) given to SD rats for 8 weeks induced a significant increase in systolic BP and microvascular changes in the kidney (Sanchez-Lozada *et*

al., 2007). In a study performed in Dr Wu's lab, male SD rats fed a 60% fructose diet for 16 weeks showed an increase in the BP from about 110 mmHg to about 155 mmHg at the end of 16 weeks of treatment, alongwith an associated increase in serum MG levels from 2.48 ± 0.3 $\mu\text{mol/L}$ in the control to 4.28 ± 0.5 $\mu\text{mol/L}$ in the fructose treated rats (Wang *et al.*, 2008). The increase in the BP in fructose treated rats was attenuated by co-treatment with metformin, a MG scavenger (Wang *et al.*, 2008). Although MG was not established as a direct causative factor for the increased BP in the study by Wang *et al* (2008), the finding assumes great significance because fructose is a known precursor of MG (Dhar *et al.*, 2008) and serum MG was significantly elevated in fructose treated rats (Wang *et al.*, 2008). Fructose is equipotent to glucose in producing MG when metabolized (Dhar *et al.*, 2008). In the present study, a high fructose-diet was fed to SD rats for 16 weeks, and it resulted in a significant increase in the BP, MG levels, angiotensin, AT₁ receptor, α_{1D} receptor protein and mRNA expression in the aorta and the kidney. There was also a significant increase in NF- κ B and RAGE protein expression, and reduction in GSH levels in both the kidney and the aorta of fructose treated rats. Our results strongly support MG as a mediator of the effects of high fructose diet on the RAAS and the BP. In one of the ongoing studies in our lab, both high glucose (60% of total calories) and high fructose (60% of total calories) diets fed to 10 week old male SD rats for 12 weeks caused a similar significant increase in the BP. Thus, the BP in high glucose treated rats was $139 \pm 3^{**}$ mm Hg, and in high fructose treated rats it was $136 \pm 3^*$ mm Hg, compared to 102 ± 11 mm Hg in the control group (* $P < 0.5$, ** $P < 0.01$ vs control group) (unpublished results). One limitation in comparing the effects of a high fructose diet versus exogenously administered MG on BP and other hypertension-associated parameters is that the amount of MG generated from fructose in

different organs and tissues can be expected to depend on the amount of fructose metabolized in a given organ/tissue. The amount of MG generated from fructose in a given organ/tissue can also be expected to be different or similar to exogenously administered MG depending on which organs/tissues receive the administered MG.

One major limitation in designing protocols for this project was the choice of a good MG scavenger. The commonly used MG scavengers include aminoguanidine, N-acetyl cysteine and metformin. N-acetyl cysteine has marked antioxidant effects which can complicate interpretation of results, especially since MG induces oxidative stress. Aminoguanidine is not suitable for *in vivo* studies because of its other effects besides MG scavenging, such as inhibition of NOS and histaminase. Aminoguanidine may prove toxic over 16 weeks of administration. Metformin, a clinically used compound for type 2 diabetes, is a known MG scavenger and would not cause toxicity in a 16 week *in vivo* study. However, the insulin sensitizing effect of metformin can make interpretation of results difficult. To overcome this dilemma to a certain extent, the MG scavenging effect of metformin was tested in cultured VSMCs where the *in vivo* hepatic and other effects of metformin would not interfere with results, and the observed results can be attributed to metformin's MG scavenging action. An *in vitro* MG scavenging assay was also performed by incubating MG with metformin (Dhar *et al.*, 2013). The results allowed to me conclude that MG is a likely mediator of the hypertensive effects of high fructose, through an up regulation of the RAAS.

Arginine seems to be an attractive potential MG scavenger because of several reasons. Arginine has been shown to have affinity for MG and other aldehydes. Arginine is an amino acid and supplements of L-Arg are used over the counter for several conditions. Its use in

hypertension is based on the assumption of increased availability of NO. If the MG scavenging potential can be realized *in vivo* then it can be an attractive supplement in diabetes. It can be argued that besides NOS, L-Arg is a substrate for several other enzymes, such as arginase. This can lead to unexpected effects when L-Arg is taken as a supplement. This fact is acknowledged and for this reason D-Arg was compared with L-Arg for its MG scavenging action. D-Arg is equally effective as a MG scavenger. Important findings will likely emerge when both L-Arg and D-Arg are tested in *in vivo* models of diabetes. Much more remains to be done to establish arginine as a safe MG scavenger.

It is difficult to gauge the relative MG scavenging ability of different MG scavengers because of their multiple direct and indirect effects. For example, NAC can scavenge MG and it is also an antioxidant and it can also increase GSH levels in the body (Jia *et al.*, 2006; Millea, 2009; Lauterberg *et al.*, 1983; Vasdev *et al.*, 1998). Thus, NAC can decrease MG levels directly by scavenging it, and also indirectly by increasing GSH which will increase degradation of MG by the glyoxalase enzymes. An *in vitro* incubation assay can be performed using different MG scavengers incubated separately with MG for different time periods and measuring free MG at timed intervals.

7.2 CONCLUSIONS

The present study strongly indicates MG as one of the causative factors in the pathogenesis of hypertension, especially in high carbohydrate- and diabetes-induced hypertension. Although several studies have reported elevated MG levels in hypertensive and diabetic conditions, it is not clear whether MG is the cause or effect of these pathological

conditions. Chronic MG administration by mini pump for 28 days up regulates components of the RAAS in the plasma, kidney and the aorta, and significantly increases BP, which was attenuated by a MG scavenger. Activation of RAGE and NF- κ B likely mediates the signal transduction triggered by MG. High fructose diet treated Sprague-Dawley rats show findings similar to MG treated rats and suggest a strong possibility of MG as the mediator of the deleterious effects of high fructose on the RAAS and BP.

The study has shown efficacy of arginine as a MG scavenger *in vitro*. Incubation of VSMCs and HUVECs with glucose and MG increases MG formation, oxidative stress and arginase I and II expression and activity, the latter being a novel finding. These effects were attenuated by amino acid L- and D-arginine.

The present study indicates that a high carbohydrate diet may cause daily abnormal elevations of MG levels in the plasma and tissues, which could result in vascular pathology. The severity and progression of these changes leading to hypertension depends on the health of the individual, any underlying oxidative stress, GSH levels and antioxidant defenses.

In conclusion, MG is one of the causative factors in the pathogenesis of hypertension and endothelial dysfunction. Development of specific MG scavengers like arginine will help prevent the progression of high carbohydrate induced hypertension and endothelial dysfunction.

7.3 LIMITATIONS OF THE STUDY

Peripheral resistance influences the BP. The resistance in small vessels such as the second and third order branches of the mesenteric artery contributes to BP regulation. Determination of vascular contractility and endothelium-dependent relaxation of small peripheral

resistance vessels is a useful parameter to study in hypertension related studies. I studied vascular contractility in aortic rings. The results showed a marginal increase in contractility to the α_1 selective agonist phenylephrine. I attempted to study contractility with the angiotensin AT₁ receptor agonist Ang II, but the vessel became desensitized to Ang II after the first dose and I was unable to obtain a dose-response curve to Ang II. Vascular smooth muscle contractility studies in small resistance vessels using a myograph would have been a useful addition to the results and will be a priority consideration in future studies.

There is a lack of availability of specific and safe MG scavengers. This has been discussed in the introduction. Aminoguanidine has other effects besides its MG scavenging action and is not an ideal MG scavenger for *in vivo* experiments. I used alagebrium, which was kindly provided by Alteon Inc., USA, in initial experiments. Alagebrium was developed as an AGEs breaker but we have shown its MG scavenging action (Dhar *et al.*, 2010a). It is not toxic *in vivo*. However, the company ceased operations and we were not able to replenish our supply and had to use aminoguanidine in later studies. Aminoguanidine being toxic *in vivo* we used metformin in high fructose treated rats. Metformin is used clinically in type 2 diabetes but is not a specific MG scavenger. For this reason the conclusions have been moderated in studies with high fructose diet fed rats and metformin. Even though I was convinced that elevated MG generation following high fructose intake mediates increased expression of RAAS components, I concluded that “we report a strong association between elevated levels of methylglyoxal, RAGE, NF- κ B, mediators of the renin angiotensin system and blood pressure in high fructose diet fed rats.”

Another argument can be made that increased BP caused by increased RAAS activity should be prevented by inhibitors of angiotensin converting enzyme such as ramipril and enalapril, or with angiotensin receptor blockers such as losartan. Therefore, a group treated with MG plus an ACE inhibitor or angiotensin receptor blocker should have been included. My explanation is that there are many factors, such as inflammation, hyperglycemia, and obesity, which can increase RAAS activity. Chronic inflammation itself can occur in many conditions. Therefore prevention of increased BP with ACE inhibitors or angiotensin receptor blockers does not provide conclusive evidence that a given factor, in this case MG, was responsible for increased RAAS expression and BP.

The BP was measured with a carotid artery catheter at the end of the treatment periods in 4 week MG treated and 16 week fructose treated rats. While it can be argued that anesthesia can affect the recorded BP, I believe that the factor of anesthesia would affect all groups equally, including the control group. Therefore, the results bear significance. I tried to measure the BP weekly during treatment with a tail cuff system (Harvard Apparatus Canada, Saint-Laurent, QC, Canada), but I got very variable results in all groups. The BP value was different in successive recordings from the same rat on the same day. I experienced that tail cuff BP recording is not a very reliable method. It can be argued that telemetry is a reliable method to measure the BP. Telemetry is a very invasive procedure with major drawbacks of expertise needed, the extra post-surgical recovery time, the increase in expense and the limitations of fewer implants available at any given time. It was decided to use the option of telemetry recording if the results were inconclusive with carotid artery recording, in a separate follow up project, which was not required.

It can be argued that 60% calories provided by fructose in the diet is higher than the 10-15% fructose seen in North American diets, but 60% is widely used in animal studies by researchers, and 60% is expected to produce results comparable to 10-15% diet in a shorter time.

7.4 SIGNIFICANCE OF THE STUDY

Hypertension is a major problem in Canada and the whole world. Adding to the burden is the recent alarming epidemic of obesity and type 2 diabetes, attributed in a large part to high dietary carbohydrates. The cases of diabetes associated hypertension are bound to increase. For practical reasons compounds targeting glucose or fructose molecule itself cannot be used. Establishing excessive reactive aldehydes produced from high carbohydrate diet metabolism in the pathogenesis of diabetes and hypertension will allow us to develop preventive strategies. Preventive strategies will play an important role because the dietary habits of billions of people worldwide are unlikely to change overnight or even over the next few years. The results with MG have given important clues for more work in high carbohydrate diet induced hypertension. Our lab will investigate the potential of arginine as a preventive supplement against MG-induced pathology.

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