ROLE OF METHYLGLYOXAL IN THE PATHOGENESIS OF HYPERTENSION

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ABSTRACT

Methylglyoxal (MG), a metabolite of glucose, causes non-enzymatic glycation of proteins to form irreversible advanced glycation end products (AGEs). Increased MG production, which in turn gives rise to AGEs, has been linked to the development of complications in diabetes. However, the role of MG and AGEs in hypertension has not been investigated widely. The previous study from our laboratory showed that the cellular levels of MG and MG-induced AGE formation are significantly higher in cultured aortic smooth muscle cells from spontaneously hypertensive rats (SHR) than those from normotensive Wistar-Kyoto rats (WKY). Using immunofluorescence staining with specific monoclonal antibodies against MG-induced AGEs, the present studies show a strong association of MG and its AGE products (N^{ε}-carboxyethyl-lysine and N^{ε}-carboxymethyl-lysine) with hypertension in SHR. The blood pressure of SHR was not different from that of WKY rats at 5 wks of age. From 8 wks onwards, blood pressure was significantly elevated compared to age-matched WKY rats. Importantly, this increase in blood pressure coincided with an elevated MG level in plasma and aorta of SHR in an age-dependent fashion compared to age-matched WKY rats, although no difference was observed in blood glucose levels between these two strains. Our data showed an increased MG level in plasma and aorta, but not in kidney or heart, in SHR at an early age of 8 wks, suggesting, in addition to diabetes/hyperglycemic or hyperlipidemic conditions, the accumulation of MG in blood vessel walls plays an

important role in the development of hypertension or its complications even in the absence of diabetes. Moreover, we observed increased blood pressure and vascular remodeling in Sprague Dawley rats which had been treated to increase endogenous MG and related AGEs. After inhibiting MG and MG-induced AGE generation in SHR, hypertension development in this genetic hypertension model was delayed and vascular remodeling was reversed. Our data indicate that increased MG and AGE formation may play an important role in the development of hypertension.

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DEDICATION

To my mother, Yuezhen Jie,

Who gave me life,

Always has unwavering faith in me

And is the inspiration for all my endeavors.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AG	Aminoguanidine
AGE	Advanced glycation endproduct
AMO	Acetol monooxygenase
CSA	Cross-sectional area
CEL	N^{ε}-carboxyethyl-lysine
CML	N^{ε}-carboxymethyl-lysine
DCF	2,7-dichlorofluorescein
DCFH	Dichlorofluorescin
3-DG	3-Deoxyglucosone
DHAP	Dihydroxyacetonephosphate
DOCA	Deoxycorticosterone
GC	Gas chromatography
G3P	Glyceraldehydes-3-phosphate
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase
GSH-Red	Glutathione reductase
GSSG	Oxidized glutathione
HbA1c	Hemoglobin A1C
HDL	High density lipoproteins
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
2K1C	Two-kidney one-clip

Met	Metformin
MG	Methylglyoxal
MOLD	1,3-di(*N*-lysino)-4-methyl-imidazolium
2-MQ	2-methylquinoxaline
5-MQ	5-methylquinoxaline
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\cdot-}$	Superoxide anions
PBS	Phosphate buffer saline
PCA	Perchloric acid
o-PD	*o*-phenylenediamine
PTB	Phenacylthiazolium bromide
RAGE	Receptor for advanced glycation endproducts
ROS	Reactive oxygen species
SD	Sprague-Dawley
SHR	Spontaneously hypertensive rats
SHRsp	Stroke-prone spontaneously hypertensive rats
SSAO	Semicarbazide-sensitive amine oxidase
TPI	Triosephosphate isomerase
VSMCs	Vascular smooth muscle cells
WKY	Wistar Kyoto

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Hypertension

Hypertension, commonly known as high blood pressure, is a medical condition in which the blood pressure is chronically elevated. Medical guidelines state that a normal blood pressure for most adults is less than 120/80 mm Hg. Drug treatment is recommended when the blood pressure is at or above 140/90 mm Hg (Chobanian et al. 2003). Persistent hypertension is a major risk factor for vascular morbidity and mortality and is a leading cause of chronic renal failure, heart failure and coronary heart disease. Stroke is also one of the most devastating consequences of hypertension. Hypertension is an important public-health challenge worldwide, which affects almost one third of the adult population. In 2000, the estimated total number of adults with hypertension was 972 million, 60% of which was in economically developing countries. It is predicted that the total number of adults with hypertension in 2025 will increase to 1.56 billion (Kearney et al. 2005). Hence hypertension is a significant health problem and will become a greater global burden in the next 20 years. Prevention, detection, treatment and control of this condition are very critical.

1.1 Types of hypertension

There are two categories of hypertension, primary (essential) hypertension and secondary hypertension. Essential hypertension, hypertension of unknown cause, is responsible for 90 to 95 percent of diagnosed hypertension. This type of hypertension is

a heterogeneous disorder, with different patients having different causal factors that lead to high blood pressure. Secondary hypertension is responsible for the rest of the percentage. It is most often due to some identifiable cause such as renal diseases or endocrine abnormalities like increased estrogen, catecholamine or aldosterone levels. It can also be caused by other preexisting medical conditions such as thyroid dysfunction, tumors or overactivity of the adrenal gland, pregnancy-related conditions. Secondary hypertension can often be corrected by treating the primary condition with medication or surgery.

1.2 Hypertension and lifestyle

The lifestyle has a significant impact on blood pressure. In some cases of borderline hypertension, changes of lifestyle may produce a reduction in blood pressure sufficient to avoid the need for the introduction of drug therapy.

1.2.1 Weight

Obesity increases the risk of developing hypertension. One possible mechanism is that sympathetic drive is increased in overweight people, thus contributing to vasoconstriction and increased renin production. Blood pressure falls with weight reduction (He et al. 2000; Stevens et al. 2001). Therefore body weight control is an

obvious early lifestyle intervention in many people. This becomes especially critical as the body mass index of individual in Western countries has increased to epidemic levels. For example, in United States, 32.9% of adults 20-74 years old are obese and more than 17% of teenagers are overweight, which contributes to the rise in blood pressure and related complications (Ogden et al. 2007).

1.2.2 Salt intake

The relationship between salt intake and blood pressure has been controversial over the past 50 years. Several clinical trials have shown that reduction of dietary salt intake is successful in reducing blood pressure (Midgley et al. 1996; Sacks et al. 2001). It has been demonstrated that the reduction of sodium intake significantly lowered systolic and diastolic blood pressure in a stepwise fashion, and there was a greater response of blood pressure to progressively lower levels of sodium intake (Sacks et al. 2001). Salt restriction induces greater reduction in blood pressure in hypertensive patients than that in normotensive subjects (Midgley et al. 1996). Therefore, limitation of salt intake is a useful adjunct to pharmacological treatment of hypertension.

1.2.3 Alcohol

There is a positive linear relationship between alcohol consumption and blood pressure in both men and women (Maheswaran et al. 1992; Xin et al. 2001). Both the chronic and acute alcohol intake increase blood pressure. Clinical trials have shown that

67% reduction in heavy alcohol consumption (3-6 drinks/day) leads to a 3.31 mmHg decrease in systolic blood pressure and 2.04 mmHg in diastolic blood pressure (Xin et al. 2001). In general, it is recommended that hypertensive patients should limit alcohol consumption to no more than 2 drinks per day.

1.2.4 Exercise

Physical activity has been associated with reduced blood pressure. It has been reported that moderately intense aerobic exercise lowers resting blood pressure. In addition, progressive resistance exercise may also reduce resting blood pressure, possibly by reducing peripheral resistance at rest (Kelley and Kelley 2000; Whelton et al. 2002).

1.3 Pathogenesis of essential hypertension

Although the cause of essential hypertension in any specific patient is usually unclear, numerous pathophysiologic factors have been associated with it. These include increased sympathetic nervous system activity, overproduction of sodium-retaining hormones and vasoconstrictors, increased renin secretion, lifestyle, insulin resistance, deficiencies of vasodilators such as NO and prostacyclin, altered cellular ion transport and abnormalities of resistance vessels (Fig. 1-1) (Oparil et al. 2003). Recently, compelling evidence has shown that structural and functional abnormalities in the vasculature, including increased oxidative stress, endothelial dysfunction and vascular

remodeling may contribute to the pathogenesis of hypertension (Koller 2002; Mulvany 2000; de Champlain et al. 2004). Many of the antihypertensive drugs interfere with one or more of the above factors.

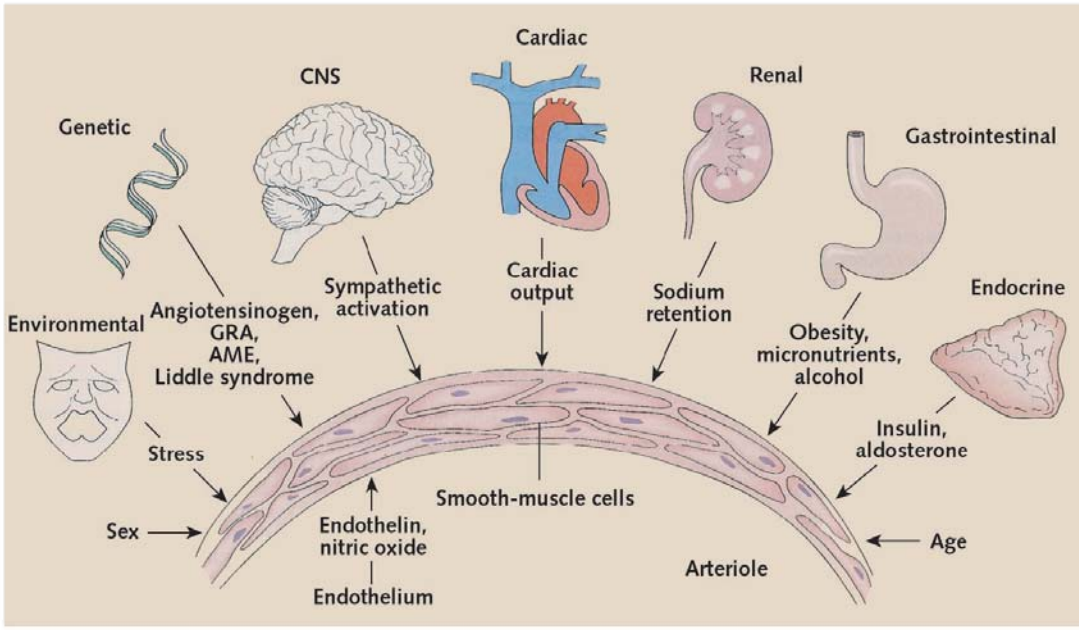


Fig.1-1. Pathophysiologic mechanisms of hypertension. (Adopted from *Ann. Intern. Med.* 139:761-776, 2003)

1.3.1 Oxidative stress

Oxidative stress is a situation of serious imbalance between the production of reactive oxygen species (ROS) and antioxidant defense mechanisms. ROS include superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2). The primary sources for ROS generation include the mitochondrial electron transport chain and oxidase enzymes such as NAD(P)H oxidase, xanthine oxidase, cytochrome P450

enzymes and lipoxygenases (Mueller et al. 2005). Under normal conditions, only small quantities of ROS are generated and ROS are safely neutralized by the antioxidant system which consists of numerous enzymes and antioxidant molecules. Under pathological conditions, the rate of ROS generation exceeds the neutralizing capacity of the antioxidant defense system, leading to oxidative stress. Nearly all animal studies performed in various models of hereditary and acquired hypertension have provided evidence to support the causative role of oxidative stress to the pathogenesis of hypertension. For example, a progressive increase in the production of superoxide anion in vascular and cardiac tissues during the development of hypertension has been demonstrated in various models of hypertension such as DOCA-salt, SHR, and the angiotensin or glucose-induced model of hypertension (de Champlain et al. 2004). There are three lines of evidence for the causal contribution of oxidative stress to hypertension. First, oxidative stress is present in various animal models of hypertension (Vaziri and Rodriguez-Iturbe 2006). Second, chronic treatment with potent antioxidant can prevent or attenuate the development of hypertension (de Champlain et al. 2004). Finally, induction of oxidative stress causes hypertension in genetically normal and healthy animals (Vaziri et al. 2000; Zhou et al. 2002).

1.3.2 Endothelial dysfunction

Vascular endothelial cells play an essential role in cardiovascular regulation by producing a number of potent local vasoactive agents, such as the vasodilator molecule

nitric oxide (NO), and the vasoconstrictor peptide endothelin. Dysfunction of the endothelium has been implicated in human essential hypertension (MacGregor and Kaplan 2006; Quyyumi 1998). It is clear that antihypertensive drugs that interrupt the renin-angiotensin system, including ACE inhibitors, angiotensin-receptor blockers and mineralocorticoid receptor antagonists, are effective in restoring endothelial dysfunction (MacGregor and Kaplan 2006). This action may at least partly account for their cardioprotective effects.

Endothelial dysfunction is a functional and reversible alteration of endothelial cells, resulting from impairment in NO availability. Endothelial cells release NO in response to several stimuli including increased shear stress during increased blood flow or muscarinic receptor stimulation (MacGregor and Kaplan 2006). Numerous studies have demonstrated a beneficial effect of acute and chronic L-arginine supplementation on NO production and endothelial function, and L-arginine has been shown to reduce systemic blood pressure in some forms of experimental hypertension (Gokce 2004). Endothelin-1 (ET-1), the main endothelin generated in the endothelium, acts in a paracrine or autocrine manner on ET_A and ET_B receptors on adjacent endothelial or smooth muscle cells (Pollock and Pollock 2005). An interaction of the NO and ET-1 systems may participate in the pathogenesis of endothelial dysfunction. It was found that the vasoconstrictor activity of ET-1 is increased along with diminished availability of NO (Haynes and Webb 1998). The imbalance between the two systems may enhance the vasoconstrictor and protrophic activity of ET-1, leading to the increase of blood

pressure.

1.3.3 Vascular remodeling

Peripheral resistance is consistently increased in hypertensive conditions (MacGregor and Kaplan 2006). Peripheral resistance is determined mainly by resistance vessels which consist of the small arteries (lumen diameters < 300 μm) and arterioles. Examination of gluteal skin biopsy specimens obtained from patients with untreated essential hypertension revealed that essential hypertension is associated with reduced lumen areas and increased media-lumen ratios without an increase in medial area in resistance vessels (Mulvany 2000). This remodeling is known as inward eutrophic remodeling. Mulvany M (Mulvany 2002) suggests that essential hypertension is associated only with eutrophic, not hypertrophic, remodeling in small arteries. Although the mechanisms by which cause eutrophic remodeling is not clear, the following process is proposed: increased neurohumoral activity leads to vasoconstriction and increased blood pressure. On the basis of the Laplace relation, the wall stress remains normal by decreasing the diameter and increasing the wall thickness of the vessels (Mulvany 2002). With time, the active vasoconstriction changes to a passive eutrophic remodeling as demonstrated by *in vitro* experiments (Bakker et al. 2000).

Several classes of antihypertensive agents such as ACE inhibitors, angiotensin-receptor blockers and calcium-channel blockers normalize the vascular remodeling (Schiffrin 2001). But not all effective antihypertensive treatments are able to

correct the abnormal vessel structure. For example, a β-blocker does not reverse resistance artery remodeling even it effectively lowers blood pressure (Thybo et al. 1995).

2. Rat models of hypertension

Hypertension is a multifactorial and polygenic disease that involves complex interactions between genetically determined homeostatic control mechanisms and environmental factors; currently no species can meet the requirement for an ideal animal model of hypertension. The choice of animal models for specific research is determined by experimental design and other constraints. There are two categories of rat models for hypertension research, nongenetic and genetic models. Nongenetic models include 2K1C and DOCA-salt rats, whereas genetic models include SHR, SHRsp and Dhal salt-sensitive rats.

2.1 Two-kidney one-clip (2K1C)

2K1C, the first animal model of hypertension was developed in dogs by clipping the renal artery in 1934 (Goldblatt et al. 1934). Five years later, this model was developed in rats (Wilson and Byrom 1939). In rats, 2K1C leads to a gradual and chronic increase in blood pressure, which plateaus after 2 weeks (Leenen and de Jong 1971). This model has some sensitivity to diet. For example, sodium restriction attenuates development of hypertension in young rats (Miksche et al. 1970). Circulating

renin and aldosterone levels are increased in 2K1C model (Koletsky et al. 1971). The blood pressure in 2K1C is very sensitive to the inhibition of the renin-angiotensin system. Calcium antagonists and direct vasodilators are also effective, but this model does not respond to β-blocker, endothelin antagonists and diuretics (Pinto et al. 1998).

2.2 Deoxycorticosterone acetate (DOCA)-salt rats

The most common endocrine method to induce hypertension is administration of mineralocorticoid, particularly DOCA (Terris et al. 1976). In rats, the development of hypertension with DOCA requires a high salt diet (Lawler et al. 1987). DOCA-salt rats have impaired endothelium dependent relaxation, increased cardiac output, proteinuria and glomerulosclerosis (Pinto et al. 1998). DOCA-salt is the only model in which renin-angiotensin inhibition does not decrease blood pressure. This model is sensitive to endothelin antagonists and diuretics (Pinto et al. 1998).

2.3 Spontaneously hypertensive rats (SHR) and stroke prone SHR (SHRsp)

SHR was obtained by inbreeding Wistar Kyoto rats with the highest blood pressure (Okamoto and Aoki 1963). The genetic mechanisms of hypertension in SHR have been attributed to both neural and vascular alterations. At least three major genes contribute to the early development of hypertension, and additional one to the development and maintenance of hypertension during aging in SHR (Yamori 1999). The blood pressure in SHR rises around 6 weeks of age and steadily increases with age until it reaches 200

mmHg. By far, SHR is the most widely used animal model of hypertension. Endothelial dysfunction is consistently found in SHR. This model has many features of hypertensive end-organ damage like cardiac hypertrophy, cardiac failure and renal dysfunction (Pinto et al. 1998). Blood pressure in this model responds to calcium antagonists, direct vasodilators and the inhibition of renin-angiotensin system (Pinto et al. 1998).

Stroke-prone SHR is a further developed substrain from SHR with even higher levels of blood pressure and a strong tendency to die from stroke. The blood pressure of SHRsp increases rapidly at a young age and finally reaches around 240 mmHg. SHRsp rats die with stroke from a few days to 24 weeks after initial symptoms of stroke or hypertension. The average life span of this model is 33 to 41 weeks in males and far longer in females (Okamoto et al. 1974).

2.4 Dahl salt-sensitive rats

Dahl salt-sensitive rats were developed by Dr. Lewis K. Dahl in 1950s (Dahl et al. 1962). The salt sensitive Dahl rats rapidly develop hypertension when treated with high salt (8% NaCl) diets at weaning and all died by the 16^{th} week of salt feeding. The magnitude of the blood pressure response in this model is partly determined by the age at which high salt diet is started. For example, when given high salt at weaning, the rats developed fulminating hypertension (above 200 mm Hg) within 6 weeks. If high salt feeding was given at 3 months of age, the hypertension developed less rapidly and blood pressure went to about 185 mm Hg by 16-20 weeks (Dahl et al. 1968). Dahl

salt-sensitive rats have a moderately increased blood pressure even on normal rat chow containing 1% NaCl, but it takes a longer time (months instead of weeks) to develop (Sustarsic et al. 1981). Inhibition of the renin-angiotensin system, diuretics and vasodilators can attenuate the increase of blood pressure in this model, while calcium antagonists and β-blockers are less effective (Pinto et al. 1998).

2.5 Fructose-induced hypertensive rats

It was first demonstrated by Hwang *et al* that a high-fructose diet can not only cause insulin resistance and hyperinsulinemia, but also induce hypertension in non-genetic Sprague-Dawley (SD) rats (Hwang et al. 1987). In Wistar rats, treatment with 10% fructose in drinking water (equivalent to a diet containing 48-57% fructose) for one week or longer is appropriate for the rapid production of fructose-induced hypertension (Dai and McNeill 1995). Fructose induced hypertension is a relatively mild hypertension compared to genetic hypertension in SHR or DOCA-induced hypertension. Irrespective of the concentration of fructose solution given or the duration of treatment, the maximum increase in systolic blood pressure is no more than 50 mmHg (Dai and McNeill 1995). The mechanism of fructose-induced hypertension is not fully understood. It is clear that fructose- induced hypertension is not related to the renin-angiotensin-aldosterone system (Hwang et al. 1989). A number of studies (Takagawa et al. 2001; Verma et al. 1996) have shown that the endothelium-dependent vascular relaxation is impaired in fructose-fed rats. Vasdev et al proposed that aldehydes,

especially methylglyoxal, may be the cause of fructose-induced hypertension (Vasdev et al. 2000).

3. Gender differences in hypertension

The incidence and the progression rate of hypertension are markedly higher in men than in age-matched, premenopausal women, and very comparable in men and age-matched menopausal women (Reckelhoff 2001). This suggests the gender differences in vascular tone and possible vascular protective effects of the female sex hormone, estrogen. Experimental and clinical data have suggested that hormone replacement therapy may reduce cardiovascular disease in postmenopausal women. Gender differences in hypertension have also been found in hypertensive animal models, such as SHR (Reckelhoff et al. 1999), Dahl salt-sensitive rats (Rowland and Fregly 1992) and DOCA-salt rats (Ouchi et al. 1987). It has been reported that estrogen can also lower total and low density lipoprotein cholesterol, increase high density lipoprotein cholesterol and reduce fibrinogen and factor VII, which may contribute to its cardioprotective effect (Knopp et al. 1994; Riedel et al. 1993). A recent study demonstrated that the expression of angiotensin II type 2 receptors, a receptor that has antiproliferative, anti-inflammatory and antioxidative effects in injured vessels, was lower in the injured arteries of male rats compared to female rats (Okumura et al. 2005).

This might be the underlying cause for the sex differences in the degree of vascular injury, being more pronounced in males than females. The receptors of sex hormone are also critical determinants of cardiovascular gender differences. For example, a recent work found that activation and expression of estrogen receptor-α, a receptor that reduces smooth muscle cell differentiation and induces microvessel dysfunction, were increased in women compared to men, which partly explains why women are at high risk of dying from cardiovascular disease after menopause (Montague et al. 2006).

Hormone-dependent gender differences exist in vascular function. Vascular contraction is greater in blood vessels of intact male than intact female rats (Crews and Khalil 1999). Moreover, the increase in vascular contraction is prevented by estrogen replacement in ovariectomized female rats (Tsang et al. 2004). It has also been demonstrated that endothelium-dependent vascular relaxation is greater in female SHR compared with male SHR (Kauser and Rubanyi 1995). In addition, endothelial dysfunction is improved by selective estrogen receptor agonists in ovariectomized SHR (Widder et al. 2003). Considerable evidence indicates that sex hormone modifies the synthesis/bioactivity of NO, an important vasodilator (Moncada et al. 1991). The gender of the animal has been shown to have an important influence on the expression/activity of endothelial nitric oxide synthase (eNOS) in the blood vessels (Ellison et al. 1989; Kauser and Rubanyi 1994) and the kidney (Neugarten et al. 1997). It has been shown in humans and animals that NO level is greater in females than in males because estrogen

not only stimulates NO production (Hayashi et al. 1992; Kauser and Rubanyi 1994) but also decreases inactivation of NO by oxygen radicals (Mendelsohn and Karas 1999). Post-menopausal females has reduced arterial NO activity which was restored to premenopausal level after two weeks of estrogen replacement therapy (Majmudar et al. 2000).

4. Methylglyoxal

Methylglyoxal (MG), also known as acetlyformaldehyde, pyruvic aldehyde, 2-ketoproprion-aldehyde etc. is a reactive α-oxoaldehyde. As shown in Fig. 1-2, MG has a simple structure with a ketone bond and an aldehyde bond. The aldehyde group is more reactive than the ketonic group. In aqueous solution, MG is present mostly in the monohydrate (71%) form, while the unhydrate (1%) and dihydrate (28%) forms account for the rest part (Creighton et al. 1988). Chemically prepared MG is a yellow liquid with a pungent odor.

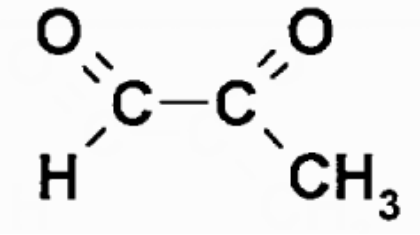


Fig. 1-2. Structure of methylglyoxal

4.1 Formation of MG

4.1.1 Main pathways of MG formation

4.1.1.1 Enzymatic MG formation

Endogenous MG is mainly formed spontaneously during glycolysis as a result of fragmentation and elimination of phosphate from glyceraldehydes-3-phosphate (G3P) and dihydroxyacetonephosphate (DHAP) (Thornalley 1996) (Fig. 1-3). Normally, 40-67% of MG is formed from G3P and 33-60% from DHAP, depending on the DHAP/G3P molar ratio (Phillips and Thornalley 1993). G3P and DHAP can be converted to MG enzymatically (Pompliano et al. 1990; Ray and Ray 1981) and nonenzymatically (Richard 1991). Triosephosphate isomerase (EC.5.3.1.1) can convert both G3P and DHAP to MG (Pompliano et al. 1990). Triosephosphate isomerase is a very effective catalyst of isomerization and is present at very high cellular concentrations (Albery and Knowles 1976). Therefore, a significant concentration of cellular MG might be generated by triosephosphate isomerase even though its catalysis of degradation of G3P and DHAP to MG is very slow. DHAP can also be catalyzed to MG by methylglyoxal synthase in goat liver (Ray and Ray 1981). Methylglyoxal synthase (EC.4.2.99.11.) cannot catalyze G3P, fructose 1,6-bisphosphate, dihydroxyacetone, and glyceraldehydes. Its activity is regulated by inorganic phosphate

which suggests that its role in the control of glycolysis depends on the availability of intracellular inorganic phosphate (Ray and Ray 1981).

4.1.1.2 Nonenzymatic MG formation

The instability of G3P at physiological pH was first reported in 1969 (Mel'nichenko et al. 1969). This was followed by a study which found two reaction products, inorganic phosphate and MG, from nonenzymatic G3P reaction at pH 7.7, in the presence of increasing concentrations of the buffer catalyst lysine (Bonsignore et al. 1973). Currently, it is accepted that the deprotonation of G3P or DHAP to an enediolate phosphate followed by the cleavage of phosphate group from the carbon skeleton results in the formation of MG (Richard 1993). An estimated rate of nonenzymatic MG formation is 0.1 mmol/L/day (Richard 1991).

4.1.2 Minor pathway of MG formation

Under physiological conditions, minor sources of MG formation include metabolism of acetone from lipolysis and metabolism of threonine from protein catabolism (Casazza et al. 1984; Lyles and Chalmers 1992). The conversion of acetone to MG involves two enzymes, acetone monooxygenase which converts acetone to acetol and acetol monooxygenase (AMO) which converts acetol to methylglyoxal. O_2 and NADPH are required for both enzyme activities. Agents like acetone and ethanol induce

the expression of acetone and acetol monooxygenases (Casazza et al. 1984). Their activities are also increased under pathological conditions such as diabetes mellitus (Gonzalez 1988). Semicarbazide-sensitive amine oxidase (SSAO, EC.1.4.3.6) is a group of enzymes containing quinine and copper. Because SSAO contains a cofactor having carbonyls, it is sensitive to semicarbazide inhibition (Lyles 1996). This enzyme is present in two forms, a soluble form in plasma and a membrane-bound form in tissues. Both forms can convert amino acetone to MG (Lyles 1996). SSAO is located only in some tissues like vascular wall, retina, kidney and the cartilage tissues, and seems to be specific to mammals (Lyles 1996; Yu 1998). Increased SSAO activities are associated with diabetic complications, vascular disorders and heart disease. Under physiological conditions vascular endothelial cells are the major source of circulating SSAO (Stolen et al. 2004). Increased activities of plasma SSAO and AMO have been suggested to be responsible for the increased circulating MG levels and diabetic vascular complications in animals (Yu et al. 2003). Increased SSAO in transgenic mice are associated with an increased endothelial cell capacity for lymphocyte binding and altered expression of redox-sensitive proteins (Stolen et al. 2004).

4.2 Exogenous formation of MG

Exogenous sources of MG include many food products, beverages, alcohol and coffee (Nemet et al. 2006). During food processing and storage, MG is formed as a

by-product. For example, MG level in honey is in the range of 0.4-5.4 mg/kg, which comes from sugar degradation during the heating processes applied in manufacturing and storage (Nemet et al. 2006). MG formation was also observed during the heating of glucose, fructose and maltulose. There are different amounts of MG in dairy products and alcoholic drinks coming from fermentation. Take wine process as an example; MG is released by *Saccharomyces cerevisiae* during alcoholic fermentation and by *Oenococcus oeni* during malolactic fermentation (Marchand et al. 2000).

In addition, cigarette smoking is also an exogenous source of MG. Gas chromatographic measurement revealed MG levels of 13.5-59.6 μg/cigarette (Fujioka and Shibamoto 2006). MG is found in plants, too. It has been reported that MG concentration is 30-75 micromolar in various plant species and it can be increased up to 6-fold by stress conditions such as salinity, drought, and cold (Yadav et al. 2005).

4.3 Metabolism of MG

4.3.1 Glyoxalase metabolism of MG

MG is mainly degraded to D-lactate via glyoxalase system (Fig 1-3). The glyoxalase system consists of two enzymes, glyoxalase I and glyoxalase II, and a cofactor, reduced glutathione (GSH) (Racker 1951). It is a relatively simple pathway comprising two consecutive enzymatic reactions. First reaction is the generation of S-D-lactoylglutathione from the hemithioacetal formed non-enzymatically from MG and

GSH, which is catalyzed by glyoxalase I (S-lactoylglutathione lysase, EC 4.4.1.5). The second reaction is the hydrolysis of S-D-lactoylglutathione to D-lactate catalyzed by glyoxalase II (hydroxyacylglutathione hydrolase, EC 3.1.2.6). It has been reported that both the maximal velocity and the affinity for its substrate of glyoxalase I are three fold greater than those of glyoxalase II (Rae et al. 1990).

Glyoxalase I activity is found in all tissues of prokaryotic and eukaryotic organisms. In human tissues, glyoxalase I activity is highest in the pancreas, kidney, lung and brain and lowest in the liver and adipose tissue (Thornalley 1993). The concentration of glyoxalase I in most human tissues is approximately 0.2 mg/mg protein (Larsen et al. 1985). Human glyoxalase I is a dimer and its molecular mass is 44,000Da. GSH is essential for glyoxalase I reaction. It not only provides specific binding to the active site of glyoxalase I, but also activates the C1 protein of the aldehydic group of MG by hemithioacetal formation (Thornalley 1993). GSH is a weak competitive inhibitor of glyoxalase I, and its estimated Ki value is 7.9 mM (Rae et al. 1990). Glyoxalase II activity is found in the cytosol and mitochondria of cells (Talesa et al. 1988). In human, glyoxalase II is a monomer and its molecular mass is 29,200 (Allen and Thornalley 1993). In cytosol the physiological substrate for glyoxalase is S-D-lactoylglutathione, but in mitochondria other substrates such as S-succinylglutathione, may be available substrate for this enzyme (Allen et al. 1993a).

4.3.2 Non-glyoxalase metabolism of MG

4.3.2.1 Aldose reductase

Aldose reductase catalyzes the conversion of MG to acetol (95%) and D-lactaldehyde (5%), with NADPH as a cofactor (Vander Jagt et al. 1992). Aldose reductase also catalyses the further reduction of acetol and D-lactaldehyde to L-1,2-propanediol and D-propanediol respectively. In most human tissues, the ratio of the rate of detoxification of MG by glyoxalase I to that by aldose reductase is *ca.* 10-40 (Allen et al. 1993b).

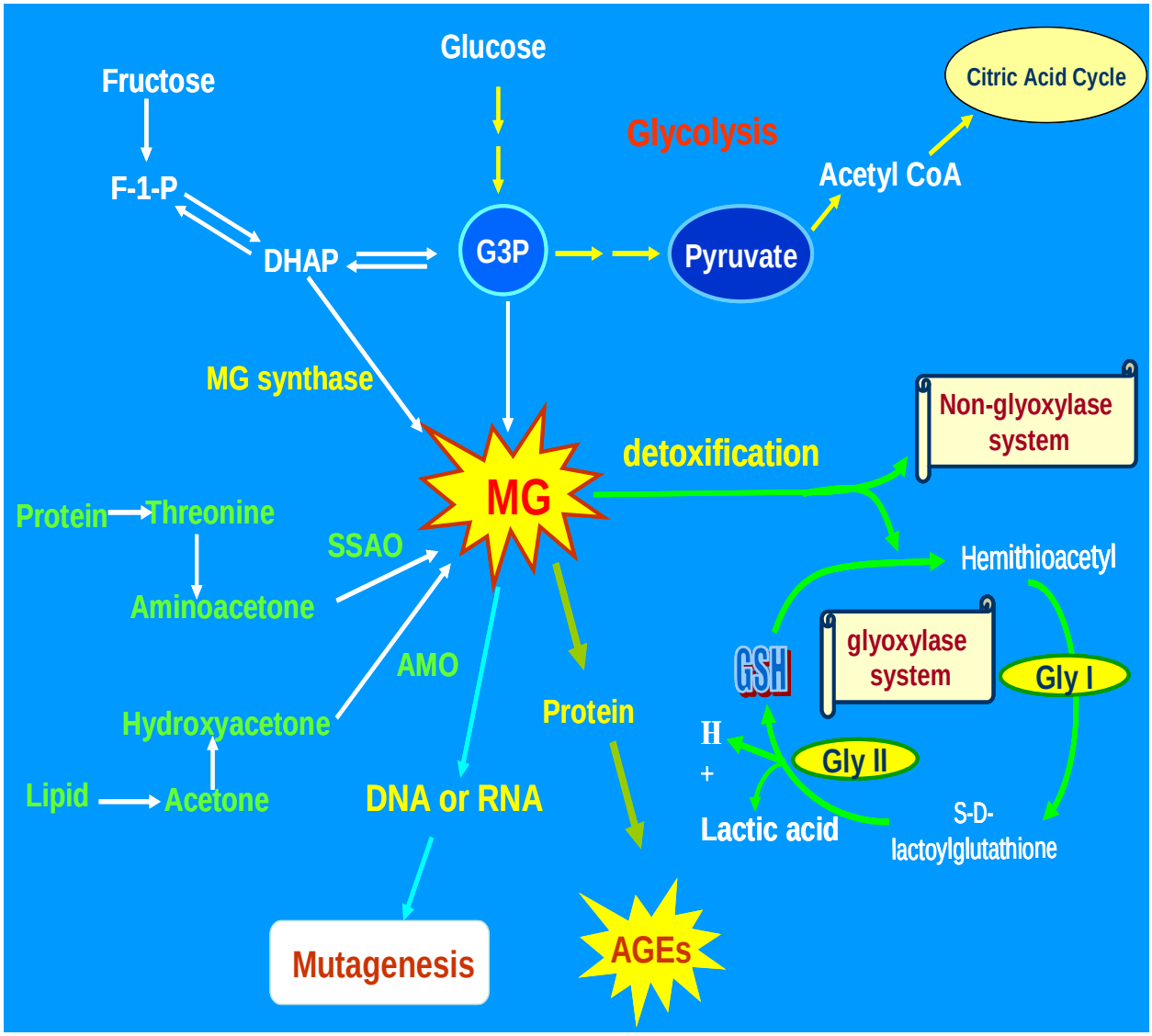
4.3.2.2 MG reductase

MG reductase catalyzes the reduction of MG to L-lactaldehyde. It has been found in rat liver homogenates (Ting et al. 1965) and purified from goat liver (Ray and Ray 1984). In mammals, the molecular weight of MG reductase is 89,000 Da. It requires NADH as cofactor and has broad substrate specificity for α-oxoaldehydes and other aldehydes, with MG the best substrate (Ray and Ray 1984). MG reductase is highly sensitive to sulfhydryl group reagents, and the pH optimum of this enzyme is 6.5. In yeast, the molecular weight of this enzyme is 43,000 Da, and it requires NADPH as a cofactor and is specific for α-oxoaldehydes (Murata et al. 1986).

4.3.2.3 MG dehydrogenase

MG dehydrogenase catalyzes the oxidation of MG to pyruvate. Two MG dehydrogenases have been purifed from goat liver, one requiring NAD and the other needing NADP as a cofactor (Ray and Ray 1982). Subsequent studies revealed that MG dehydrogenase requires an activator for its activity. Vicinal aminoalcohols have been shown to be the best activators. In human liver, this enzyme is an important detoxification enzyme for protection against MG (Vander Jagt and Hunsaker 2003).

Fig. 1-3. Methylglyoxal formation and detoxification



AGE, advanced glycation endproducts;
AMO, acetol monooxygenase;
DHAP, dihydroxyacetone phosphate;
F-1-P, fructose-1-phosphate;
G3P, glyceraldehyde-3-phosphate;
Gly, glyoxalase;
GSH, reduced glutathione;
MG, methylglyoxal;
SSAO, semicarbadize-sensitive amine oxidase;

4.4 Determination of MG in biological systems

It is difficult to determine MG content in biological systems. There are several major problems with MG quantification. One difficulty is that MG can react with other components of the biological matrix. Therefore, it is impossible to detect MG directly. Most high performance liquid chromatography (HPLC) methods for MG measurement are based on MG derivatization into more stable compounds. Another difficulty with MG analysis is interferences from other metabolites. During sample processing involving alkalinization, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate are converted to MG through spontaneous phosphate elimination (Richard 1991). Moreover, the low concentration of MG in biological samples also makes MG difficult to detect.

To date, various techniques have been worked out to detect MG levels in biological systems. An early approach for MG determination involved derivatization with 2,4-dinitrophenylhydrazine, with or without chromatographic separation of the resultant osazone and spectrophotometric detection (Fodor et al. 1978; Riddle and Lorenz 1968). This method was used to measure non-enzymic formation of MG from glyceraldehydes (Riddle and Lorenz 1968) and to detect the presence of MG in beef liver (Fodor et al. 1978). A disadvantage of this approach is the interference from glycolytic metabolites which react with 2,4-dinitrophenylhydrazine to form the same osazone as MG.

In the last two decades, several types of derivatization methods for MG have been developed with the use of HPLC. The most common technique is the generation of quinoxaline derivatives. In 1980s, 1, 2-diamino-4, 5-dimethoxybenzene was used to derivatize MG, and this quinoxaline adduct formed by derivatization has a high extinction coefficient and is fluorescent (Hara et al. 1988; Ohmori et al. 1987; Yamaguchi et al. 1989). It can be easily detected by UV or fluorescent detectors of HPLC or by a mass spectrometry detector. A big problem with this technique is the interference from other metabolites. Because the sample was prepared under alkaline conditions, not only the glyceraldehyde-3-phosphate and dihydroxycetone phosphate spontaneously eliminate phosphate to form MG, but also the hydroxyacetone and lactaldehyde can be oxidized to form MG (Richard 1991; Thornalley 1985). These reactions can be avoided by acidic sample preparation. Therefore, in the improved method, MG was derivatized in deproteinized perchloric acid extracts in order to minimize the interference from glycolytic metabolites (McLellan et al. 1992). However, a few years later, a study carried by Chaplen's group (Chaplen et al. 1996a) demonstrated that there were a few problems with the method described by McLellan *et al* (McLellan et al. 1992). Firstly, perchloric acid used to deproteinize the sample and to maintain an acidic pH can significantly increase MG level due to its oxidative degradation of nucleic acid (RNA and DNA) to MG. DNA is 12 fold more susceptible to degradation than RNA (Chaplen et al. 1996a). Secondly, DNA degradation can also form quinoxaline derivative of 2,3-butandione which was used as an internal standard.

Based on these problems, a new method was developed (Chaplen et al. 1996b). In this new method, perchloric acid-precipitated material was removed by centrifugation before derivatization in order to reduce the nucleic acid interference. An additional C-18 solid-phase extraction step was added before derivatization to further reduce the amount of DNA and RNA in the samples. 5-methylquinoxaline was used as an internal standard because it cannot be formed by nucleic acid degradation and it is also commercially available. Sample derivatization was done at 20^{o}C instead of 37^{o}C because at low temperature the formation of MG from nucleic acid degradation was reduced and the rate of reaction between derivative agent, *o*-phenylenediamine, and MG was lower (Chaplen et al. 1996a). Thus, this newly developed method is widely accepted to use for the measurement of MG levels in biological systems. In addition, the HPLC method where MG was derivatized into the corresponding fluorescent pteridinic derivative with 6-hydroxy-2,4,5-triaminopyrimidine has also been described (Espinosa-Mansilla et al. 1998).

Gas chromatography (GC) has been used for MG qualification. After derivatization with o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride, MG level can be measured on MS/SIM detector or electron-capture detector (Bao et al. 1998; Lapolla et al. 2003b). Electrospray ionization liquid chromatography mass spectrometry (ESI/LC/MS) is also used to detect MG concentrations in human plasma or animal tissues (Odani et al. 1999; Randell et al. 2005).

4.5 Cellular toxicity of MG

4.5.1 Modification of protein

Under physiological conditions, MG may reversibly and irreversibly bind and modify proteins. It has been showed that at physiological concentration (< 5 μM), more than 90% of MG is reversibly bound to protein (Lo et al. 1994c). MG reacts with protein by initial reversible reactions: with arginine and lysine residues it forms glycosylamine, with cysteine it forms hemithioacetal. Further irreversible reaction of MG with protein yields irreversible advanced glycation endproducts (AGEs), leading to cross-linking and denaturation of protein. The irreversible reaction of MG with lysine residues of protein forms N^{ε}-carboxyethyl-lysine (CEL), N^{ε}-carboxymethyl-lysine (CML) and 1,3-di(*N*-lysino)-4-methyl-imidazolium (MOLD) (Ahmed et al. 1997; Degenhardt et al. 1998; Shamsi et al. 1998). MG reacts with arginine residues of protein to form the non-fluorescent products 5-hydro-5-methylimidazolone and tetrahydropyrimidine, and the major fluorescent products argpyrimidine and hydroimidazolone (Ahmed et al. 2003; Oya et al. 1999; Shipanova et al. 1997) (Fig. 1-4).

At physiological concentrations of MG, the major irreversible modification of protein by MG was of arginine residues forming the hydroimidazolone (Westwood et al. 1997). When human serum albumin was modified minimally with MG, tryptic peptide mapping indicated a hotspot of modification at Arg-410 located in drug-binding site II and the active site of albumin-associated esterase activity. Other sites of minor

modification were: Arg-114, Arg-186, Arg-218, and Arg-428 (Ahmed and Thornalley 2005).There are three structural isomers of MG induced hydroimidazolone, MG-H1, MG-H2 and MG-H3. Among them, MG-H1 predominates in protein residues (Ahmed and Thornalley 2002). MG-H1 residues were found in relatively high amounts in cellular and excellular proteins (Thornalley et al. 2003). It is predicted that MG can modify up to 13 percent of all proteins (Ahmed et al. 2005a), which suggests that MG derived AGEs have significant effects on protein structure and function, contributing to protein dysfunction. For example, by hydroimidazolone formation, MG modification of critical arginine residues could disrupt protein-ligand interactions and inactivate enzyme activity (Ahmed et al. 2005a).

These glycation effects of MG on protein function are expected to be of little physiological significance because the glyoxalase system maintains a very low concentration of MG under normal physiological conditions. However, chronic exposure to increased concentrations of MG or impairment of the glyoxalase system may lead to significant deterioration of protein structure and function. MG-induced AGEs have been linked to microvascular and macrovascular complications of diabetes, aging and Alzheimer's disease (Kuhla et al. 2005; McNulty et al. 2007; Thomas et al. 2005).

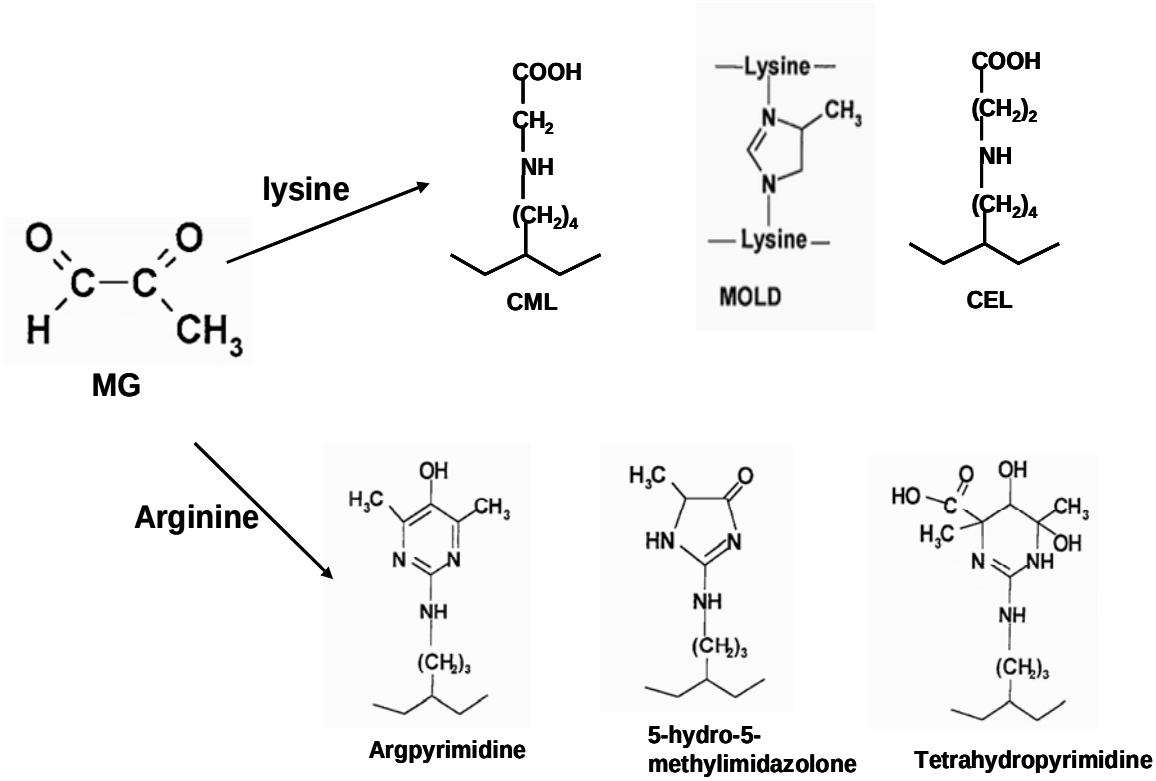


Fig.1- 4. MG-induced advanced glycation endproducts (AGEs)

CEL, N^{ε}-carboxyethyl-lysine; CML, N^{ε}-carboxymethyl-lysine; MOLD, 1,3-di(*N*-lysino)-4-methyl-imidazolium

4.5.2 Modification of nucleic acid

MG binds to guanine and guanyl nucleosides and nucleotides. With radioactively labeled MG, it was found that the reactivity ratio of MG binding to poly-G, poly-A and poly-C was 100:7:3, but MG was not reactive with poly-U (Krymkiewicz 1973). MG also binds reversibly to t-RNA, and can inhibit the translation of natural and chemically

decapped mRNAs (Lozano and Mezl 1984). MG has mutagenic activity, and in mammalian cells, MG-induced mutation mainly occurred at G:C pairs. MG also caused multi-base deletions and base-pair substitutions (Murata-Kamiya et al. 2000). MG can bind to DNA, leading to the formation of so called DNA-bound advanced glycation end-products (DNA-AGEs). Among the nucleobases, guanine and its derivatives readily react with MG (Papoulis et al. 1995). With the LC-MS/MS technique, a recent study (Frischmann et al. 2005) showed that in the presence of high concentrations of MG (100 mM), four adducts were formed which were identified to be two diastereomers of N^2-(1-carboxyethyl)-2'-deoxyguanosine ($CEdG_{A,B}$) and two diastereomers of N^2-(1-carboxyethyl)-2'-deoxyadenosine ($CEdA_{A,B}$). With physiological concentrations of MG, only $CEdG_{A,B}$ was detected. From literature, many studies have shown that MG-induced DNA-AGEs may contribute to the age-related decrease of genomic functionality, diabetes kidney dysfunction or other diseases (Baynes 2002; Roberts et al. 2003; Thornalley 2003a).

5. Advanced glycation endproducts (AGEs)

AGEs are formed by the non-enzymatic Maillard reaction. The Maillard reaction, first described by Louise Camille Maillard, is also known as “browning reaction” because of the brown colour of its products (Maillard 1916). It begins when the reactive

aldehyde or ketone carbonyl group of a reducing sugar interacts with the nucleophilic amino group of the amino acid, protein or lipid to produce an unstable Schiff base. This step is dependent on the concentration of the available sugars and is reversible. In the late phase of the reaction, the Schiff base undergoes further chemical rearrangement to form a more stable Amadori product. With time, Amadori products tend to accumulate and undergo more complex rearrangements or oxidation or degradation to form the AGEs.

5.1 Endogenous AGEs

Under physiological conditions, the Maillard reaction is very slow, which means that AGE formation occurs mainly on long-lived proteins such as extracellular matrix components. The extracellular matrix and vascular basement membrane (BM) are highly susceptible to AGE modification. AGE-mediated crosslinks in BM are known to cause reduced solubility and decreased enzymatic digestion (Charonis and Tsilbary 1992). It has been demonstrated that AGE formation can cause structural and functional abnormalities of BM protein by impairing their ability to form precisely assembled three dimensional matrix aggregates (Vlassara and Palace 2002). Free amino groups on collagen fibrils are also very vulnerable to glycation. The AGE-collagen crosslinking causes collagen to lose its normal elasticity, strength and flexibility (Zieman and Kass

2004). In addition to the crosslinking of collagen in the vascular wall matrix, AGEs may crosslink elastin fibrils, further reducing arterial distensibility (Winlove et al. 1996).

The formation of AGEs occurs at a faster rate under conditions of hyperglycemia, dyslipidemia and oxidative stress. For example, it has been demonstrated *in vitro* that the rate of the formation of intracellular AGEs may be 14-fold faster in 30 mM glucose compared to 5 mM glucose (Giardino et al. 1994). Under pathological conditions, not only the long-lived proteins are heavily modified, but also the short-lived molecules such as plasma protein and lipids, become the targets of AGE modification. AGE modification of short-lived molecules is involved in oxidation of proteins and lipids, disruption of molecular conformation, abnormal clearance by receptors, and reduction of degradative capacity (Zieman and Kass 2004). Take low density lipoproteins (LDL) as an example, AGE formation on the apoprotein part of LDL makes this molecule more vulnerable to crosslink formation with the collagen fraction of the vessel wall and reduces the ability of LDL uptaken by its receptors, thus, leading to increased serum LDL levels (Bucala et al. 1993).

AGEs are a chemically heterogeneous group of compounds, most of which are structurally unidentified. About twenty-five AGEs have been characterized and they primarily are the products of reactions involving glyoxal, MG and 3-deoxyglucosone. Some of these identified AGE products are non-cross-linking AGEs such as CML (Ahmed et al. 1986), CEL (Ahmed et al. 1997), pyralline (Miyata and Monnier 1992)

and carboxymethylvaline (Cai and Hurst 1999). Other more complex AGEs form cross-links between and within modified proteins. These AGEs include MOLD (Degenhardt et al. 1998), glyoxal lysine dimer (GOLD) (Frye et al. 1998), pentosidine (Sell and Monnier 1989), argpyrimidine (Shipanova et al. 1997) and hydroimidazolone (Oya et al. 1999). Because many of these AGE cross-link moieties have intrinsic fluorescence, fluorescence can be used as a marker to detect the presence of these AGEs. For example, collagen-linked fluorescence has been shown to increase with age (Dyer et al. 1993). A recent study (Lutgers et al. 2006) found that skin autofluorescence, as a measure of tissue accumulation of AGEs, was significantly higher in patients who have type 2 diabetes with both micro- and macrovascular disease and it was associated with the severity of diabetes-related complications. The authors concluded that skin autofluorescence might serve as a rapid and useful tool in the outpatient clinic for identifying diabetic patients who are at risk for developing vascular complications.

5.2 Exogenous sources of AGEs

Numerous studies suggest that AGEs from exogenous sources such as diet and smoking may have significant impact on disease mechanisms. Diet is the major source of exogenous AGEs. AGEs are responsible for the brown color that develops in food during cooking and the toughness of food after storage. Foods of the fat group have the

highest amount of AGE content, followed by meat (Goldberg et al. 2004). Formation of AGEs in food depends on the cooking temperature, length of the cooking time, and the presence of moisture (Goldberg et al. 2004). A recent study found that cola drinks contain low concentrations of AGEs whereas pasteurised and sterilized milk are rich in heat-stable AGEs (Ahmed et al. 2005b). The content of CML in raw milk was 337 ± 94 nM, but its concentration increased 3-fold during pasteurisation and 6-fold during sterilization. CEL content in milk was also increased 3- fold during pasteurisation and sterilization (Ahmed et al. 2005b).

Conventional diets contain significant amounts of AGE-modified substances. Oral bioavailability studies of such substances found that approximately 10% of ingested AGEs are absorbed and about two-thirds of absorbed AGEs are deposited in tissues, where they remain biologically active and exert their pathological effects (Koschinsky et al. 1997). The renal excretion of AGEs in human adults is normally poor (only about 30% of the amount absorbed) and is markedly suppressed in the presence of renal disease (e.g. diabetic nephropathy) (Koschinsky et al. 1997; Lin et al. 2002). It has becoming increasingly evident that dietary AGEs are an important source of circulating and tissue AGEs and manifest similar pathogenic effects to their endogenous counterparts. In animal models, restriction of AGEs intake provides significant protection against postinjury restenosis, atherosclerosis and nephrophathy and improves wound healing and insulin sensitivity (Hofmann et al. 2002; Lin et al. 2002; Peppa et al.

2003; Zheng et al. 2002). In type 2 diabetic patients, a high AGE diet over 6 weeks induced a significant increase in markers of inflammation including C-reactive protein, tumor necrosis factor-α and vascular cell adhesion molecule 1 (VCAM-1) (Vlassara et al. 2002). Furthermore, LDL pooled from patients on a high AGE diet was more glycated and more oxidized and markedly stimulated NF-κB activity (Cai et al. 2004). A recent study also demonstrated that a single high AGE meal induces a profound impairment of both macro and microvascular function in type 2 diabetic patients (Negrean et al. 2007).

The curing of tobacco involves conditions conducive to Maillard reaction. Both aqueous extracts of tobacco and cigarette smoke contain glycotoxins, highly reactive glycation products that can rapidly induce AGE formation on proteins. During the process of smoking, high concentrations of glycotoxins are inhaled into the alveoli, where they may be both absorbed into the blood stream and taken up into the lung parenchymal cells. Studies have shown that total serum AGEs and AGE-apoprotein B levels in cigarette smokers were significantly higher than in nonsmokers (Cerami et al. 1997). In addition, smokers, especially diabetic smokers had an elevated level of AGEs in their arteries and ocular lens (Vlassara and Palace 2002).

5.3 Precursors of AGE formation

5.3.1 Glucose

In principle, all reducing sugars such as glucose, fructose, lactose, arabinose and maltose as well as certain molecules related to sugar, for example, ascorbic acid (Ortwerth and Olesen 1988) can cause non-enzymatic glycation of protein. Since glucose is the most abundant sugar in blood, to date, glycation by glucose is the focus of most studies. However, glucose has the slowest glycation rate because the reactivity of each sugar is dependent on the percentage of sugar in the open chain (carbonyl) structure (Bunn and Higgins 1981). In addition to direct glyation reactions, glucose autoxidation also contributes to the formation of stable AGEs (Wolff and Dean 1987). By metal-catalyzed reaction, glucose itself can be slowly autoxidized, resulting in the formation of ketoaldehyde and hydrogen peroxide. Ketoaldehyde formed by the reaction of ketoaldehyde and amino groups on proteins is linked to AGE formation (Hunt et al. 1988; Wolff and Dean 1987). Hydrogen peroxide can produce highly reactive hydroxyl radicals, which, in turn, further induces oxidative protein degradation. It has been reported that the increased glucose level in diabetic patients causes accelerated non-enzymatic glycation that affects some proteins such as LDL apoprotein, hemoglobin and collagen (Thomas et al. 2005).

5.3.2 Fructose

Fructose is a highly reactive reducing sugar. It also undergoes the Maillard reaction with proteins and amino acids, producing reactive intermediates, cross-linking of proteins, and forming brown and fluorescent polymeric materials (Gaby 2005). With respect to human health, the contribution of fructose as an effective glycating agent is very important for two main reasons. One is that the fructose consumption has been increasing steadily in the last two decades. An elevated concentration of fructose and its metabolites, caused by the increased fructose intake from the diet, can potentiate the Maillard reaction. The other reason is that fructose participates in the glycation reaction at a much faster rate than glucose because it is much more reactive in glycating proteins (Schalkwijk et al. 2004). It has been found that fructose is 7.5 times faster than glucose in glycation of hemoglobin, and 10 times greater in the rate of protein cross-linking (Bunn and Higgins 1981; McPherson et al. 1988). It was also revealed that after incubation with fructose, not only the intensity of fluorescent albumin but also the protein oxidation was 3 to 10 fold greater than that with glucose under the same conditions (Takagi et al. 1995). Thus, the large percentage increases in plasma fructose concentrations that happen after ingestion of fructose or sucrose may have clinical consequences, even though the circulation level of fructose is much lower than that of glucose (35 μmol/L fructose vs 5 mmol/L glucose). One animal study showed that long term consumption of fructose accelerated glycation (Levi and Werman 1998). In this

study, after one year treatment, levels of the early glycation products, glycated hemoglobin and fructosamine, were significantly higher in fructose-fed rats compared to glucose and sucrose treated groups. AGEs evaluated by collagen-linked fluorescence in bones were also significantly elevated in fructose treated rats.

5.3.3 α-oxoaldehydes

The α-oxoaldehydes include glyoxal, MG and 3-deoxyglucosone (3-DG). The α-oxoaldehydes are very reactive. They can react with amino groups of protein up to 100 times more rapidly than does glucose (Beisswenger et al. 2003). They react with arginine and lysine residues of proteins to form AGEs, as mentioned earlier. Under physiological conditions, the concentrations of these AGE precursors are very low because they are metabolized and inactivated by enzymatic conversion. However, the accumulation of α-oxoaldehydes may occur when their formation is increased and/or their degradation is decreased. Hyperglycemia, lipid peroxidation and oxidative stress may increase the formation of α-oxoaldehydes (Rahbar and Figarola 2003).

5.3.3.1 3-Deoxyglucosone

3-Deoxyglucosone (3-DG) mainly comes from fructosamine 3-phosphate (Beisswenger et al. 2003). Other sources of 3-DG formation include decomposition of

fructose 3-phosphate, direct auto-oxidation of glucose and reverse aldol, rearrangement and hydrolysis of Amadori product (Zyzak et al. 1995). The concentration of 3-DG in plasma of healthy subjects is 60 nmol/L and increased two to three fold in diabetes mellitus and four fold in uraemia (Knecht et al. 1992). 3-DG is detoxified by both reductive and oxidative pathways. The reductive pathway results in the production of 3-deoxyfructose catalyzed by NADPH-dependent aldehyde reductase and aldose reductase. The oxidative pathway leads to the production of 3-deoxygluconate catalyzed by NAD(P)-dependent 2-oxyaldehyde dehydrogenase (Abordo et al. 1999). 3-DG is a potent and rapidly acting glycated agent. The reaction of 3-DG with the lysine residue of protein forms pyrraline and the 3-DG-derived bis(lysyl)imidazolium crosslink DOLD. The reaction of 3-DG with arginine produces hydroimidazolone (Thornalley et al. 1999).

5.3.3.2 Glyoxal

Glyoxal is formed from several reactions such as spontaneous oxidative degradation of glucose, lipid peroxidation, oxidative fragmentation of Schiff's base etc. (Abordo et al. 1999). Recently, a study found a new pathway for glyoxal formation, which is through peroxynitrite mediated oxidation of glucose (Nagai et al. 2002). Glyoxal is mainly detoxified by the glyoxalase system. It is converted to glycolate with reduce glutathione as a cofactor. Glyoxal can react with protein to form CML, GOLD and hydroimidazolone (Thornalley et al. 1999).

5.3.3.3 Methylglyoxal

MG, a metabolite of glucose, is a highly reactive dicarbonyl compound formed spontaneously during glycolysis as a result of transformation of triose phosphates (Kalapos 1999). Other sources of MG formation include intermediates of protein metabolism and fatty acid metabolism (Casazza et al. 1984),(Lyles and Chalmers 1992). MG is detoxified by the glyoxalase system that uses glutathione as a cofactor (Phillips and Thornalley 1993). MG is the most reactive AGE precursor (Thornalley 1990), and it can react with selective proteins to yield irreversible AGEs, leading to cross-linking and denaturation of protein (Lo et al. 1994b) (See section 4).

5.4 Agents that inhibit AGE formation

Based on the sites of action of inhibitors in the pathways of AGEs formation, there are six groups of AGE inhibitors (Khalifah et al. 1999). Type A inhibitors are sugar competitors that compete with sugar for attachment to amino groups of protein. Aspirin is an example of this group of inhibitors (Reiser 1998). Type B consists of compounds that react with aldose and ketose sugars to inactivate them and prevent their reaction with proteins. These compounds inhibit AGE formation at more than one step. Aminoguanidine, pyridoxamine and other vitamin B6 derivatives belong to this group (Booth et al. 1996). Type C inhibitors are antioxidants and metal chelators. These inhibitors are non-specific. Vitamins C and E are antioxidants that react with reactive oxygen species (ROS) generated by AGEs and their intermediates (Kutlu et al. 2005).

Desferoxamine and penicillamine are two examples of metal chelators (Khalifah et al. 1999). Type D inhibitors are compounds that trap reactive dicarbonyl intermediates such as MG, glyoxal, glycoaldehyde and glucosones to form substituted triazines and thus inhibit AGE formation. This group includes aminoguanidine (Thornalley 2003b), metformin (Beisswenger and Ruggiero-Lopez 2003), L-arginine (Lubec et al. 1997), OPB-9195 (Miyata et al. 1999), D-Penicillamine (Khalifah et al. 1999) The mechanism of AGE inhibition by Type E group is by inhibiting products such as amadoriase (Vlassara et al. 1994) and human fructosamine-3-kinase (Saxena et al. 1996). Type F is AGE breakers or cross-link breakers. This group includes thiazolium compounds such as phenacylthiazolium bromide (PTB) (Vasan et al. 1996) and ALT-711 (Vasan et al. 2003). These compounds mostly break α-dicarbonyl cross-links.

5.4.1 Aminoguanidine

A variety of agents have been developed to inhibit AGEs formation. Among them, aminoguanidine is the best known and most widely used. It was the first agent reported to prevent the diabetes-induced arterial wall protein crosslinking (Brownlee et al. 1986). Aminoguanidine is a nucleophilic hydrazine compound. It exerts its inhibitory effect mostly at the Amadori stage with little effect on post-Amadori products (Khalifah et al. 1999). Aminoguanidine inhibits the formation of Amadori products by reacting with reactive carbonyl metabolites particularly α-oxoaldehydes such as MG, glyoxal and 3-DG. Aminoguanidine is mostly efficient in scavenging MG (Thornalley 2003b).

Aminoguanidine is also a potent and irreversible inhibitor of semicarbazide-sensitive amine oxidase (SSAO), an enzyme that catalyzes aminoacetone to MG (Casazza et al. 1984). Thus, by scavenging AGE precursors and inhibiting SSAO activity, aminoguanidine could decrease the formation of AGEs. The direct antioxidant effect of aminoguanidine provides an additional protection against the formation of AGEs. Aminoguanidine can not only inhibit catalase activity (Ihm et al. 1999) but also have the ability to scavenge peroxynitrite (Szabo et al. 1997). Amiguanidine is also a selective inducible nitric oxide synthase (NOS) inhibitor (Misko et al. 1993). Therefore, the use of aminoguanidine to inhibit AGEs formation would affect NO production and a number of processes associated with it.

Numerous studies have shown that aminoguanidine has beneficial effect in preventing the development of diabetic complications such as nephropathy, neuropathy and retinopathy (Thornalley 2003b). In STZ-induced diabetic rats treated with aminoguanidine for 32 weeks, not only the glomerular and renal tubular fluorescence increase was attenuated, but also the rise of urinary albumin excretion and mesangial expansion were retarded (Soulis et al. 1996). In another diabetic animal model (OLEFT rats), aminoguanidine (1 g/l in drinking water) also prevented the development of albuminuria, mesangial expansion and glomerular basement membrane thickening (Yamauchi et al. 1997). It has been reported that aminoguanidine prevents the development of experimental diabetic retinopathy in both normotensive and hypertensive diabetic rats (Hammes et al. 1991; Hammes et al. 1994). In normotensive

diabetic rats, aminoguanidine prevented the accumulation of AGEs at branching sites of precapillary arterioles and attenuated the increase in the number of acellular capillaries and formed capillary microaneurysms, characteristic pathologic features of background diabetic retinopathy (Hammes et al. 1991). In diabetic spontaneously hypertensive rats, arteriolar deposition of periodic acid-Schiff (PAS)-positive material and abnormal microthrombus formation were completely prevented by aminoguanidine (Hammes et al. 1994). Aminoguanidine can also prevent cardiovascular changes associated with age. There was a decreased level of age-induced AGE formation in cardiac, aortic and renal tissues of rats treated with aminoguanidine, in comparison with that from untreated rats. Age-related aortic stiffening and cardiac hypertrophy were also prevented by aminoguanidine administration (Li et al. 1996).

Clinical trials have also been performed to test the effect of aminoguanidine in overt diabetic nephropathy (ACTION): ACTION I was conducted in patients with type 1 diabetes mellitus; and ACTION II in patients with type 2 diabetes mellitus. The results of ACTION I showed that aminoguanidine did not attenuate the rate of progression of renal disease in type 1 diabetic patients, although it reduced the levels of triglycerides, LDL cholesterol, and urinary protein. In ACTION II, 599 patients were enrolled. However, this trial was terminated early because of the lack of efficacy and the safety concerns (Freedman et al. 1999). Adverse effects of aminoguanidine included gastrointestinal disturbance, abnormalities in liver function tests and flu-like symptoms (Freedman et al. 1999).

In another clinical study, a group of 690 patients with type 1 diabetes mellitus were treated with aminoguanidine to determine if it can ameliorate nephropathy. The study found that inhibiting AGE formation can attenuate serious complications of type 1 diabetes mellitus (Bolton et al. 2004). A one year trial with erythropoietin and aminoguanidine on 12 patients on dialysis observed that the combination of erythropoietin and aminoguanidine restored red blood cell-deformability to nearly normal levels in these patients (Brown et al. 2001).

5.4.2. Metformin

Metformin (dimethylbiguanide) is widely used as an oral antihyperglycaemic agent for the management of type 2 diabetes mellitus. It is a guanidine compound with some structure similarity to aminoguanidine, which suggests that metformin may also inhibit the glycation process. A number of studies have shown that metformin treatment may protect against diabetic complications through the mechanisms independent of its antihyperglycemic effect (Beisswenger and Ruggiero-Lopez 2003; Ruggiero-Lopez et al. 1999; Tanaka et al. 1999). *In vitro* studies have shown that metformin is able to significantly inhibit AGEs formation (Kiho et al. 2005; Ruggiero-Lopez et al. 1999). An *in vivo* study (Tanaka et al. 1999) reported that chronic treatment with metformin can reduce AGE levels in lens, kidney and nerves in diabetic animals. After 10 wks treatment of STZ-induced diabetic rats with metformin (500-650 mg/kg/day), the AGE levels in lens, sciatic nerve and renal cortex were reduced by 72%,

42% and 33%, respectively. In patients with type 2 diabetes, both high dosage (1,500-2,500 mg/day) and low dosage (< 1,000 mg/day) of metformin significantly reduced MG levels by decreasing MG production or increasing MG detoxification. Moreover, it was found that metformin reduced MG in a dose-dependent fashion (Beisswenger et al. 1999). Using mass spectrometry technique, MG-metformin end product, triazepinone was identified in plasma and urine from type 2 diabetic patients treated with metformin (Ruggiero-Lopez et al. 2000). Several mechanisms by which metformin inhibit glycation processes have been proposed. One mechanism is suggested that metformin traps reactive carbonyl species like MG and glyoxal. An *in vitro* study (Ruggiero-Lopez et al. 1999) demonstrated that metformin directly reacts with MG to form stable triazepinone derivatives. Another mechanism is proposed that metformin reduces MG levels via enhancement of MG detoxification through the glyoxalase pathway with unclear mechanism (Beisswenger et al. 1999). Metformin may also react with post-Amadori products (Rahbar et al. 2000).

5.4.3. AGE breakers

Phenacylthiazolium bromide (PTB) was the first AGE breaking compound reported (Vasan et al. 1996). PTB was synthesized based on a hypothesis that the carbon-carbon bond of α-diketones can be selectively cleaved with some thiazolium salts. The nucleophilic centre of PTB should be able to react with a carbonyl group and facilitate its spontaneous cleavage at physiological pH. PTB was successfully tested as

an AGE breaker on 1-phenyl-1,2-propane-dione, an α-dicarbonyl compound. In diabetic animals, PTB reduced the accumulation of AGEs in blood vessels and attenuated the diabetes induced mesenteric vascular hypertrophy (Cooper et al. 2000). Because PTB is not stable, a more stable derivative ALT 711 (4,5-dimethylthiazolium) was developed (Vasan et al. 2003). A number of studies have demonstrated that ALT 711 can significantly improve the elasticity of large vessels and decrease the artery stiffness and peripheral resistance, although the mechanism of cross-link breaking is not clear (Vasan et al. 2003). A clinical trial has shown that ALT 711 significantly attenuated large artery stiffness and decreased arterial pulse pressure in patients with isolated systolic hypertension (Liu et al. 2003).

5.5 Quantification of AGEs

Although there is experimental evidence that AGE accumulation is associated with age and diabetes, it is difficult to compare results among laboratories due to different techniques used in the determination of AGEs in different laboratories. Since AGEs are structurally heterogeneous, it is difficult to ensure that the AGE detected is relevant to the complications observed *in vivo* or *in vitro*. Currently, there is no commonly accepted or widely used method to detect AGEs. Moreover, the lack of internal standards leaves assays open to error which require a high degree of accuracy and reproducibility for each sample run.

To date, the use of immunoassay is most common to detect AGEs (Ahmed et al.

1997; Hammes et al. 1999; Horiuchi et al. 1991; Makita et al. 1992). The assessment of high-titer polyclonal and monoclonal anti-AGE antibodies has been applied successfully to ELISA and immunohistochemical studies. Although immunoassay procedures have provided important analytical evidence for the presence and ultrastructural location of AGEs, they have significant disadvantages and limitations. The first disadvantage is the specificity of the antibodies. For example, the conventionally prepared anti-CML antibody (6D12) cross-reacts with CEL (Koito et al. 2004). The second disadvantage is the background interference. This is because proteins containing glycation adduct are added in order to reduce non-specific binding of the antibody during immunoassay procedure. For instance, there are AGEs in the blocking solution prepared from dried milk powder (Ahmed et al. 2005b). The third disadvantage is temperature interference. The high temperatures during sample processing may cause artificial AGEs formation (Hayashi et al. 2002). The last disadvantage is inaccuracy. This immunoassay does not provide absolute concentrations or amounts but rather arbitrary units with or without normalization to a reference AGE protein standard (Thornalley et al. 2003).

Total AGE fluorescence has also been used to determine AGE levels. Many AGEs like argpyrimidine and pentosidine crosslink have intrinsic fluorescence which can be measured with excitation and emission wavelengths of 350 nm and 450 nm respectively. The problem with this method is that major AGEs like CML and CEL are not fluorescent and are, therefore, not detected (Thornalley et al. 2003). Recently a new method has been developed to detect different AGEs in enzymic hydrolysates of protein

by HPLC with derivatization by 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate (AQC) (Ahmed et al. 2002). The AQC chromatographic assay was deployed successfully for the quantification of CML and CEL in CML-and CEL-modified human serum albumin. Structural isomers of hydroimidazolones derived from glyoxal, MG and 3-DG were also determined for the first time. The limitation of this method is that it cannot detect AGEs *in vivo* because of sensitivity and/or poor chromatographic resolution. To date, the best analytical method available to quantify glycation adducts in biological systems is liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) and quantification using stable isotope substituted standards (Lieuw-A-Fa et al. 2004; Thornalley et al. 2003).

5.6 AGE receptors

Many effects of AGEs are mediated by their interactions with specific AGE-receptors. These receptors have been discovered in macrophages, endothelial cells and several other cell types (Schmidt et al. 2000). Over the last decade, a number of specific AGE-receptors have been identified including AGE-R1, AGE-R2, AGE-R3 and the receptor for AGEs (RAGE). AGE-R1 (oligosaccharyl transferase-48) and AGE-R2 (80K-H phosphoprotein) were initially identified (Yang et al. 1991). AGE-R1 is a single transmembrane integral protein and has a small extracellular *N*-terminal domain and a cytoplasmic *C*-terminal domain (Vlassara 2001). AGE-2, which contains a tyrosine-phosphorylated section in the plasma membrane, is involved in the intracellular

signaling of various receptors (Goh et al. 1996). AGE-R3, also known as Galectin-3, Mac-2 or carbohydrate binding protein-35, has a high-affinity binding for AGE ligands (Vlassara et al. 1995). Studies have found that AGE-R1 and –R3 are largely responsible for AGE-recognition and high-affinity binding, but they have not been shown to transduce cellular signals after binding with AGE. Instead, they may cause the clearance and possible detoxification of AGEs (Bucciarelli et al. 2002).

RAGE, a multiligand member of the immunoglobulin superfamily, has clearly been demonstrated to function as a signal transduction receptor (Hudson et al. 2003). It has a single transmembrane domain followed by a highly charged 43-amino acid cytosolic tail (Schmidt et al. 2001). In homeostasis, RAGE is expressed at a very low level, however, biologic stress such as mechanical vascular injury, diabetes and inflammation lead to striking upregulation of RAGE expression (Park et al. 1998). RAGE may be involved in a broad array of diseases like diabetic complications, inflammation and wound healing (Hudson et al. 2003).

6. The association of MG and diseases

Under physiological conditions, with its low concentration (<5 μM) (Westwood and Thornalley 1995), the effect of MG on biological systems are expected to be of little physiological significance. However, an increased formation of MG may occur when the availability of its precursors is increased. There is a link between overproduction of MG and over-consumption of food high in carbohydrates and fat and/or alcoholic drinks.

Accumulation of glucose, fat or alcohol, precursors of MG, may eventually cause an overproduction of MG. Because MG level depends on the balance of its synthesis and degradation, an increased MG level also occurs when the rate of its detoxification is decreased even with normal levels of glucose or other MG precursors. Since MG is a major sources of intracellular and plasma AGEs (Thornalley 1990), overproduction of MG gives rise to elevated level of AGEs. Increased levels of MG and AGEs have been linked to many diseases including diabetes mellitus, aging, and Alzheimer's disease.

6.1 Diabetic complications

MG formation is accelerated in hyperglycemic conditions (Beisswenger et al. 2001). Plasma MG is highly elevated, reaching ~400μmol/L in poorly controlled type 2 diabetic patients (Lapolla et al. 2003a). Biochemical and clinical evidence shows that increased formation of MG is linked to the development of chronic complications of diabetes, such as retinopathy, nephropathy, neuropathy and also macrovascular diseases like atherosclerosis even though the exact role of this dicarbonyl in these processes is still unclear. There are three lines of evidence for the role of MG in diabetic complications: (i) increased MG concentration at the sites of complications development (Kalapos 1999); (ii) Induction of diabetes-like vascular disease by experimental exposure to MG (Berlanga et al. 2005); (iii) suppression of the development of diabetic complications by scavenging MG in experimental diabetes and clinical trial (Thomas et al. 2005; Thornalley 2003b).

The first indication that MG formation was increased in hyperglycaemia came from a study of human red blood cells cultured under high glucose concentration (Thornalley 1988). The concentration of MG and the flux of MG metabolized by the glyoxalase system were proportional to the glucose concentration. An *in vivo* study showed increased concentrations of MG in the kidney cortex and medulla, lens and blood in diabetic rats (Phillips et al. 1993). Then a clinical study was initiated to survey the MG concentrations in patients with type 1 or 2 diabetes and healthy control subjects (McLellan et al. 1994). The concentration of MG was increased 2-3-fold in blood samples of type 1 diabetic patients and 5-6-fold in blood samples of type 2 patients.

In endothelial cells, the hyperglycemia-induced intracellular AGE formation was significantly increased when glyoxalase-I was partially inhibited. In addition, overexpression of glyoxalase-I completely prevented hyperglycemia-induced AGE formation, indicating that hyperglycemia increased AGE formation primarily by enhancing the concentration of MG (Shinohara et al. 1998). MG-induced AGEs level is also increased in diabetic animal models. Studies using rats rendered diabetic after STZ injection found that the AGE content was increased in lens tissue, kidney, skin and vascular tissues after only a few weeks of the injection (Baynes and Thorpe 1999; Nakayama et al. 1991). A recent study (Thornalley et al. 2003) showed that in STZ-induced diabetic rats, the concentration of CML and CEL was increased in renal glomeruli, retina, sciatic nerve and plasma proteins compared to healthy controls. Moreover, the increased levels of MG-induced hydroimidazolones were found in renal

glomeruli (295%), retina (279%), nerve (211%) and plasma proteins (154%), in comparison with that from control group. In human diabetic subjects, levels of MG-induced AGEs are significantly higher compared to healthy controls. It had been reported that the MOLD level was higher in diabetic plasma compared with that of control (273 ± 62.67pmol per milligram of protein vs 172.5 ± 32.53 pmol per milligram of protein) (Chellan and Nagaraj 1999). Serum hydroimidazolone level was also increased in diabetic patients (Fosmark et al. 2006; Kilhovd et al. 2003). With LC-MS/MS technique, quantitative screening of MG-induced AGEs was performed in diabetic patients. It was found that the level of free CML was increased approx. 4-fold in peritoneal dialysis (PD) subjects and 8-fold in haemodialysis (HD) subjects. The level of free CEL was also increased markedly, approx. 10-fold in PD subjects and 22-fold in HD subjects. In addition, the concentrations of MG-induced hydroimidazolone in plasma proteins were also increased 16-fold in PD and 50-fold in HD subjects (Thornalley et al. 2003). The argpyrimidine concentrations in diabetic serum proteins was two- to threefold higher than in nondiabetic controls (9.3 ± 6.7 vs 4.4 ± 3.4 pmol/mg). Moreover, there is a significant correlation between serum protein argpyrimidine and glycosylated hemoglobin, which suggested that argpyrimidine synthesis in serum proteins, occurs in relation to the degree of glycemia (Wilker et al. 2001). The increased formation of MG modified proteins may initiate processes leading to vascular dysfunction and development of diabetic complications.

Oral administration of MG to mice may induce renal pathology similar to that

found in human diabetic nephropathy (Golej et al. 1998). After 5-month treatment with MG, the mice had elevated level of collagen in kidney and increased glomerular basement membrane thickness. Another study showed that prolonged exposure of rats to MG by intraperitoneal administration induces diabetes-like microvascular changes in rats (Berlanga et al. 2005). After 7 wk treatment with MG (50-75 mg/kg of body weight), serum creatinine was increased, hypercholesterolaemia was induced and vasodilation was impaired compared with saline controls. Degenerative changes in cutaneous microvessels with loss of endothelial cells, basement membrane thickening and luminal occlusion were also detected. There is also evidence that administration of AGE-modified proteins may induce diabetes-like vascular damage (Vlassara et al. 1992; Vlassara et al. 1994). In this study, AGE-modified albumin was administrated intraperitoneally in healthy nondiabetic rats. Within 2-4 weeks of AGE treatment, it was found that AGE administration was associated with a significant increase in vascular permeability, and markedly defective vasodilatory responses to acetylcholine and nitroglycerin, consistent with marked NO inactivation or/and downstream pathways (Vlassara et al. 1992). Chronic administration of *in vitro* prepared protein-AGE to normal rats also leads to advanced pathologic changes in renal glomerular structure and function consistent with focal glomerulosclerosis and albuminuria (Vlassara et al. 1994).

Further support for the involvement of MG in the development of diabetic complications comes from the observations that the pharmaceutical agents, which prevent the accumulation of MG and MG-related AGEs, attenuate the development of

diabetic complications. Therefore, prevention of protein damage by inhibiting AGE formation at critical functional sites of proteins in diabetes is expected to contribute to the preventive therapy of diabetic complications. Many agents inhibit AGE formation by scavenging or trapping MG. Aminoguanidine, one of the most widely used AGE inhibitor, scavenges MG and other α-oxoaldehydes and thereby prevents the formation of α- oxoaldehydes-modified proteins (Lo et al. 1994a). Metformin, another AGE inhibitor, can significantly reduce MG levels by decreasing MG production or increasing MG detoxification (Beisswenger and Ruggiero-Lopez 2003). Other AGE inhibitors like pyridoxamine, LR-90, or LR-9, have also been reported to interact with MG (Rahbar and Figarola 2003).

MG also interferes with insulin action. It has been reported that intracellular MG can impair insulin signalling pathways by inhibiting insulin-stimulated phosphorylation of protein kinase B and extracellular-regulated kinase 1/2, without affecting insulin receptor tyrosine phosphorylation (Riboulet-Chavey et al. 2006). A recent study from our lab (Jia et al. 2006) found that MG modifies insulin molecule by attaching to the internal arginine in the β-chain of insulin. This MG-modified insulin has a significantly reduced capacity in stimulating [^{3}H]-2-deoxyglucose uptake by 3T3-L1 adipocytes and L8 skeletal muscle cells, compared with native insulin (Jia et al. 2006).

6.2 Aging

Aging is characterized by structural and functional changes of different tissues or organs, especially, cardiovascular and renal systems. It has been proposed that most of these cardiovascular and renal modifications were related to glycation of proteins (Vlassara et al. 1992). A recent study found that prolonged intraperitoneal administration of MG can induce premature aging of the animals (Berlanga et al. 2005). In this study, after 7-wk treatment with MG, the rats developed an emaciated and wasted phenotype involving the whole body and, particularly, the carcass. Cachexia was associated with a loss of adipose tissue and a reduction in muscle mass, along with a decrease in skeletal compactness. Moreover, MG-treated rats exhibited an aged cutaneous phenotype characterized by thinner skin with abundant wrinkles. The formation of AGEs progressively increases with aging, even in the absence of disease. For example, in human, tissue CML concentration increased five-fold from 20 to 85 years of age (Dyer et al. 1993). Accumulation of AGEs may cause decreased central arterial compliance, increased myocardial stiffness and endothelial dysfunction (Zieman and Kass 2004).

6.3 Atherosclerosis

There is abundant *in vitro* and *in vivo* evidence that MG and AGEs play a role in the pathogenesis of atherosclerosis. (i) MG induced platelet-derived growth factor

(PDGF) β modification and altered mitogenic signaling in response to PDGF-β and subsequent cell proliferation, leading to fragile thin cap in atherosclerotic lesion (Cantero et al. 2007). (ii) AGE formation alters the functional properties of several important matrix molecules. For instance, AGEs can cause collagen cross-linking and high resistance to collagenases (Sell and Monnier 1989), enhance synthesis of extracellular matrix components (Sims et al. 1996) and trap circulating serum proteins, such as lipoproteins (Meng et al. 1998). (iii)By binding to AGE receptors on endothelial cells, AGEs increase vascular permeability (Wautier et al. 1996), intracellular oxidative stress (Yan et al. 1994), and expression of adhesion molecules (Schmidt et al. 1995). (iv) Tissue-bound AGEs can chemically quench cell-derived nitric oxide activity, thereby inhibiting the nitric oxide-dependent vascular relaxation (Bucala et al. 1991). (v) AGEs also increase proliferation and fibronectin production of smooth muscle cells (Sakata et al. 2000).

7. Rationale and hypothesis

The association of MG and AGEs with hyperglycemic states such as diabetes mellitus has been intensively studied. Increased MG production, which in turn gives rise to AGEs, has been linked to the development of diabetic complications although the exact role of this dicarbonyl in this process remains unclear (Goldin et al. 2006; Singh et

al. 2001). The association of MG and related AGEs with hypertension, however, is still largely unsettled. It has been reported that the systolic blood pressure was significantly increased after Wistar Kyoto (WKY) rats were treated with MG in drinking water. This increase was associated with an increase of aldehyde conjugate levels in kidney, and a decrease in serum levels of circulating NO (Vasdev et al. 1998). An enhanced AGE formation was found in the aorta of stroke-prone spontaneously hypertensive rats (Mizutani et al. 1999). Our previous study (Wu and Juurlink 2002) demonstrated increased MG and AGEs in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) in comparison with that from WKY rats. We also showed that MG increased NF-κB p65 and ICAM-1 expression in VSMCs from SHR (Wu and Juurlink 2002). **These observations lead us to hypothesize that *an increased cellular level of MG is one of the causative factors of essential hypertension.* MG causes the development of hypertension by enhancing AGEs formation in VSMCs, increasing oxidative stress, causing endothelial dysfunction and inducing vascular remodeling.**

8. Objectives and experimental approaches

I. *To investigate the correlation between increased intracellular MG levels and hypertension development in spontaneously hypertensive rats.* We investigated whether MG and MG-induced AGEs were increased in plasma, aorta and kidney

of SHR and whether an increased formation of MG and related AGEs was correlated with the development of high blood pressure in SHR. We used different ages (5-, 8, 13, and 20-weeks) of SHR and age-matched normotensive WKY rats to carry out this part of the study. Blood pressure in 5-week old SHR is still in the normal range (pre-hypertensive stage). At 8 wks of age, SHR start to develop hypertension and 13-week old SHR have fully developed hypertension. SHR of 20-wks age represents the aged hypertension model.

II. *To investigate gender-related changes in AGEs and NOS in Sprague-Dawley (SD) rats and stroke-prone SHR (SHRsp).* There is as yet no data on gender differences in regard to AGEs. Also there is no study correlating gender related changes in CEL, CML and NOS with hypertension development. In this part of the study, gender related changes in AGEs and NOS were investigated through an immunohistochemical characterization in Sprague-Dawley (SD) rats and SHRsp.

III. *To investigate whether decreased MG levels in SHR will prevent or slow down the development of hypertension.* We chronically treated young SHR with aminoguanidine, an inhibitor of AGEs formation, before their blood pressure started to increase. We determined if aminoguanidine attenuated the increase of blood pressure in SHR and the possible underlying mechanisms.

IV. *To test whether accumulated MG and its AGEs in blood vessel walls may constitute a causative factor for hypertension development.* We chronically

treated young SD rats with fructose, and/or metformin. Then we determined whether increased formation of MG and MG-induced AGEs caused blood pressure increase, insulin resistance and vascular remodeling.

CHAPTER 2

GENERAL METHODOLOGY

ANIMALS

All the rats were purchased from Charles River laboratories (St-Constant, Quebec, Canada), and housed in a temperature-regulated animal facility, exposed to a 12 h light/dark cycle with free access to food and water. The standard lab rat chow, Prolab® RMH 3000, contains 60% starch, 22% crude proteins, 5% crude fat, 5% crude fiber, 6% ash, and 2% added minerals (PMI® Nutrition Intl., MO. USA). Rats were treated in accordance with guidelines of the Canadian Council on Animal Care and the experimental protocols were approved by the Animal Care Committee at the University of Saskatchewan. Systolic blood pressure was determined weekly using a standard tail cuff noninvasive BP measurement system (Model 29-SSP, Harvard Apparatus, St. Laurent, QC, Canada). At the end of each study, rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight) intraperitoneally. Blood was collected from the heart using anti-heparin coated tubes, and plasma was separated by centrifuging blood samples at 1,000 g for 10 min at 4ºC. Tissues including heart, kidney, liver, aorta and mesenteric arteries were isolated in ice-cold phosphate buffer saline, cleaned and snap-frozen in liquid nitrogen immediately and stored at -80ºC until processing.

MG MEASUREMENT

Quantitation of MG was done by the widely accepted o-phenylenediamine (o-PD)-based assay (Chaplen et al. 1996) with some modifications. Earlier assays for MG measurement employed dinitrophenylhydrazine for derivatization of MG. This was

not specific since other intermediates of glycolysis also reacted with dinitrophenylhydrazine (Richard 1991). The method we have used is specific for MG and is also very sensitive. In this method the reagent, o-PD, a 1,2-diaminobenzene derivative, rapidly reacts with MG to form a quinoxaline which can be easily quantified with reverse-phase high-performance liquid chromatography.

Sample preparation

Frozen tissues were pulverized with a Mikro-Dismembrator and kept on ice. Then phosphate buffer was added to tissue samples. The amount of tissue and volume of buffer depend on the quantity of tissue and protein content. Tissues were sonicatied on ice (3 times, 10 seconds each), followed by addition of about 1/4 or 1/5 volume of 1 mol/L perchloric acid (PCA) to each sample. The samples were mixed up, incubated on ice for 10 minutes and centrifuged at 15,000 g for 10 min at 4 ^{o}C to remove the PCA-precipitated material. The supernatant of the tissue homogenate or plasma were derivatized with 100 mmol/L o-PD (derivatizing agent) for 3 h at room temperature, followed by centrifuge at 15,000 g for 10 min to remove any particles. 252 µl supernatant was taken out and mixed up with 28 µl of the 100 µmol/L internal standard (5-methylquinoxaline, 5-MQ). Then 130 µl of mixture was transferred into glass inserts of HPLC.

HPLC of quinoxalines

The quinoxaline derivative of MG (2-methylquinoxaline, 2-MQ) and the quinoxaline internal standard (5-MQ) were measured using a Hitachi D-7000 HPLC system (Hitachi Ltd., Mississauga, ON, Canada). The column was a Nova-Pak® C18 column (3.9x15 mm, and 4 um particle diameter; Waters, MA). The mobile phase was composed of 80 vol% of 10 mmol/L NaH_2PO_4 (pH 4.5) and 20 vol% of HPLC grade acetonitrile. The analysis conditions were as follows: detector wavelength, 315 nm; the flow rate of mobile phase, 1.0 ml/min; typical sample size, 130µl; and colume temperature, room temperature. Duplicate injections of each sample were made. Samples were calibrated by comparison with 2-MQ standards.

IMMUNOHISTOCHEMISTRY

Immunostaining

Immunostaining was performed using the method described in our previous study (Ndisang et al. 2003). In brief, paraformaldehyde-fixed and OCT-embedded renal tissues derived from WKY and SHR at different ages were prepared. Sections of 8-10 µm thickness were cut using cryostat. Sections on poly-l-lysine coated slides were permeabilized with 0.1% triton X-100 in PBS for 5 min after 3 washes in 0.1 mol/L phosphate buffer. Nonspecific antibody binding was blocked by incubation for 30 min with normal goat serum diluted 1:30. Sections were then incubated overnight at room temperature in a humid atmosphere in the presence of either monoclonal anti-CML

antibody or anti-CEL antibody (Novo Nordisk, A/S, Denmark) diluted 1:100 in PBS containing 0.1% bovine serum albumin. Control sections were exposed to the diluting solution without the primary antibody added. After the reaction with the primary antibody, the slides were washed 3 times with PBS and incubated with anti-mouse IgG-FITC (Sigma, St. Louis, USA) diluted 1:200 for 1 h at room temperature, followed by another 3 washes. Thereafter, the slides were mounted in PBS-glycerin (7:3 v/v), coverslipped and examined under a fluorescence microscope (Olympus 1×70) with the appropriate filters.

Double staining

For double immunofluorescence staining, aortic sections were incubated overnight in a cocktail solution containing mouse anti-CEL antibody (1:100) and rabbit anti-eNOS antibody (1:200; Chemicon International, Temecula, CA, USA). After multiple washes in PBS, sections were incubated for 2 h in goat anti-mouse IgG-FITC (1:200) and Alexa Fluor 568–conjugated anti-mouse secondary antibody (1:400; Invitrogen, Burlington, ON, Canada). Sections were mounted on gelatinized slides and coverslipped. Immunofluorescence images were obtained under a confocal laser-scanning microscope (LSM 510 META; Zeiss).

Quantification of staining

The intensities of staining were quantified using GeneTools image analysis

software (PerkinElmer®, MA, USA). For each animal, thirty spots of each micrograph, with 10 micrographs of each section and three sections of each animal were analyzed and the average value was used to express the intensity of staining.

MEASUREMENT OF REDUCED GLUTATHIONE LEVELS

The reduced glutathione (GSH) levels in plasma, aorta or kidney were determined by derivation with 5, 5'-dithio-bis (2-nitrobenzoic acid), and reverse-phase HPLC using ultra-violet detection, as described in our previous study (Wu et al. 2004).

MEASUREMENT OF HYDROGEN PEROXIDE

The formation of H_2O_2 was measured by a dichlorofluorescin (DCFH)-assay as described previously (Wu and Juurlink 2002). Briefly, cleaned aortic rings 5 mm long were loaded with a membrane permeable and nonflurescent probe DCFH-DA (10 μmlo/L) in Kreb's buffer bubbled with O_2 and protected from light, for 2 hrs at 37°C. The washed aortic tissues were transferred to a 96-well plates containing 200 μl Kreb's buffer in each well, incubated for 2 hrs at 37°C and detected. Once inside cells, DCFH-DA becomes the membrane impermeable $DCFH_2$ in the presence of cytosolic esterases and further oxidized by H_2O_2 to form oxidized-DCF with detectable fluorescence. Oxidized-DCF was quantified by monitoring the DCF fluorescence intensity with excitation at 485 nm and emission at 527 nm with Fluoroskan Ascent plate-reader and Ascent software (Thermo Labsystem, Helsinki, Finland). The level of

oxidized –DCF was expressed using arbitrary units.

DETECTION OF SUPEROXIDE PRODUCTION

O_2^- production was measured using the lucigenin-enhanced chemiluminescence method as described previously (Wu et al. 2002). Briefly, cleaned aortic rings in Kreb's buffer were incubated for 30 min at 37°C, and then mixed with lucigenin at 50 μmol/L for 15 min before subjecting to detection. MG-induced O_2^- was measured by the intensity of chemiluminescence detected with a luminometer (TD-20/20, Tunner Designs, CA) and expressed using arbitrary units.

MEASUREMENT OF ENZYME ACTIVITIES

The activities of GSH-Px and GSH-Red were measured according to methods described previously (Wu and Juurlink 2001). The measurement of GSH-Px was coupled to the reduction of GSSG formed in the peroxidase reaction. The activity of GSH-Red was determined by monitoring the decrease in absorbance at 340 nm based on the oxidation of NADPH which is coupled to the reduction of one GSSG back to two GSH. The enzyme activity for both GSH-Px and GSH-Red was expressed as nmole NADPH oxidized/min/mg protein. Protein concentrations were determined by bicinchoninic acid procedure using bovine serum albumin standards.

CHAPTER 3

INCREASED METHYLGLYOXAL AND ADVANCED GLYCATION ENDPRODUCTS IN KIDNEY FROM SPONTANEOUSLY HYPERTENSIVE RATS

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ABSTRACT

Background: Methylglyoxal (MG), a metabolite of glucose, causes non-enzymatic glycation of proteins to form irreversible advanced glycation endproducts (AGEs). The role of MG in the development of essential hypertension is unknown though MG has been extensively studied in relation to diabetes.

Methods: Blood pressure of spontaneously hypertensive rats (SHR) and paired Wistar Kyoto (WKY) rats was measured at 5, 8, 13 and 20 wks of age. HPLC was used to determine the levels of plasma and kidney MG as well as reduced or oxidized glutathione in the kidney. MG-induced AGEs, N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-carboxymethyl-lysine (CML), in the kidney were detected by immunohistochemistry.

Results: Plasma MG levels were significantly elevated in SHR, but not in WKY rats, at 8, 13 and 20 wks of age in parallel with blood pressure increase. Kidney MG levels in SHR were increased by 21% and 38% at 13 and 20 wks, respectively, compared to age-matched WKY rats. There were no differences in blood pressure and MG levels in plasma and kidney between SHR and WKY rats at 5 wks of age. Immunohistochemistry revealed more intense staining for CML and CEL in kidneys from SHR compared to WKY rats from 8 wks onwards. Most of the staining was localized to renal tubules with some staining in the glomerular vessels.

Conclusions: MG and AGEs formation was significantly elevated in kidney from SHR, which may cause local vascular and tubular damage, contributing to the development and complications of hypertension.

Key Words: methylglyoxal ■ advanced glycation endproducts ■ kidney ■ hypertension

INTRODUCTION

Methylglyoxal (MG), a metabolite of glucose, is a highly reactive dicarbonyl compound formed spontaneously during glycolysis as a result of transformation of triose phosphates[1]. This dicarbonyl molecule can be also formed during various metabolic processes including metabolism of acetone from lipolysis and metabolism of threonine from protein catabolism[2,3]. MG is detoxified by the glyoxalase system that uses glutathione (GSH) as a cofactor[1]. MG is the most reactive AGE precursor[4] and can react with selective proteins to yield irreversible advanced glycation endproducts (AGEs), leading to cross-linking and denaturation of protein[5]. The irreversible reaction of MG with lysine residues of protein forms N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-carboxymethyl-lysine (CML).

MG and related AGEs such as CEL have been recognized as indicators of carbonyl overload *in vivo*[6,7], and their formations are correlated with age[8,9]. The association of MG and AGEs with hyperglycemic states such as diabetes mellitus has been intensively studied. Increased MG production, which in turn gives rise to AGEs, has been linked to the development of nephropathy in diabetes although the exact role of this dicarbonyl in this process remains unclear[10]. The associations of MG and related AGEs with hypertension, however, are still largely unsettled. It has been reported that the systolic blood pressure was significantly increased after Wistar Kyoto (WKY) rats were treated with MG in drinking water. This increase was associated with an increase of aldehyde conjugate levels in kidney, not in heart or liver[11]. An enhanced AGE formation was

found in the aorta of stroke-prone spontaneously hypertensive rats[12]. Our previous study[13] demonstrated increased MG and AGEs in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) in comparison with that from WKY rats. We also showed that MG increased NF-κB p65 and ICAM-1 expression in VSMCs from SHR[13].

The aim of the present study was to investigate whether MG and MG-induced AGEs were increased in plasma or kidney of SHR and whether an increased formation of MG and related AGE was correlated with the development of high blood pressure in SHR. To this end, MG levels in plasma and kidney were determined in SHR and WKY at different hypertension development stages. The MG-induced CML and CEL in kidney were evaluated in both strains. Since glutathione (GSH) plays a role in the metabolism of MG, GSH levels, oxidized glutathione (GSSG) and related enzyme activities including glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Red) were also evaluated.

MATERIALS AND METHODS

Animals

Male SHR and WKY rats were purchased from Charles River laboratories (St-Constant, Quebec, Canada), and were treated in accordance with guidelines of the Canadian Council on Animal Care and the experimental protocols were approved by the Animal Care Committee at the University of Saskatchewan. SHR and paired WKY rats

were compared at 4 different time points ranging from 5 to 20 wks of age. Systolic blood pressure was determined weekly using a standard tail cuff noninvasive BP measurement system (Model 29-SSP, Harvard Apparatus, St. Laurent, QC, Canada). At the end of the study, rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight) intraperitoneally. Blood was collected from the heart, and plasma was separated by centrifuging blood samples at 1,000 g for 10 min at 4ºC. Kidney was isolated in ice-cold phosphate buffer saline (PBS), cleaned and snap-frozen in liquid nitrogen immediately and stored at -80ºC until processing.

MG Measurement

Quantitation of MG was done by the widely accepted o-phenylenediamine (o-PD)-based assay as described by Chaplen *et al.*[14] with some modifications. Briefly, the supernatant of kidney homogenate or plasma was incubated with 100 mmol/L o-PD (derivatizing agent) for 3 h at room temperature. The quinoxaline derivative of MG (2-methylquinoxaline) and the quinoxaline internal standard (5-methylquinoxaline) were measured using a Hitachi D-7000 HPLC system (Hitachi Ltd). The column was a Nova-Pak® C18 column (3.9x15 mm, and 4 μm particle diameter; Waters, MA). The mobile phase was composed of 80% (vol) of 10 mmol/L NaH_2PO_4 (pH 4.5) and 20% (vol) of HPLC grade acetonitrile. Duplicate injections of each sample were made. Samples were calibrated by comparison with a 2-MQ standard.

Measurement of reduced and oxidized glutathione levels

The reduced glutathione (GSH) and oxidized glutathione (GSSG) levels in kidney were determined by derivation with 5, 5'-dithio-bis (2-nitrobenzoic acid), and reverse-phase HPLC using ultra-violet detection, as described in our previous study.[15]

Measurement of enzyme activites

The activities of GSH-Px and GSH-Red were measured according to methods described previously[16]. The measurement of GSH-Px was coupled to the reduction of GSSG formed in the peroxidase reaction. The activity of GSH-Red was determined by monitoring the decrease in absorbance at 340 nm based on the oxidation of NADPH which is coupled to the reduction of one GSSG back to two GSH. The enzyme activity for both GSH-Px and GSH-Red was expressed as nmole NADPH oxidized/min/mg protein. Protein concentrations were determined by bicinchoninic acid procedure using bovine serum albumin standards.

Immunohistochemistry

Immunostaining was performed using the method described in our previous study[17]. In brief, paraformaldehyde-fixed and OCT-embedded renal tissues derived from WKY and SHR at different ages were prepared. Sections of 7-10 μm thickness were cut on a cryostat and picked up on poly-l-lysine coated slides. Sections were permeabilized with 0.1% triton X-100 in PBS for 5 min after 3 washes in 0.1 mol/L PBS.

Nonspecific antibody binding was blocked by incubation for 30 min with normal goat serum diluted 1:30. Sections were then incubated overnight at room temperature in a humid atmosphere in the presence of either monoclonal anti-CML antibody or anti-CEL antibody (Novo Nordisk, A/S, Denmark) diluted 1:100 in PBS containing 0.1% bovine serum albumin. Control sections were exposed to the diluting solution without the primary antibody added. After the reaction with the primary antibody, the slides were washed 3 times with PBS and incubated with anti-mouse IgG-FITC (Sigma, St. Louis, USA) diluted 1:200 for 1 h at room temperature, followed by another 3 washes. Thereafter, the slides were mounted in PBS-glycerin (7:3 v/v), coverslipped and examined under a fluorescence microscope with the appropriate filters. The sections were independently scored by two observers in a blinded fashion. Staining intensities of the renal tissue were graded semiquantitatively as follows: (-) negative staining, (–/+) occasional, (+) weak, (++) moderate, (+++) intense staining.

Data Analysis

Data are expressed as mean±SEM and analyzed using Student's *t*-test in conjunction with the Newman-Keuls test where applicable. Significant difference between treatments was defined at a level of $P<0.05$.

RESULTS

Basal parameters of SHR

As shown in Table 1, the systolic blood pressure was significantly higher in SHR at 8 wks and onwards, compared to age-matched WKY. During the observation period, blood pressure increased significantly above the initial value recorded at 5 wks in SHR but remained unchanged in WKY rats. In young rats at 5 wks of age there was no difference in the blood pressure and body weights between SHR and WKY rats. The body weight of SHR was significantly lower than age-matched WKY rats from 8 wks onward. No significant difference was found in kidney weight and the ratio of kidney weight to body weight in both groups of rats at different ages. The plasma glucose levels (mean±SEM) of 7 and 12 wks old SHR were 4.1±0.1 mM (n=5) and 5.2±0.2 mM (n=10), respectively, which were comparable to those in age-matched WKY rats, 4.2±0.1 mM (n=5) at 7 wks and 5.1±0.1 mM (n=6) at 12 wks.

Increased MG levels in plasma and kidney of SHR

As shown in Figure 1A, plasma MG level was age-dependent and progressively increased in SHR, compared to WKY rats. The plasma levels (nmol/ml) of WKY rats / SHR at 5, 8, 13 and 20 wks were 11.2±0.44 / 11.3±0.65 (n=3), 9.1±0.81 / 13.8±0.72 (n=4), 18.5±2.71 / 30.3±2.05 (n=5), and 14.2±3.48 / 33.6±2.16 (n=4), respectively.

In young WKY and SHR rats (5 and 8 wks old), there was no significant difference in kidney MG levels. However, from 13 wks onwards the renal MG level was

significantly higher in SHR than in age-matched WKY rats (Figure 1B). The renal MG levels (nmol/mg protein) in WKY rats / SHR were 0.67±0.15 / 0.67±0.12 (*n*=3) at 5 wks, 0.79±0.20 / 0.82±0.10 (*n*=4) at 8 wks, 0.71±0.06 / 0.91±0.09 (*n*=5) at 13 wks, and 0.22±0.04 / 0.30±0.06 (*n*=4) at 20 wks, respectively.

Increased MG-induced CEL and CML in kidney of SHR

With immunohistochemical staining method, we further investigated whether MG-induced AGEs were increased in kidney from SHR. Figure 2 shows more intense or positive staining for CEL in kidney from SHR, compared to WKY rats at 8, 13 and 20 wks of age (*n*=3 for each age group), respectively (Table 2). Positive CEL staining in kidney of SHR was observed occasionally even at the age of 5 wks (Table 2). Most of the positive staining was localized to renal tubules in both strains. There was some staining observed in glomerular vessels, too. Staining for CML was also more marked in kidney from SHR as opposed to WKY rats at 8, 13 and 20 wks of age (*n*=3 for each group) (Figure 3 and Table 2). The control sections revealed no staining.

GSH and related enzyme activities in the kidney of SHR

As shown in Figure 4A, GSH level in kidney was significantly decreased by 16.5% in SHR at the age of 20 wks (50.7±4.3 nmol/mg protein) in comparison with that from age-match WKY rats (60.7±3.0 nmol/mg protein). The GSH levels (nmol/mg protein) in WKY rats / SHR at 5, 8, and 13 wks of age were 95.9± 9.9 / 94.6±6.1, 82.5±6.2 /

76.6±2.6, and 67.6±11.0 / 62.0±11.0, respectively. The ratio of GSH/GSSG was also significantly decreased in 20 wks old SHR than that in age-matched WKY rats (Figure 4B). There was no significant difference in renal GSH levels and GSH/GSSG ratio between SHR and WKY rats at the relatively younger age (Figure 4).

The renal GSH-Px activity was significantly decreased by 14% in SHR at the age of 20 wks (1.62±0.1 nmol/mg protein), compared with age-matched WKY (1.87±0.18 nmol/mg protein) (n=3 for each group, P<0.05). There was no significant change in renal GSH-Px activities between SHR and WKY rats at the age of 5, 8, and 13 wks (n=3 for each group, P>0.05). No significant difference in GSH-Red activity was observed between these two strains in different age groups (data not shown).

DISCUSSION

Studies on MG and AGEs have been mainly focused on their roles in diabetes mellitus and its complications. A state of hyperglycemia as a precedent for MG and AGE formation stimulates such studies. However, an association of MG and AGEs with disease conditions with an apparently normoglycemic state has not been investigated intensively. Our study shows a strong association of MG and its AGE products, CML and CEL, with hypertension in SHR. The blood pressure of SHR was not different from that of WKY rats at 5 wks of age. From 8 wks onwards, it was significantly elevated compared to age-matched WKY rats. Importantly, this increase was associated with a progressive increase in plasma MG levels, which was significantly higher in SHR than

in WKY rats. MG levels in SHR kidney were also significantly higher than in WKY rats from 13 wks onwards. Moreover, the levels of MG-induced AGEs, CML and CEL, in the kidney were higher in SHR than in WKY from 8 wks onwards, in parallel with increased plasma MG levels and blood pressure in SHR. On the other hand, no hyperglycemia developed in SHR at different ages. It has been shown that the glucose levels and insulin sensitivity in SHR and WKY rats are comparable[18]. Our study suggests that in addition to diabetes/hyperglycemic or hyperlipidemic conditions[19], MG is associated with the development of high blood pressure in SHR, and indeed MG may be a causative factor for the development of hypertension or its complications in this non-diabetic model. This hypothesis is supported by the evidence, including coincidental increase in plasma MG level with blood pressure development, observed in our current study and the induction of hypertension in WKY rats by chronic MG feeding reported previously[11]. However, further studies are needed to clarify the causative role of MG in hypertension. In comparison with glucose-induced glycation that undergoes a reversible Maillard reaction with N-terminal amino groups or lysyl chains to form fructosamine, an early glycation adduct, MG induces glycation through an oxidation process. This reaction is also called glycoxidation and it generates irreversible end glycation adducts[6] including CEL and CML. With their stable chemical property, CEL and CML have been interpreted as a measure of the status of oxidative stress and of cumulative oxidative damage to proteins induced by MG in aging and diabetes[4,20]. In our study, antibodies against CEL and CML were used. The antibody against MG-induced

CML is less selective since this antibody can also react with glyoxal (another dicarbonyl compound)-induced AGE[20]. In contrast, anti-CEL antibody is specifically against MG-induced CEL and has been suggested to be a good indicator for MG-induced AGE or glycoxidation product[21].

The kidney plays a major role in blood pressure homeostasis[22] and possibly the pathogenesis of hypertension in SHR[23] and other models of hypertension such as DOCA-salt sensitive hypertension[24]. The afferent and efferent arterioles and the glomerular capillaries along with the renal proximal tubules and the thick ascending limb of the loop of Henle regulate salt and water absorption as part of the homeostatic mechanism. A number of enzymes including the cytochrome P450s and various ion pumps are involved. An alteration in the function of one or more of these enzymes/proteins can readily disturb the homeostatic mechanisms operating within the kidney and lead to increased blood pressure. AGE-induced renal injury was demonstrated *in vivo* when diabetic mice were randomized to receive either a regular diet or diet that had less AGE content. Mice on the regular diet developed changes typical of diabetic nephropathy, while mice on the low AGE diet did not, even in the face of persistent hyperglycemia[25]. Immunohistochemical studies of kidney from normal and diabetic rats have suggested that glomerular basement membrane, mesangium, podocytes, and renal tubular cells accumulate high levels of AGEs. Ultrastructural studies have indicated that AGE peptides may be reabsorbed by the renal proximal tubular cells[26,27]. AGE deposition can lead to glomerulosclerosis and widespread

dysfunction, independent of diabetes. This is evidenced by the development, *in nondiabetic animals* infused with AGE-albumin, of glomerular pathology resembling diabetic nephropathy, including glomerular hypertrophy, BM thickening, mesangial extracellular matrix expansion, and albuminuria[28]. Our immunohistochemical studies revealed a strong staining for CML and CEL in the renal tubules and the glomerular vessels of SHR compared to WKY rats from 8 wks onwards. This suggests that an increased renal MG level and MG-glycated proteins in kidney may induce local micro-vascular and tubular damage, consequently impairing renal function. A chronically increased MG and related AGE formation may lead to the development of high blood pressure in SHR.

MG levels in plasma or tissues including kidney are dependent on the balance between its anabolism and catabolism. When the formation of MG exceeds its degradation, accumulation of MG occurs even with normal glucose levels. As mentioned earlier, the main source of MG in mammals is glycolysis. The estimated rate of MG formation in normal tissues is approximately 125 μM/day, largely resulting from the fragmentation of triosephosphates[1]. Approximately 0.1-0.4% of glucose is metabolized to D-lactate in human red blood cells and promyelocytic leukaemia cells, which represents the maximum flux of MG formation[29]. Other sources of MG are aminoacetone or acetone catalyzed by semicarbadize-sensitive amine oxidase (SSAO) or acetol mono-oxygenase (AMO)[2,3], with former enzyme found in high amounts within VSMC and in human plasma[30,31]. Increased activities of plasma SSAO and AMO have

been suggested to be responsible for the increased circulating MG and endothelial dysfunction in diabetes patients[30]. Moreover, SSAO enzyme levels have been shown to be significantly elevated in *nondiabetic patients* with congestive heart failure[32] and obesity[33], compared to controls. In our study, MG levels in plasma and kidney were significantly and age-dependently increased in SHR at different ages in comparison with the age-matched WKY (Fig.1). A lower MG level in kidney, but not in plasma, was also observed from both SHR and WKY rats at the age of 20 wks in comparison with their relative younger age groups tested. The exact mechanism for this age-dependent difference is unclear yet. One possible explanation for this might be a reduced MG formation in kidney in the aged rats. MG formation in plasma is more complicated, linked to different circulating enzymes such as SSAO and AMO. An age-dependent change in SSAO and AMO might be responsible for the alteration in circulating MG at different ages in diabetes[30] as well as in hypertension. These speculations need to be validated in future.

Normal catabolism of MG is largely dependent on the availability of cellular GSH and the related enzyme activities including GSH-Px and GSH-Red. In our study, the elevated levels of MG in plasma or kidney and MG-induced CEL and CML in kidney of SHR were observed at as early as the age of 8 wks. These changes preceded the decreased GSH level and suppressed activity of GSH-Px in kidney of SHR, which appeared at the age of 20 wks. At the same time the GSH-Red activity also increased, albeit not significant. This increase may be a compensatory response to increased GSSG

levels. The delayed decrease of GSH in SHR indicates that the primary reason for the early onset of MG increase could be the abnormally increased MG production rather than a dysfunctional MG degradation. Blood pressure of young SHR begins to increase as MG level and its associated oxidative stress level are progressively increased and the anti-oxidant GSH system fails to compensate. Eventually, GSH level will drop and hypertension in aged SHR enters a sustained stage.

In conclusion, MG levels in the plasma and kidney of SHR, but not WKY rats, increased in an age-dependent manner associated with the development of hypertension. Increased MG and AGE formation in kidney may cause local damage, contributing to both development and complications of hypertension.

Table 3-1. Basal parameters of SHR and WKY at different ages.

Age (wks)	Rats	BP (mmHg)	BW (g)	KW (g)	KW/BW (mg/g)
5	WKY	123±1	107±3	1.33±0.08	12.43±0.30
	SHR	131±2	98±2	1.27±0.01	12.96±0.17
8	WKY	121±1	197±6	1.91±0.09	9.64±0.40
	SHR	190±3 **	180±4 *	1.81±0.01	10.1±0.08
13	WKY	120±1	339±5	2.45±0.05	7.23±0.08
	SHR	203±1 **	281±7 **	2.36±0.02	8.4±0.30
20	WKY	119±1	353±11	2.22±0.03	6.3±0.10
	SHR	202±1 **	308±4*	2.52±0.08	8.15±0.20

BP: systolic blood pressure, BW: body weight, KW: kidney weight. *P<0.05, SHR *vs*. WKY of the same age group. **P<0.001, SHR *vs*. WKY of the same age group.

Table 3-2. CEL and CML staining in kidney from SHR and WKY at different ages (*n*=3 for each group).

	Rats	5 wks	8 wks	13 wks	20 wks
CEL	WKY	-	++	+	+
	SHR	-/+	+++	+++	++
CML	WKY	-	+	-/+	-/+
	SHR	-	++	++	+

Staining: (-) negative, (-/+) occasional, (+) weak, (++) moderate, (+++) intense

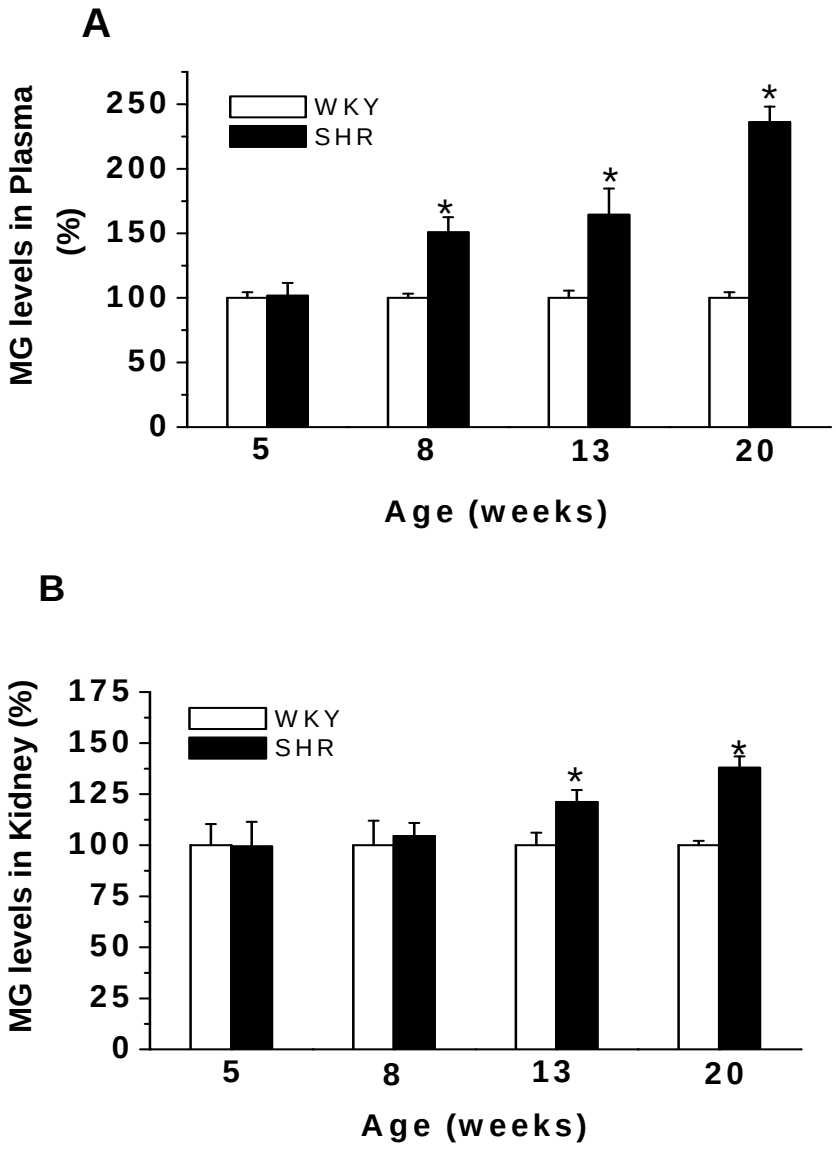

Figure 3-1. MG levels in kidney and plasma from WKY and SHR at different ages. MG levels were measured in plasma (A), and kidney (B) from SHR and age-matched WKY rats at 5 wks (*n*=3), 8 wks (*n*=4), 13 wks (*n*=5) and 20 wks (*n*=4) of age. MG levels in SHR are presented as the percentage of that in age-matched WKY. **P*<0.05, SHR *vs*. WKY of the same age group.

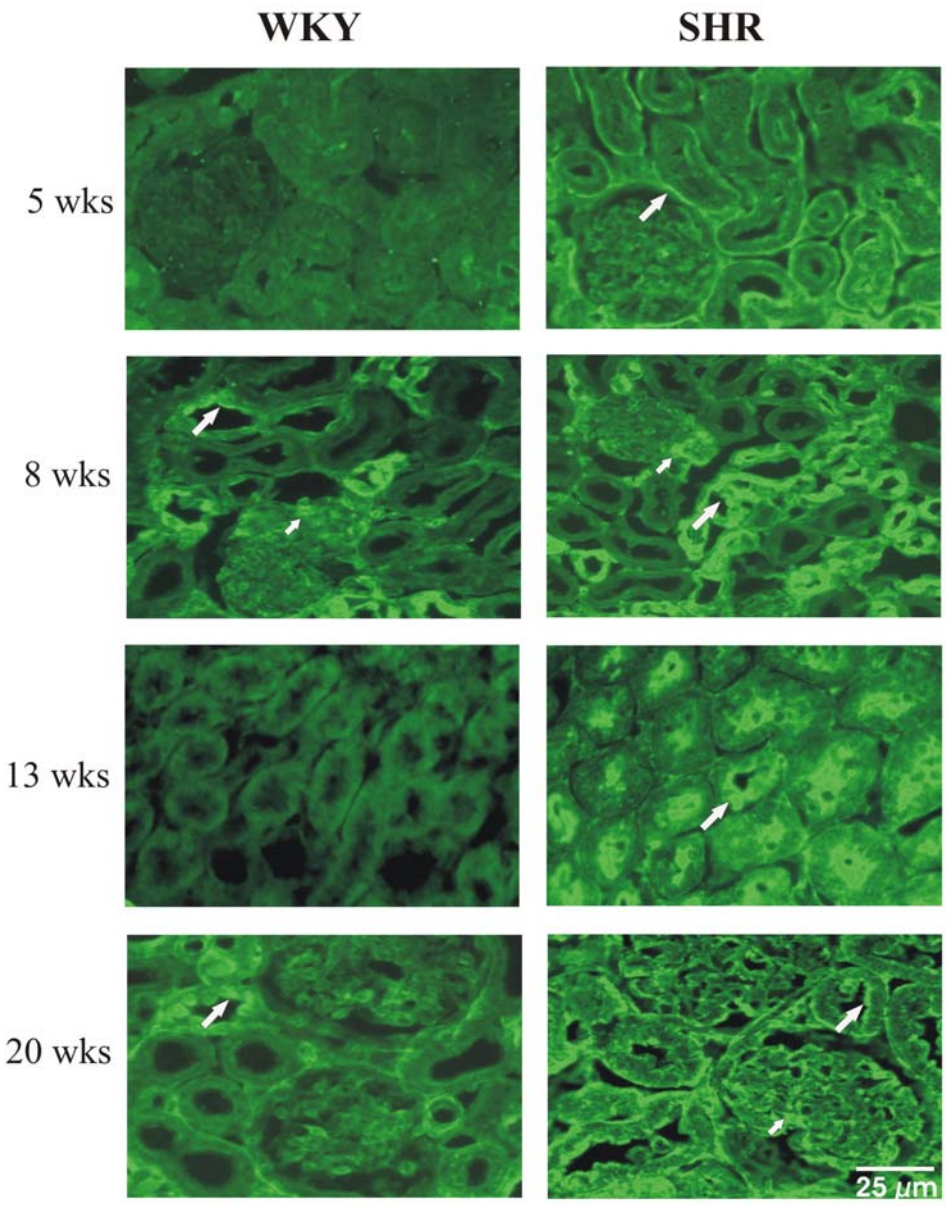

Figure 3-2. Immunohistochemical detection of CEL in kidney from WKY rats and SHR at different ages. Kidney sections from SHR (right panel) show more extensive staining than age-matched WKY rats (left panel). Big arrows indicate the representative immunoreactivity in renal tubules, and small arrows show staining in the glomerulus.

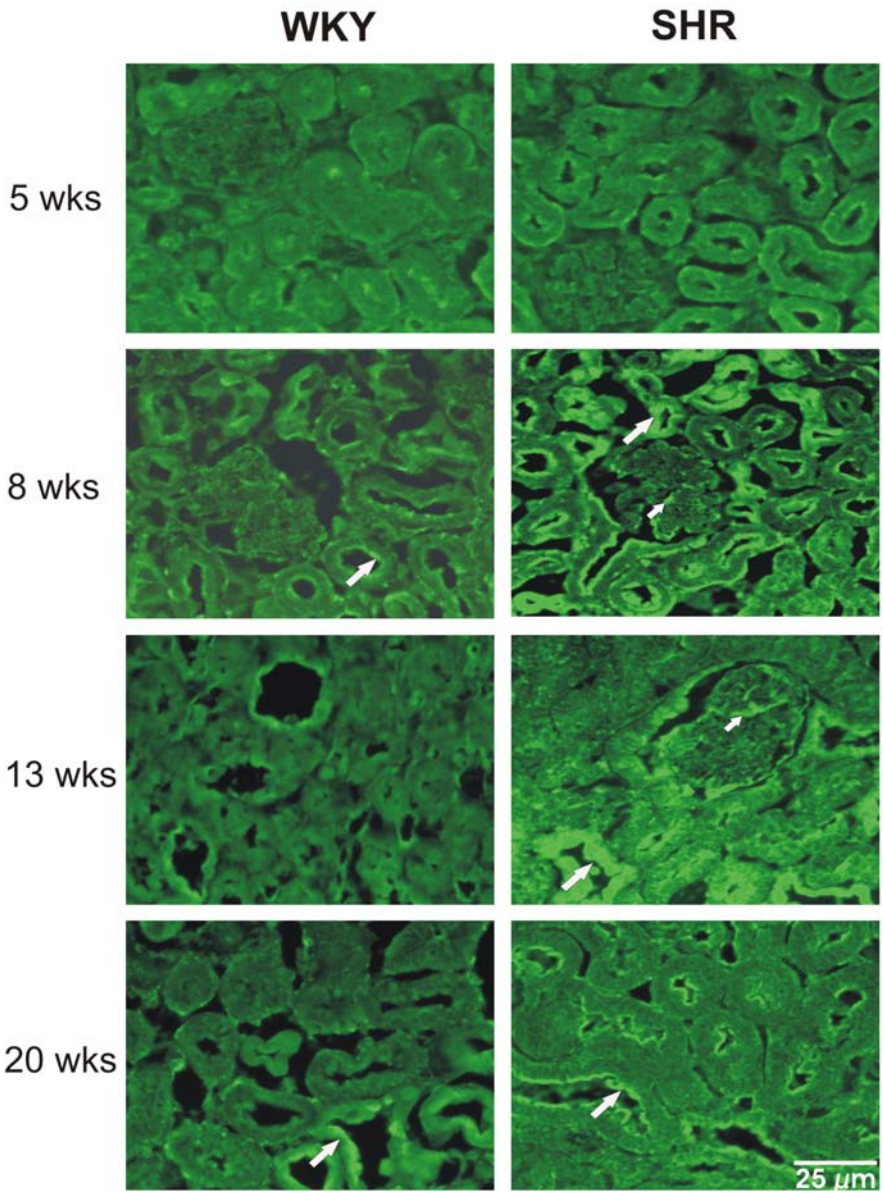

Figure 3-3. Immunohistochemical detection of CML in kidney from WKY rats and SHR at different ages. Kidney sections from SHR (right panel) show more staining than age-matched WKY rats (left panel). Big arrows indicate representative immunoreactivity in renal tubules, and small arrows show staining in the glomerulus.

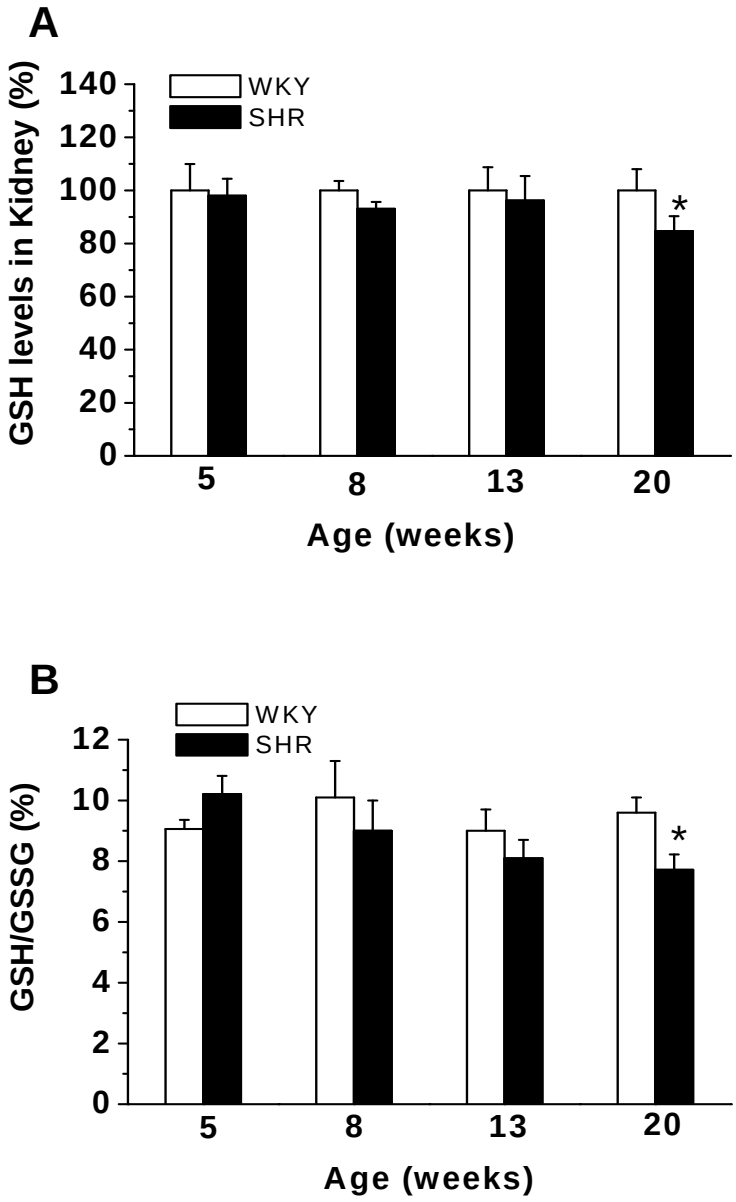

Figure 3-4. GSH levels and oxidative stress in kidney from WKY rats and SHR at different ages. A. Renal levels of GSH. *n*=4 for 5 and 20 wks and *n*=7 for 8 and 13 wks. GSH levels in SHR are presented as the percentage of that in age-matched WKY. B. Oxidative stress in kidney. *n*=3 in each group. **P*<0.05, SHR *vs*. WKY of the same age group.

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CHAPTER 4

GENDER-RELATED DIFFERENCES IN ADVANCED GLYCATION ENDPRODUCTS, AND OXIDATIVE STRESS MARKERS IN RATS

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ABSTRACT

An age- and blood pressure-associated increase in methylglyoxal (MG) and MG-induced advanced glycation endproducts (AGEs), including N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-carboxymethyl-lysine (CML), in the kidney of spontaneously hypertensive rats (SHR) has been shown. In the present study, gender-related changes in AGEs and NOS were investigated in Sprague-Dawley (SD) rats and stroke-prone SHR (SHRsp). Immunohistochemical analyses were conducted on kidneys from 24-week old male and female SD rats as well as SHRsp. The systolic blood pressure of SHRsp was significantly higher than that of SD rats. Male SD rats had more intense kidney staining for CEL than female SD rats. Both male and female SHRsp had more marked CEL and CML staining localized to kidney tubules, as opposed to SD rats. Female rats showed more staining in glomerular vessels than male rats in both SD and SHRsp. Nuclei containing NF-κB p65 and activated macrophages were seen in the kidney from SHRsp, not so much in SD rats, localized to renal tubules in male and glomerular vessels in female SHRsp. Higher protein level of NF-κB p65 was found in SHRsp than in SD rats. SD rats had more intense kidney nNOS staining than SHRsp. Intensity of iNOS staining was significantly higher in SHRsp than in SD rats with no gender differences in either strain. SHRsp and male rats exhibit higher AGEs and oxidative stress than SD and female rats, respectively. These differences might partly account for development of hypertension in SHRsp and higher vulnerability of male animals to renal pathology.

INTRODUCTION

The kidney plays a major role in blood pressure homeostasis (1) and possibly the pathogenesis of hypertension in spontaneously hypertensive rats (2) (SHR) and other models of hypertension such as DOCA-salt sensitive hypertension (3). A number of enzymes including the nitric oxide synthase (NOS) isoforms, cytochrome P450s and various ion pumps are involved in the modulation of renin secretion and secretion/absorption of ions. An oxidative stress-induced alteration in the function of one or more of these enzymes/proteins can readily disturb the homeostatic mechanisms operating within the kidney and lead to increased blood pressure. Nitric oxide (NO), synthesized by NOS is an important vasodilator (4) and renin secretion modulator (5) and can be considered as an anti-hypertensive mediator. The gender of the animal has been shown to have an important influence on the expression/activity of endothelial nitric oxide synthase (eNOS) in the blood vessels (6-8) and the kidney (9), conferring protection to the female gender against development of hypertension, atherosclerosis and cardiovascular mortality (10,11). It has been shown in humans and animals that NO level is greater in females than in males because estrogens not only stimulate NO production (6,7) but also decrease inactivation of NO by oxygen radicals (12). Post-menopausal females and males have reduced arterial NO activity which was restored to premenopausal levels in females after two weeks of estrogen replacement therapy (13).

The impact of gender on renal disease has also been observed in rats. Aging male

rats show a reduction in renal NOS compared to females (14) and develop age-dependent kidney damage, proteinuria and glomerulosclerosis, whereas females and orchidectomized males are resistant to the development of renal injury (15). Male rats have a greater tendency, an androgenic effect, than female rats, to develop proteinuria after chronic NOS inhibition (16). Also, male stroke-prone spontaneously hypertensive rats (SHRsp) have more frequent renal lesions and more severe renal damage compared to female rats (17). The incidence and prevalence of end-stage renal disease are higher in men than in women (18).

The expression/activity of NOS are also altered in hypertensive animal models such as SHR. Earlier studies have shown increased reactive oxygen species, enhanced NOS expression and NO production in SHR. These findings support the role of oxidative stress in the genesis and/or maintenance of hypertension and compensatory upregulation of the expression of eNOS, iNOS (19) and nNOS (20) in SHR.

There is evidence that oxidative stress is enhanced in males compared with females (21-23). Plasma thiobarbituric acid-reactive substances and urinary 8-isoprostaglandin $F_2\alpha$ were higher in men than in women (22) and male rats produced more superoxide anions than females (23). We have recently shown an age and blood pressure-associated increase in methylglyoxal (MG), a metabolite of glucose, and MG-induced irreversible advanced glycation endproducts (AGEs), N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-carboxymethyl-lysine (CML), in the plasma and kidney of SHR (24). However, gender differences in regard to these AGEs are not

known.

There is as yet no data on gender related changes in CEL and CML, which are precursors of oxidative stress (25). Also there is no study correlating gender related changes in CEL, CML and NOS with hypertension development. In the present study, gender related changes in AGEs and NOS were investigated through an immunohistochemical characterization in Sprague-Dawley (SD) rats and SHRsp.

MATERIALS AND METHODS

Animals

Male and female Sprague-Dawley (SD) rats and SHRsp (24 wks old) were used to carry out this work. SD rats were used to fulfill our primary aim to investigate gender differences within a given strain with a normal blood pressure profile that was not genetically related to SHRsp. SHRsp were used instead of SHR because the SHRsp strain develops a severe form of hypertension and shows a high incidence of injuries in different vascular beds, especially in renal vasculature and parenchyma. Such lesions are milder or even absent in the closely related SHR. Moreover, male SHRsp have more frequent lesions and more severe renal damage compared to female rats (17). These animals were housed in a temperature-regulated animal facility and exposed to a 12 h light/dark cycle with free access to food and water. Rats were treated in accordance with guidelines of the Canadian Council on Animal Care, and the experimental protocols

were approved by the Animal Care Committee at the University of Saskatchewan. Systolic blood pressure was determined weekly using a standard tail cuff noninvasive BP measurement system (Model 29-SSP, Harvard Apparatus, St. Laurent, QC, Canada).

Tissue preparation

At the end of the study, rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight) intraperitoneally. Rats were perfused with heparinized phosphate buffered saline (PBS) followed by PBS containing 4% paraformaldehyde. Kidney tissues were dissected out immediately and kept in the same fixative solution overnight. Samples were then incubated in a 30% sucrose solution for 3 days at 4°C for cryoprotection. After embedding in O.C.T. compound (Somagen Diagnostics, Edmonton, AB, Canada), 8 μm thick cross sections were cut using a cryostat, collected on gelatin-chrome alum-coated slides, and stored at -20°C until further processing.

Immunohistochemistry

CEL and CML Staining

Immunostaining was performed using the method described in our previous study (26). In brief, frozen sections were permeabilized with 0.1% triton X-100 in PBS for 5 min after 3 washes in 0.1 M PBS. Nonspecific antibody binding was blocked by incubation for 30 min with normal goat serum diluted 1:30. After overnight incubation with either monoclonal anti-CML antibody or anti-CEL antibody (Novo Nordisk, A/S,

Denmark) diluted 1:100, the slides were washed 3 times and incubated with anti-mouse IgG-FITC (Sigma, Oakville, ON, Canada) diluted 1:200 for 1 h at room temperature, followed by another 3 washes. The slides were mounted in PBS-glycerin (7:3 v/v), coverslipped and examined under a fluorescence microscope with the appropriate filters.

ED-1 and NF-κB

Frozen sections were immunostained by using primary antibody ED-1 (Serotec, Raleigh, NC), a marker for activated macrophages, or anti-NF-κB p65 (Transduction Laboratories, Lexington, KY, USA) that recognized the nuclear localization signal on p65. ED-1 staining was detected by Cy3-conjugated affiniPure goat antimouse IgG (Jackson ImmunoResearch, WestGrove, PA, USA), and NF-κB staining was demonstrated by the avidin–biotin–peroxidase (Vector Laboratories, Burlington, ON, Canada) detection system.

NOS Staining

Kidney tissue sections (8 μm) were incubated with either monoclonal nNOS antibody or polyclonal iNOS antibody (Calbiochem, San Diego, CA, USA) diluted at 1:200. Anti-rabbit or anti-mouse IgG-FITC diluted 1:200 was the secondary antibody.

Quantification of staining

The intensities of staining were quantified using GeneTools image analysis

software (PerkinElmer®, MA, USA). For each animal, thirty spots of each micrograph, with 10 micrographs of each section and three sections of each animal were analyzed and the average value was used to express the intensity of staining.

Western Blot Analysis

Proteins were separated on polyacrylamide gels by using a minivertical electrophoresis system (Bio-Rad) and transblotted onto polyvinyldifluoridene membranes, and membranes were then incubated with the appropriate primary antibodies, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad) as done before (27). Proteins were visualized by using chemiluminescence substrate kit (Amersham Biosciences) and quantified by using the UN-SCAN-IT GEL automated digitizing system (version 5.1, Silk Scientific, Orem, UT). The membrane was reprobed with anti-ß-actin (Sigma). Quantification was relative to the ß-actin. For quantification of nuclearly localized NF-κB p65, nuclear extracts were obtained by using the NE-PER nuclear and cytoplasmic extraction kit (Pierce) as described (28).

Data analysis

Data are expressed as mean ± SEM and analyzed using Student's *t*-test or one-way analysis of variance (ANOVA) in conjunction with the Newman-Keul's test where applicable. Significant difference between treatments was defined at a level of

P<0.05.

RESULTS

Basal parameters

The systolic blood pressure of SHRsp was significantly higher than that of age-matched SD rats (Table 1). There is no difference in blood pressure between male and female rats of the same strain. Body weight of males was more than that of females within each strain ($p < .001$). In addition, SD rats weighed more than age-matched SHRsp of the same gender ($p < .001$). The plasma glucose levels of male and female SHRsp were 5.4±0.3 mM (n=10) and 5.1±0.2 mM (n=6), respectively, which were comparable to those in age-matched SD rats, 4.9±0.1 mM (n=10) of male and 5.8±0.6 mM (n=6) of female.

Increased MG-induced CEL and CML in kidney of SHRsp

With immunohistochemical staining method, we investigated whether MG-induced AGEs were increased in kidney from SHRsp. Figure 1A shows more intense or positive staining for CEL in kidney from male and female SHRsp, compared to age-matched SD rats of the same gender (n = 3 for each age group). The staining intensity in male SD rats was significantly higher than that in female SD rats (Figure 1B). There was no difference between male and female SHRsp. Both male and female SHRsp had more marked CML staining in kidney as opposed to SD rats of the same gender

(Figure 2). Most of the positive staining for CEL or CML was localized to renal tubules in both strains. There was very little staining observed in glomerular vessels of male rats, and female rats had more positive staining in glomerular vessels than male rats for both SD and SHRsp. The negative (no primary antibody) and positive (control immune globulin) control sections revealed no staining (not shown).

ED-1 and NF-κB

In SHRsp activated macrophages could be identified in the kidney, indicating an inflammatory state in its structure. Positive staining of ED-1 was only detected in the renal tubules of male SHRsp. As to female SHRsp, ED-1 staining was only observed in glomerular vessels (Figure 3A). Activated macrophages were not detectable in the kidney from SD rats. Immunohistochemical staining revealed nuclei containing NF-κB p65 in the kidney from SHRsp, and very little staining was detected in the kidney from SD rats (Figure 3B). Positive staining was localized in the renal tubules of male SHRsp, while in the female SHRsp it was localized to the glomerular vessels.

Western blot analysis demonstrated that nuclearly localized NF-κB p65 was significantly higher in male SD rats and SHRsp than female rats of the same gender (Figure 4).

nNOS immunoreactivity

SD rats had more intense kidney nNOS staining than age-matched SHRsp (n = 3

for each age group) (Figure 5). Intensity of nNOS staining was significantly higher in female rats than in age-matched male rats of the same strain for both SD rats and SHRsp. Positive staining was localized to the glomerular vessels in female SD rats and SHRsp, whereas in male rats of both strains the staining was only observed in renal tubules. Negative and positive control sections revealed no staining (data not shown).

iNOS immunoreactivity

Both male and female SHRsp had more iNOS staining than age-matched SD rats of the same gender, but there was no gender difference within either strain (Figure 6). Most of positive staining was localized to renal tubules in SHRsp. There was very little staining observed in glomerular vessels.

DISCUSSION

Here we show gender differences in markers of oxidative stress and enzymes involved in blood flow regulation and renin secretion in the kidneys of male and female SD rats and SHRsp. These differences possibly contribute to the reported greater protection of the female gender against development of hypertension, atherosclerosis, renal damage and disease, and cardiovascular mortality (10,11, 15-18).

SHRsp showed marked staining for AGEs, CEL and CML, in the kidney, despite normal blood glucose levels. AGEs are precursors of oxidative stress (25) and our findings support elevated oxidative stress levels in SHRsp and SHR (24). More importantly, male SD rats had an elevated level of CEL compared to age-matched

female. This gender difference of CEL formation has not been reported before. This also supports findings of elevated superoxide anion levels and oxidative stress in male Wistar rats (23) and gender differences in terms of oxidative stress levels in humans, using plasma thiobarbituric acid-reactive substances and urinary 8-isoprostaglandin $F_2\alpha$ as markers (21,22). An elevated oxidative stress has been associated with hypertension (19,28,29) and can readily disturb the homeostatic mechanisms in the kidney which contribute to blood pressure regulation (1). In our study, antibodies against CEL and CML were used. The antibody against MG-induced CML is less selective since this antibody can also react with glyoxal (another dicarbonyl compound)-induced AGE (30). In contrast, anti-CEL antibody is specifically against MG-induced CEL and has been suggested to be a good indicator of MG-induced AGE or glycoxidation product (31). Gender difference in CEL and CML staining was lost in SHRsp with an associated severe hypertension in both male and female SHRsp (Table1, Figures 1,2).

NF-κB p65, a marker of inflammation was detected in SHRsp but not in SD rats. However, Western blot analysis demonstrated gender differences in nuclearly localized NF-κB p65, even though significantly less in SD than in SHRsp. Thus, NF-κB p65 was significantly higher in male SD rats and SHRsp than female rats of the same strain (Figure 3B) and may be one of the reasons for greater susceptibility of the male gender to cardiovascular and kidney disease, including male SHRsp (17). Moreover, activated macrophages were detected in the kidney of SHRsp but not in SD rats. These findings are consistent with the possibility that activation of NF-κB p65 and renal interstitial

infiltration of immune cells play a part in the pathophysiology of hypertension (32). We have previously shown that oxidative stress is likely involved in MG-induced activation of NF-κB in vascular smooth muscle cells of SHR (28). However, gender differences in this regard were not known. An association of inflammation and hypertension has been reported by elevated C-reactive protein, another marker of inflammation (33). Lack of NF-κB p65 staining and hypertension in the SD rats supports this association. Gender differences in terms of the localization of NF-κB p65 and ED-1, a marker of activated macrophages, in the renal tubules of male SHRsp and in the glomerular vessels of female SHRsp, are notable and can be speculated to indicate a more progressed inflammation in the males, having originated from the blood vessels.

iNOS is normally associated with inflammatory states (4) and this is supported by our observation of more intense iNOS staining in SHRsp compared to SD rats. Increased expression of iNOS in the kidney of SHR has been reported (34). Also, AGEs induce iNOS in glomerular mesangial cells (35). An association of iNOS and hypertension is strongly supported by our results.

Level of nNOS was higher in SD rats compared to SHRsp. Increased (36) as well as decreased (37) expression of nNOS in the kidney of SHR, compared to Wistar rats, has been reported. While the role of nNOS in renin secretion is controversial, a number of studies have implicated NO, derived from nNOS, stimulating renin secretion, especially after chronic sodium restriction (5,38,39). Staining intensity of nNOS was greater in female compared to male rats for both SD and SHRsp. Moreover, SHRsp had

lower levels of nNOS compared to SD rats for both male and female, which supports a previous report of reduced nNOS in the SHR (37). This can be speculated as a feedback inhibition of nNOS expression due to high blood pressure in SHRsp. In the study by Kumar *et al.* (37) gender differences were not reported. The reason for gender difference in nNOS within a strain is not clear at present. Another important difference between male and female rats, noted in our study, was localization of nNOS staining. Most of the staining in male rats was in the tubules whereas in female rats it was in the glomerular vessels. While tubular expression of nNOS has been reported (20,36,37) localization of nNOS to the glomerular vessels in female rats was a surprise and is intriguing. Functional significance of this difference needs to be determined by separate studies. Gender differences in expression of eNOS in the kidney and the vasculature are well established and increased vascular NO production has been proposed as affording more protection to females against cardiovascular morbidity and mortality (6-11).

While differences in the NOSs have been described above it might be worth pointing out that the age of rats used in our study, 24 weeks, is within the reproductive age and an influence of menopause, reportedly occurring after 18 months or about 72 weeks of age, can be ruled out (40).

In conclusion, increased expression of CEL, NF-κB p65, ED-1, and iNOS in male rats compared to female rats, or in SHRsp compared to SD rats, strongly supports the contention that female gender has better biochemical protection against cardiovascular and renal morbidity and mortality.

Table 4-1. Systolic blood pressure and body weight of SHRsp and age-matched SD rats

Rats	Gender	BP (mmHg)	BW (g)
SD	Male	120 ± 3	583 ± 10
	Female	116 ± 2	452 ± 8$^{\#\#}$
SHRsp	Male	184 ± 5***	386 ± 5***
	Female	180 ± 3***	275 ± 4***$^{\#\#}$

Values are mean ± SEM. BP, systolic blood pressure; BW, body weight.

***P < 0.0001, SHRsp *vs.* SD rats of the same gender, $^{\#\#}P$ < 0.001, Female *vs.* male of the same strain. (n = 6 each group)

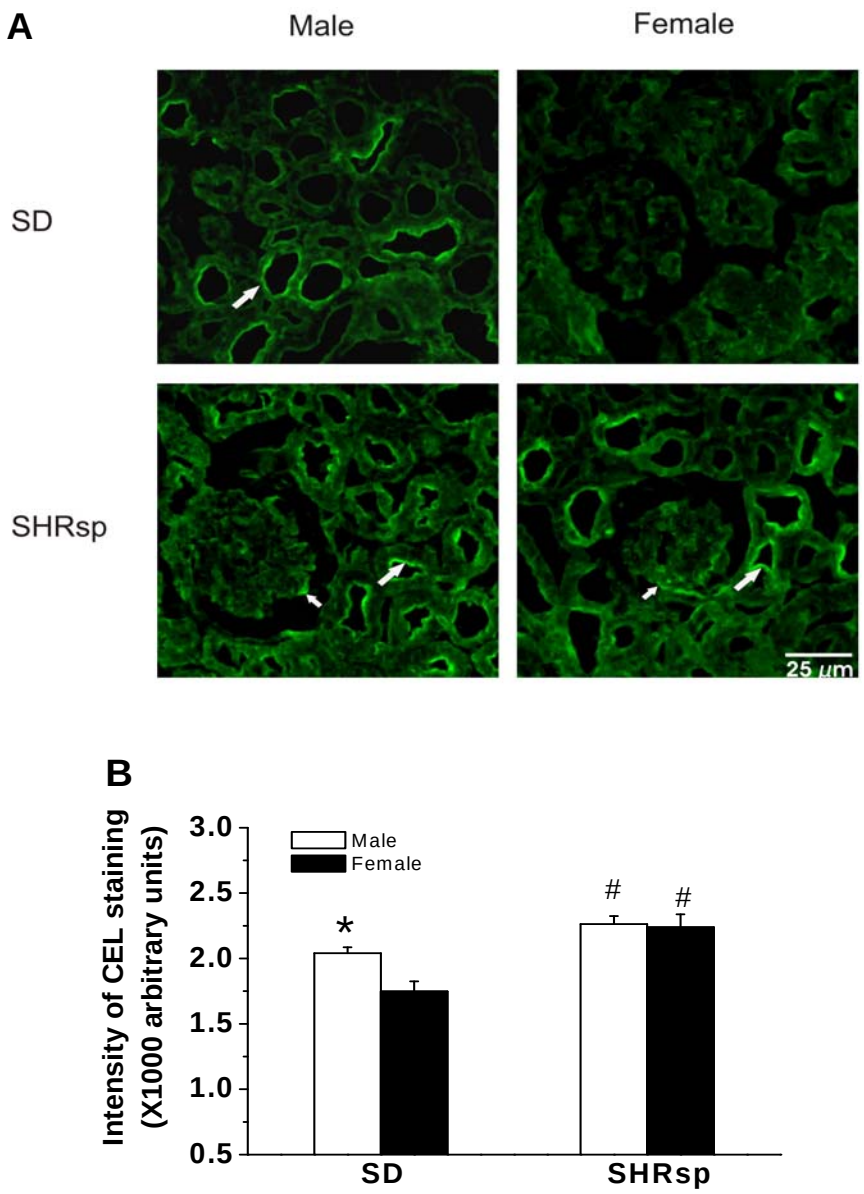


Figure 4-1. Immunohistochemical detection of CEL in the kidney from SHRsp and age-matched SD rats. (A). CEL staining was detected in kidney sections from male (left panel) and female (right panel) SHRsp and SD rats. Big arrows indicate the representative immunoreactivity in renal tubules, and small arrows show staining in the glomerulus. (B). Intensity of CEL staining (n = 3 in each group). *$P < 0.05$, male compared to female rats of the same strain. $^{\#}P < 0.05$, SHRsp compared to SD rats of the same gender.

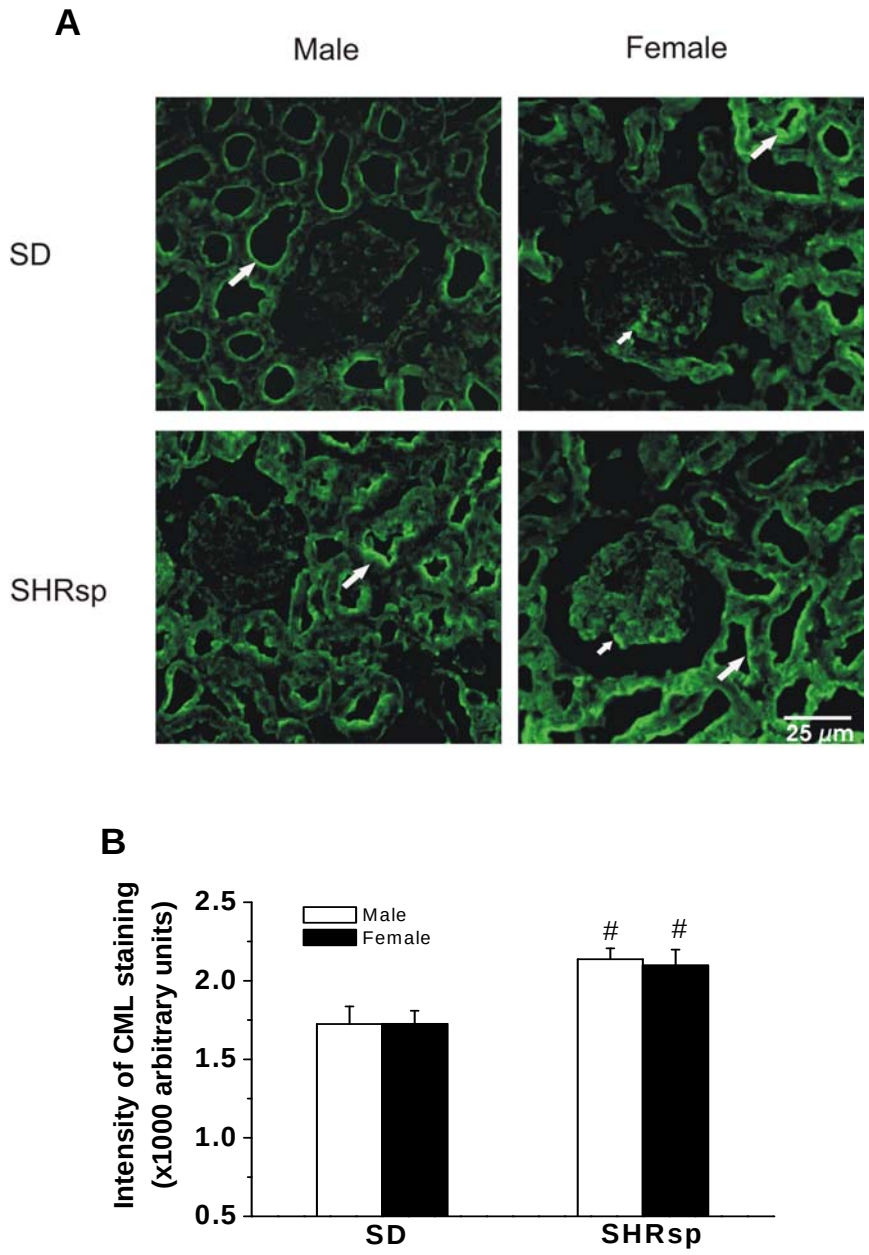
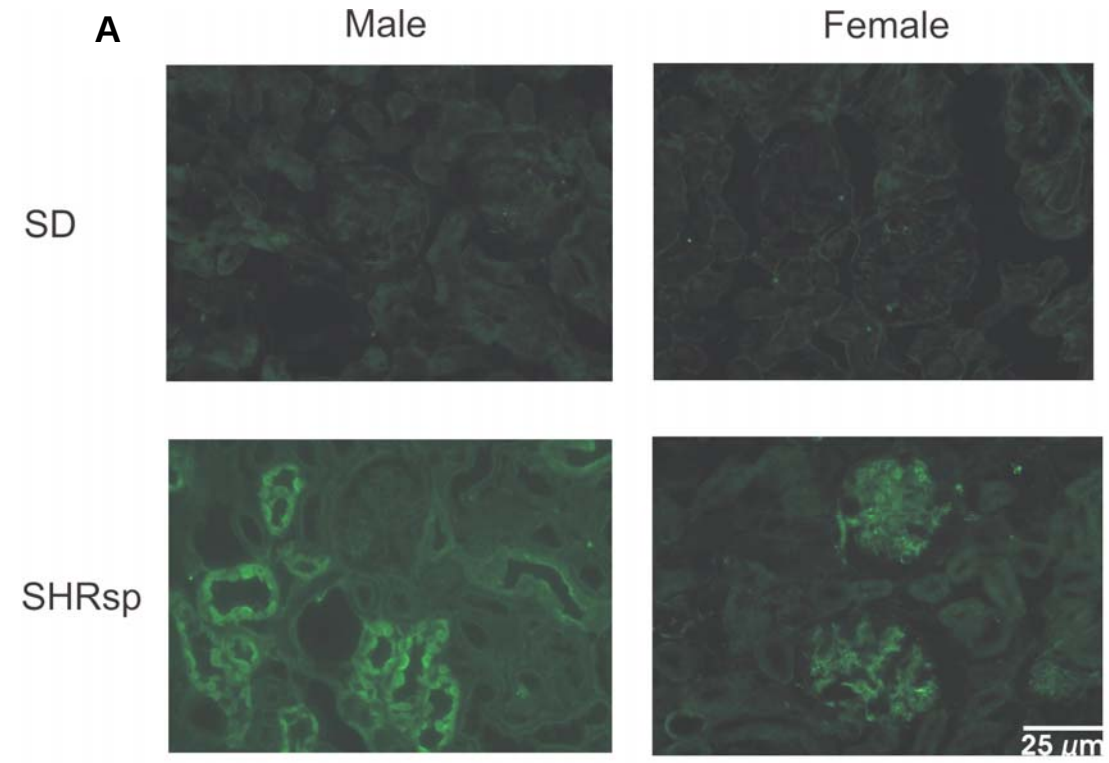


Figure 4-2. Immunohistochemical detection of CML in the kidney from SHRsp and age-matched SD rats. (A). CML staining was detected in kidney sections from male (left panel) and female (right panel) SHRsp and SD rats. Big arrows indicate the representative immunoreactivity in renal tubules, and small arrows show staining in the glomerulus. (B). Intensity of CML staining (n = 3 in each group). $^{\#}P < 0.05$, SHRsp compared to SD rats of the same gender.


A
Male
Female
SD
SHRsp
25 μm

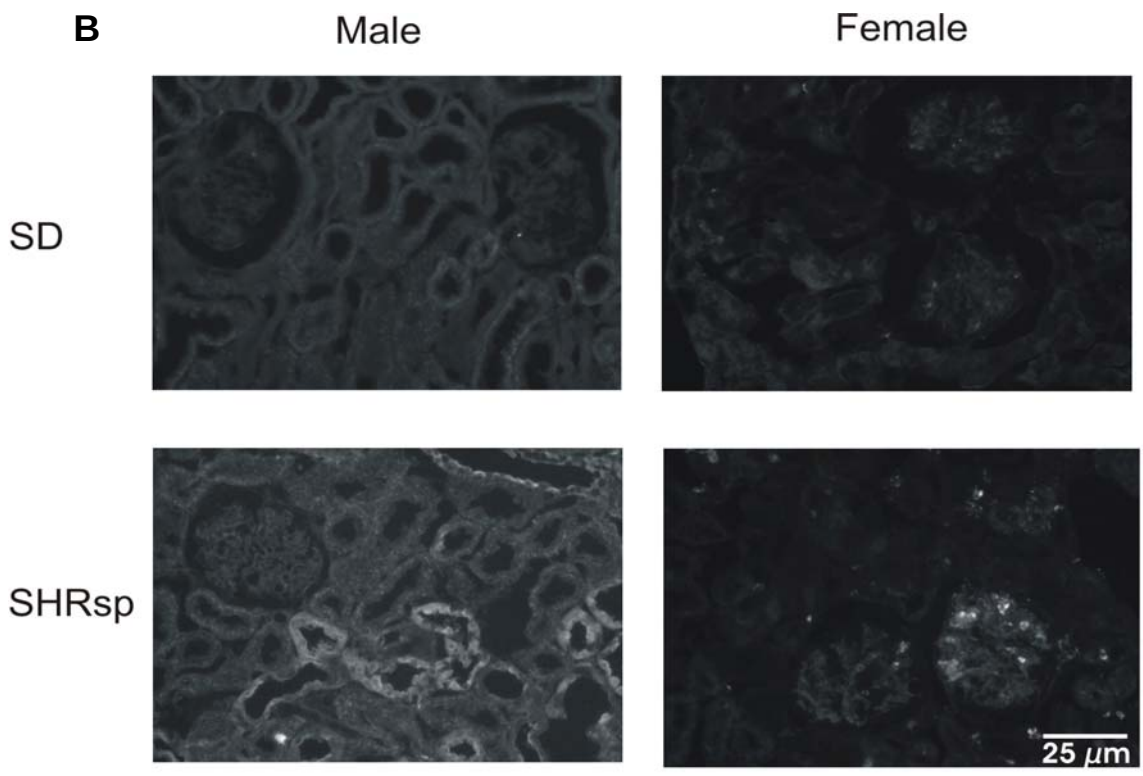

B
Male
Female
SD
SHRsp
25 μm

Figure 4-3. Immunohistochemical detection of ED-1 (A) and NF-κB (B) in the kidney from SHRsp and age-matched SD rats. Positive staining of ED-1 was only detected in the renal tubules of male SHRsp. In female SHRsp, the staining was only observed in glomerular vessels (A). Micrographs show that positive nuclear NF-κB p65 signal was observed in male and female SHRsp; and very little staining was detected in the kidney from SD rats (B). (n = 3 for each group).

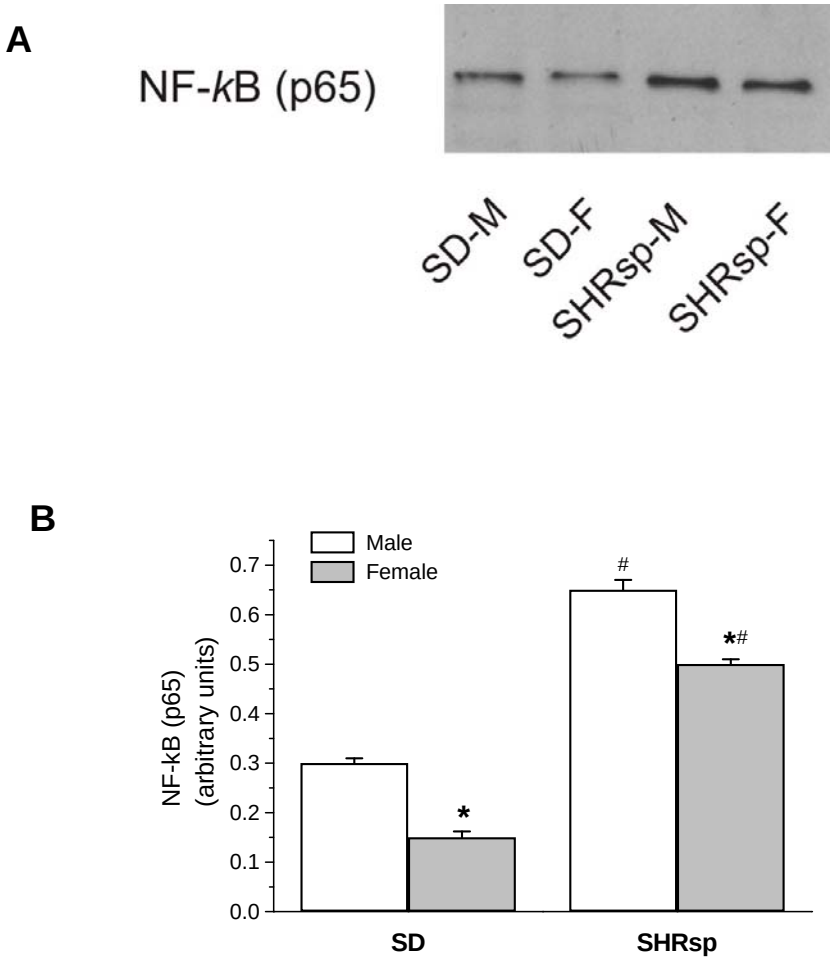


Figure 4-4. Western blot showing the localization of activated NF-κB in the kidney from SHRsp and age-matched SD rats. (A). A representative Western blot of nuclearly localized NF-κB p65. (B). Quantification of NF-κB p65 relative to ß-actin (*y* axis depicts relative units). Data were taken from kidney tissues of 3-4 animals. $^{*}P < 0.05$, male compared to female rats of the same strain. $^{\#}P < 0.05$, SHRsp compared to SD rats of the same gender.

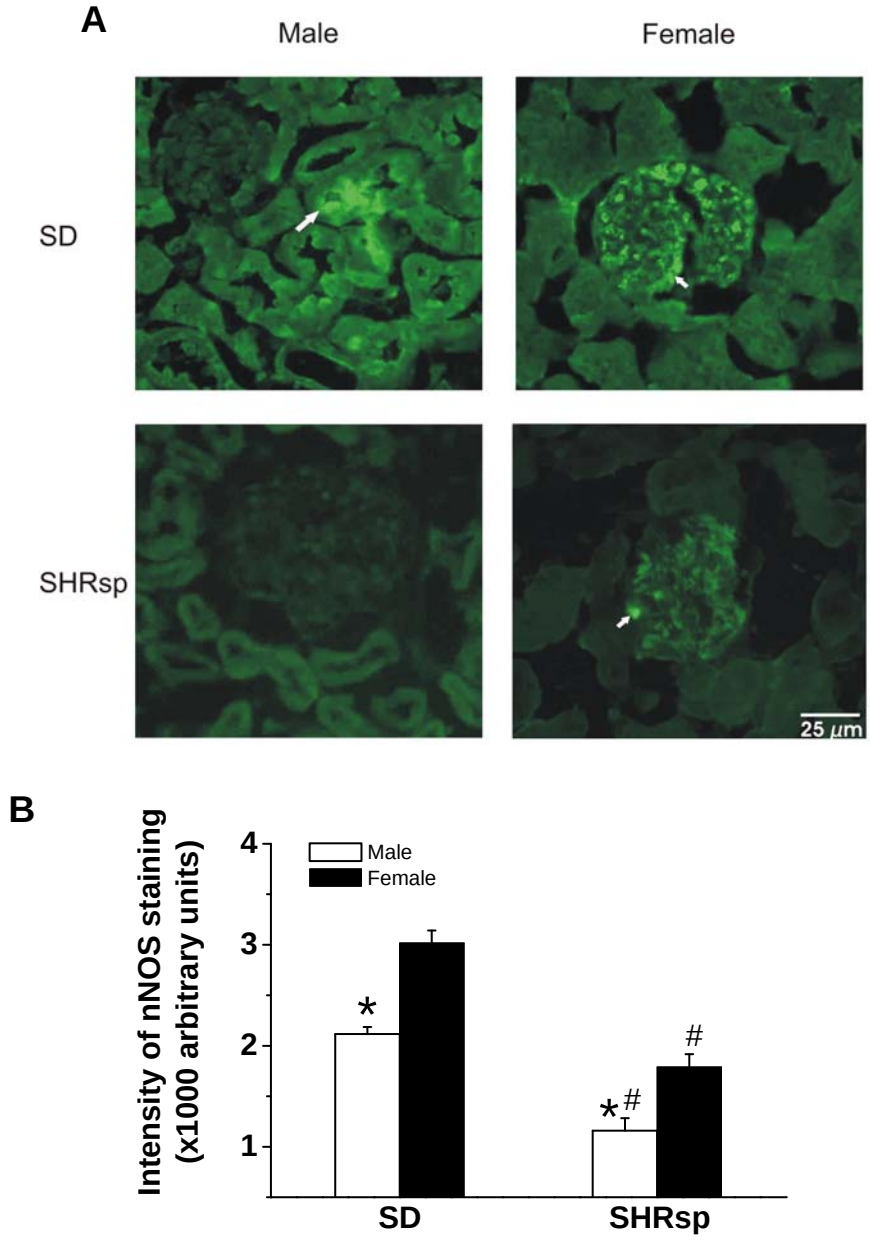


Figure 4-5. Immunoreactivity of nNOS in renal tissues from male and female SHRsp and age-matched SD rats. (A). Immunostaining of nNOS. Female rats (right panel) show more positive nNOS staining than age-matched male rats of the same strain (left panel). Big arrows indicate representative immunoreactivity in renal tubules, and small arrows show staining in the glomerulus. (n = 3 for each group). (B). Intensity of nNOS staining. *P < 0.05, male compared to female rats of the same strain. $^{\#}P$ < 0.05, SHRsp compared to SD rats of the same gender.

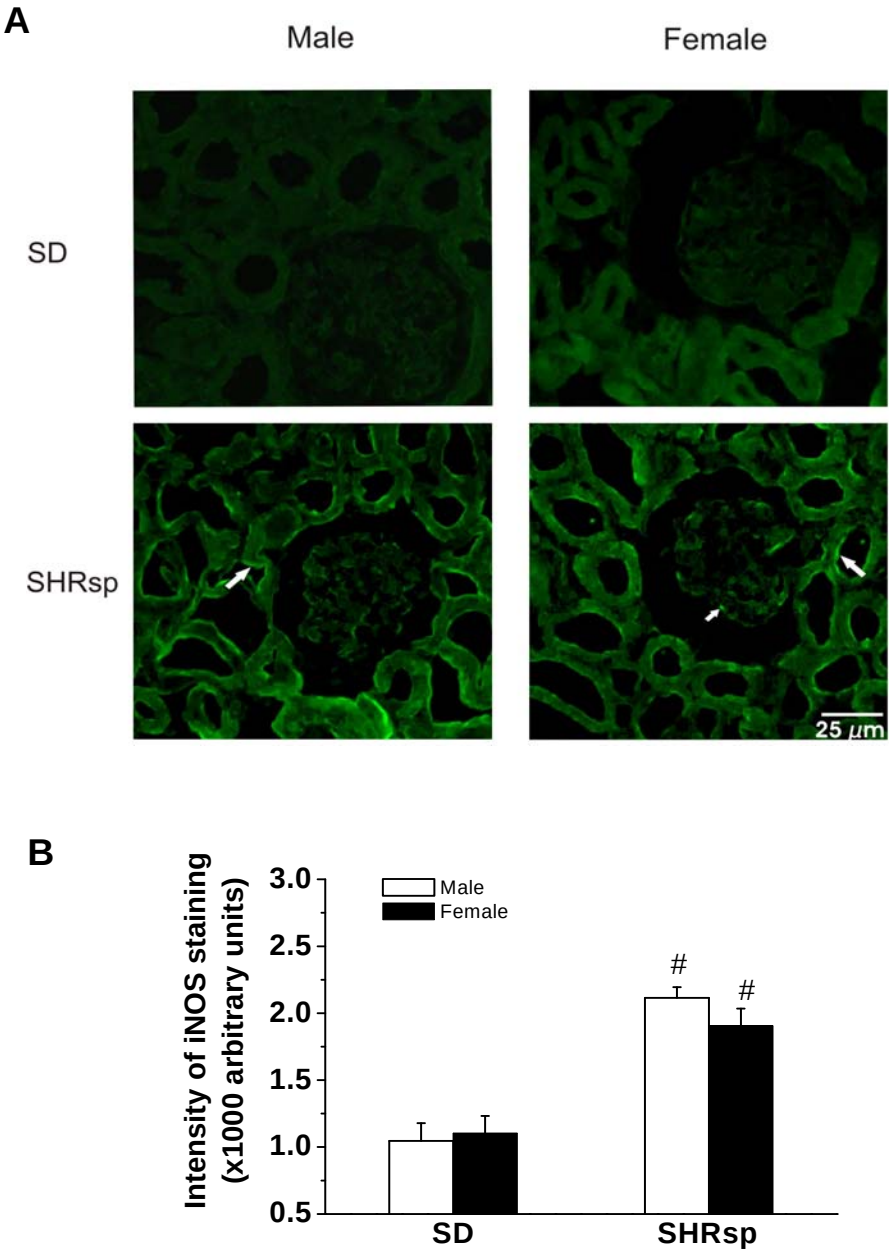


Figure 4- 6. Immunohistochemical detection (A) and intensity (B) of iNOS staining in renal tissues from male and female SHRsp and age-matched SD rats. Big arrows indicate the representative immunoreactivity in renal tubules, and small arrows show staining in the glomerulus. (n = 3 for each group). *$P < 0.05$, male compared to female rats of the same strain. #$P < 0.05$, SHRsp compared to SD rats of the same gender.

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CHAPTER 5

VASCULAR METHYLGLYOXAL METABOLISM AND THE DEVELOPMENT OF HYPERTENSION

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ABSTRACT

Objectives: The pathogenic process of diabetes mellitus is associated with increased methylglyoxal (MG). MG causes non-enzymatic glycation of proteins to form irreversible advanced glycation endproducts (AGEs). However, the correlation between MG and essential hypertension is unknown. The aim of the present study was to investigate whether MG, MG-induced AGEs, and oxidative stress were increased in the aorta of SHR and whether an increased formation of MG and related AGEs was correlated with the development of high blood pressure in spontaneously hypertensive rats (SHR).

Methods: HPLC was used to determine MG and reduced glutathione levels in plasma and aorta. MG-induced AGEs, N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-carboxymethyl-lysine (CML), in aorta were determined using immunohistochemistry. Hydrogen peroxide and superoxide levels in aorta and glutathione peroxidase and reductase activities were also determined.

Results: Aortic and plasma MG levels were significantly elevated in SHR, but not in Wistar Kyoto (WKY) rats, at 8, 13 and 20 wks of age in parallel with blood pressure increase. Immunohistochemistry revealed more intense staining for CML and CEL in aorta from SHR than that of WKY rats from 8 wks onwards. Most of the staining was localized to endothelial cells. Superoxide and hydrogen peroxide levels were significantly elevated in aorta of SHR at 13 wks whereas reduced glutathione levels, glutathione peroxidase and glutathione reductase activities were significantly decreased

compared to WKY rats.

Conclusions: Increased aortic MG, AGEs formation and oxidative stress were associated with blood pressure increase in SHR, which may cause endothelial dysfunction and altered vascular reactivity.

Key Words: methylglyoxal ■ advanced glycation endproducts ■ aorta ■ hypertension

INTRODUCTION

One of the by-products of glucose metabolism during glycolysis is methylglyoxal (MG), a highly reactive dicarbonyl compound formed spontaneously due to the transformation of triose phosphates [1]. MG is also generated from primary amines and from acetone during lipolysis, catalyzed by semicarbazide-sensitive amine oxidase (SSAO) or acetol mono-oxygenase (AMO) [2,3]. SSAO are capable of deaminating primary amines to produce aldehyde, ammonia, and hydrogen peroxide. Under physiological conditions vascular endothelial cells are a major source of circulating SSAO [4]. Increased activities of plasma SSAO and AMO have been suggested to be responsible for the increased circulating MG levels and diabetic vascular complications in animals [2,5]. Increased SSAO in transgenic mice are associated with an increased endothelial cell capacity for lymphocyte binding and altered expression of redox-sensitive proteins [4].

MG is detoxified by the glyoxalase system that uses glutathione (GSH) as a cofactor [1]. MG can react with selective proteins to yield irreversible advanced glycation endproducts (AGEs), leading to cross-linking and denaturation of protein [6]. The irreversible reaction of MG with lysine residues of protein forms N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-carboxymethyl-lysine (CML) [7].

MG and related AGEs such as CEL are inducers of the increased oxidative stress *in vivo* [7,8], and their levels are correlated with tissue damage and aging [9]. The hyperglycaemic state in diabetes mellitus is strongly and causatively associated with

increased MG and AGEs, potentially contributing to the development of nephropathy, retinopathy, neuropathy and other complications of diabetes [7,8]. The associations of MG and related AGEs with the development and maintenance of hypertension, however, are still largely unsettled. Systolic blood pressure was significantly increased after Wistar Kyoto (WKY) rats were treated with MG in drinking water, with an increase in aldehyde conjugate levels in kidney, but not heart or liver [10]. An enhanced AGE formation was found in the aorta of stroke-prone spontaneously hypertensive rats [11]. In our previous studies on the spontaneously hypertensive rats (SHR) we have shown elevated MG and AGEs levels as well as increased NF-κB p65 and ICAM-1 expression in cultured vascular smooth muscle cells (VSMCs) [12].

The aim of the present study was to investigate whether MG, MG-induced AGEs, and oxidative stress were increased in the aorta or plasma of SHR and whether an increased formation of MG and related AGEs was correlated with the development of high blood pressure in SHR. To this end, MG levels and SSAO activity in the aorta and plasma were determined in SHR and WKY rats at different hypertension development stages. The MG-induced CML and CEL in the aorta were evaluated in both strains. Since glutathione plays a role in the metabolism of MG, and MG can increase oxidative stress by inactivating antioxidant enzymes such as glutathione reductase [13] and glutathione peroxidase [14], we also measured the levels of GSH, superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), and the activities of glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Red) in the aorta.

MATERIALS AND METHODS

Animals

A total of 36 rats, including 18 male SHR and 18 male WKY rats, were purchased from Charles River laboratories (St-Constant, Quebec, Canada). Rats were treated in accordance with guidelines of the Canadian Council on Animal Care, and the experimental protocols were approved by the Animal Care Committee at the University of Saskatchewan. Systolic blood pressure was determined weekly using a standard tail cuff noninvasive BP measurement system (Model 29-SSP, Harvard Apparatus, St. Laurent, QC, Canada). At the end of the study, rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight) intraperitoneally. Blood was collected from the heart, and plasma was separated by centrifuging blood samples at 1,000 g for 10 min at 4ºC. Tissues cleaned in ice-cold phosphate buffer saline (PBS) were snap-frozen in liquid nitrogen immediately and stored at -80ºC until processing.

MG measurement

Quantitation of MG was done by the widely accepted o-phenylenediamine (o-PD)-based assay as described previously [15]. Earlier assays for MG employed dinitrophenylhydrazine for derivatization of MG. This was not specific since other intermediates of glycolysis also reacted with dinitrophenylhydrazine [16]. The method we have used is specific for MG and is also very sensitive. In this method the reagent, o-PD, a 1,2-diaminobenzene derivative, rapidly reacts with MG to form a quinoxaline

which can be easily quantified with reverse-phase high-performance liquid chromatography [15]. For the assay, the supernatant of the aortic homogenate or plasma was incubated with 100 mmol/L o-PD (derivatizing agent) for 3 h at room temperature. The quinoxaline derivative of MG (2-methylquinoxaline) and the quinoxaline internal standard (5-methylquinoxaline) were measured using a Hitachi D-7000 HPLC system (Hitachi Ltd., Mississauga, ON, Canada). Samples were calibrated by comparison with 2-MQ standards.

Measurement of reduced glutathione levels

The reduced glutathione (GSH) levels in the aorta were determined by derivation with 5, 5'-dithio-bis (2-nitrobenzoic acid), and reverse-phase HPLC using ultra-violet detection, as described in our previous study [17].

Measurement of hydrogen peroxide levels

The formation of H_2O_2 was measured by a DCFH-assay as described previously [12]. The membrane permeable and nonfluorescent probe DCFH-DA was used to load aortic rings. H_2O_2 oxidized-DCF was quantified by monitoring the DCF fluorescence intensity with excitation at 485 nm and emission at 527 nm with Fluoroskan Ascent plate-reader (Thermo Labsystem, Franklin, MA, USA) using Ascent software and expressed using arbitrary units.

Measurement of superoxide production

$O_2^{-\cdot}$ production was measured using the lucigenin-enhanced chemiluminescence method as described previously [18]. Chemiluminescence intensity was detected with a luminometer (TD-20/20, Tunner Designs, CA, USA) and expressed using arbitrary units.

Measurement of Enzyme Activities

The activities of GSH-Px and GSH-Red were measured according to methods described previously [19]. The enzyme activity for both GSH-Px and GSH-Red was expressed as nmol NADPH oxidized/min/mg protein. Protein concentrations were determined by bicinchoninic acid procedure.

Fluorometric measurement of SSAO

SSAO activity was measured according to methods described by Storer *et al.* [20] with some modifications. Briefly, an aliquot of aorta homogenate or plasma was incubated with 0.5 mmol/L homovanillic acid and 0.5 mmol/L clorgyline. To this 0.5 mmol/L benzylamine and 30 U/ml horseradish peroxidase were added in a total volume of 250 µl. After incubation fluorescence was recorded at excitation/emission wavelengths of 310/440 nm using H_2O_2 standards.

Immunohistochemistry

Immunostaining was performed using the method described in our previous study [21]. In brief, paraformaldehyde-fixed and OCT-embedded 8 μm aortic tissue sections were incubated with either monoclonal anti-CML antibody or anti-CEL antibody (Novo Nordisk, A/S, Denmark) diluted 1:100. Anti-mouse IgG-FITC (Sigma, Oakville, ON, Canada) diluted 1:200 was the secondary antibody. The sections were quantified using GeneTools image analysis software (PerkinElmer®, Wellesley, MA, USA). Twenty fields along the endothelium of each section were analyzed and the average value was used to express the intensity of staining.

Data Analysis

Data are expressed as mean ± SEM and analyzed using Student's *t*-test. Significant difference between treatments was defined at a level of $P < 0.05$.

RESULTS

Basal parameters of SHR

As shown in Table 1, in young rats at 5 wks of age there was no difference in the systolic blood pressure and body weights between SHR and WKY rats. However, the blood pressure increased in the subsequent wks in SHR but remained unchanged in WKY rats. At 8, 13 and 20 wks of age the blood pressure was significantly higher in SHR than that of age-matched WKY rats, while the body weight of SHR was significantly lower than that of age-matched WKY rats. No significant difference was

found in the heart and kidney weights between the two strains at different ages.

MG levels in plasma and tissues from SHR

Plasma MG level was age-dependently and progressively increased in SHR, compared to WKY rats (Figure 1A). Between young 5-wk old SHR and WKY rats there was no significant difference in aortic MG levels. However, from 8 wks onwards the aortic MG level was significantly higher in SHR than in age-matched WKY rats (Figure 1B). The MG levels in the kidney and the liver, but not in the heart, were significantly higher in the SHR compared to age-matched WKY rats at 13 wks (Figure 1C). No significant change in renal, hepatic and heart MG levels was observed between SHR and age-matched WKY rats at 8 wks of age (data not shown).

Increased MG-induced CEL and CML in aorta of SHR

With immunohistochemical staining method, we further investigated whether MG-induced AGEs were increased in the aorta from SHR. Figure 2 shows more intense or positive staining for CEL in the aorta from SHR, compared to WKY rats at 8, 13 and 20 wks of age (n = 3 for each age group). Positive CEL staining in the aorta of SHR was observed occasionally even at the age of 5 wks. From 8 wks onwards, the intensity of CEL staining was significantly higher in SHR than in age-matched WKY rats (Table 2). Most of the positive staining was localized to the endothelial layer in both strains. Some staining was observed in smooth muscle cells too. Staining for CML was

also more intense in the aorta from SHR as opposed to WKY rats at 8, 13 and 20 wks of age (n = 3 for each group) (Figure 3 and Table 2). The negative (no primary antibody) and positive (control immune globulin) control sections revealed no staining (not shown).

Increased superoxide and hydrogen peroxide levels in the aorta, and semicarbazide-sensitive amine oxidase (SSAO) activity in plasma of SHR

Superoxide anion ($O_2^{\cdot-}$) level was significantly higher in the aorta of SHR at 13 wks of age (Figure 4A). Hydrogen peroxide (H_2O_2) level was also significantly elevated in the aorta of SHR at 13 wks of age (Figure 4B). The SSAO activity was significantly elevated in the plasma, but not in the aorta, of SHR at 8 and 13 wks compared to age-matched WKY rats (Figure 5). There was no significant difference in SSAO activity between 5 wk-SHR and age matched WKY rats.

GSH level and the activities of the related enzymes in the aorta of SHR

As shown in Figure 6A, GSH level in the aorta was significantly decreased by 34% in SHR at the age of 13 wks compared to age-matched WKY rats. There was no difference between the plasma GSH levels between SHR and WKY rats at 5, 8 and 13 wks (Figure 6B).

The aortic GSH-Px activity was significantly decreased by 38% in SHR at the age of 13 wks compared to age-matched WKY rats (Figure 7A). The GSH-Red activity

was also significantly reduced by 16% in SHR at 13 wks, compared to WKY rats (Figure 7B).

DISCUSSION

The pathogenesis of essential hypertension has been under ongoing intensive investigations by clinicians and basic science researchers. The aorta, a common site for atherosclerotic changes, is frequently the focus of such studies [11]. The aortic endothelium and smooth muscle host a number of mediators involved in a delicate homeostatic balance. Amongst the factors that upset this balance is oxidative stress. MG and AGEs are precursors of oxidative stress, and potently alter the structure and functions of proteins and nucleic acids [7,13,14,22]. Elevated MG and AGEs are commonly associated with hyperglycemic conditions such as diabetes mellitus. The role of MG and AGEs in hypertension under an apparently normoglycemic state has not been investigated widely. Our present study showed that the age-dependent increase in blood pressure was associated with a progressive increase in the aortic and plasma MG levels in SHR, whereas the MG levels remained unchanged in age-matched WKY rats. The levels of MG-induced AGEs, CML and CEL, in the aorta were higher in SHR than in WKY rats from 8 wks onwards, in parallel with increased plasma MG levels and blood pressure in SHR. These changes in MG and AGEs in SHR are clearly not due to increased glucose metabolism since the latter shows no hyperglycemia and the glucose levels and insulin sensitivity in SHR and WKY rats are comparable [23]. Thus, in

addition to diabetes/hyperglycemic or hyperlipidemic conditions [24], essential hypertension is associated with abnormal MG metabolism. MG may play an important role in the development of hypertension in this non-diabetic model. This hypothesis is supported by our current study showing synchronized increases in aortic and plasma MG levels and blood pressure in SHR. Moreover, chronic MG feeding induces hypertension in WKY rats [10]. Given these important advances in our understanding of the putative pathogenic role of MG, however, further studies are needed to substantiate a causative role of MG in hypertension development.

While glucose induces glycation via a reversible Maillard reaction with N-terminal amino groups or lysyl chains to form fructosamine, MG induces glycation through an oxidation process. This reaction is also called glycoxidation and it generates irreversible end glycation adducts [7], including CEL and CML. With their stable chemical property, CEL and CML have been interpreted as a measure of the status of oxidative stress and of cumulative oxidative damage to proteins induced by MG in aging and diabetes [25]. In our study, antibodies against CEL and CML were used. The antibody against MG-induced CML is less selective since this antibody can also react with glyoxal (another dicarbonyl compound)-induced AGE [25]. In contrast, anti-CEL antibody is specifically against MG-induced CEL and has been suggested to be a good indicator of MG-induced AGE or glycoxidation product [26].

When the formation of MG exceeds its degradation, accumulation of MG occurs even with normal glucose level. As mentioned earlier, the main source of MG in

mammals is glycolysis. Other sources of MG are aminoacetone or acetone catalyzed by semicarbadize-sensitive amine oxidase (SSAO) or acetol mono-oxygenase (AMO) [2,3], with the former enzyme found in high amounts in the serum, endothelium, adipose tissue and vascular smooth muscle cells in mammals [4,27]. SSAO in smooth muscle cells mediates MG formation in rat aorta and may be responsible for diabetic vascular complications [5]. A recent paper pinpointed the endothelium as being the major source of circulating plasma SSAO in mice [4]. In our study, MG levels in plasma and the aorta were significantly and age-dependently increased in SHR at different ages in comparison with the age-matched WKY (Figure 1). An age-dependent change in the activities of SSAO and AMO might be responsible for the alteration in circulating MG at different ages in diabetes [5] as well as in hypertension. We have found an age-related increase in plasma SSAO activity at 8 and 13 wks in SHR, possibly coming from the endothelium [4], even though the aortic SSAO activity did not change in SHR and WKY rats. Immunocytochemistry revealed most of the staining for CEL and CML in the endothelium and very little in the smooth muscle of the aorta, indicating the plasma or the endothelium itself to be the source of the elevated MG.

Normal catabolism of MG is largely dependent on the availability of cellular GSH and the activities of the GSH-related enzymes, including GSH-Px and GSH-Red. In our study, the elevated levels of MG in plasma or the aorta and MG-induced CEL and CML in the aorta of SHR were observed as early as 8 wks of age. These changes preceded the decreased GSH level and suppressed activities of GSH-Px and GSH-Red in

the aorta of SHR, which appeared at the age of 13 wks. The delayed decrease of GSH in SHR indicates that the primary reason for the early onset of MG increase could be an abnormally increased MG production rather than a dysfunctional MG degradation.

Blood pressure of young SHR begins to increase as the levels of MG and its associated oxidative stress are progressively increased and the anti-oxidant GSH system fails to compensate. The latter event could also putatively result from the action of MG to inactivate antioxidant enzymes such as glutathione reductase [13] and glutathione peroxidase [14]. Eventually, GSH level will drop and hypertension in aged SHR enters a sustained stage.

In conclusion, MG, AGEs, and oxidative stress in the aorta and plasma of SHR, but not WKY rats, increased in an age-dependent manner associated with the development of hypertension. Increased MG and AGEs formation in the aorta may cause endothelial dysfunction and altered vascular reactivity, contributing to the development of hypertension in the SHR.

Table 5-1. Basal parameters of SHR and WKY at different ages

Age (wks)	Rats	BP (mmHg)	BW (g)	HW (g)	KW (g)
5	WKY	123 ± 1	107 ± 3	0.66 ± 0.10	1.33 ± 0.08
	SHR	130 ± 2	98 ± 2	0.49 ± 0.01	1.27 ± 0.01
8	WKY	121±1	202 ± 5	1.13 ± 0.20	1.91 ± 0.09
	SHR	168 ± 1***	180 ± 4**	0.93 ± 0.01	1.81 ± 0.01
13	WKY	121 ± 1	315 ± 6	1.06 ± 0.03	2.45 ± 0.05
	SHR	204 ± 1***	282 ± 4**	1.03 ± 0.02	2.36 ± 0.02
20	WKY	119 ± 1	361 ± 5	1.17 ± 0.06	2.22 ± 0.03
	SHR	202 ± 1***	308 ± 4***	1.37 ± 0.10	2.52 ± 0.08

Values are mean ± SEM. BP, systolic blood pressure; BW, body weight; HW, heart weight; KW, kidney weight; WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats.

**$P < 0.01$, ***$P < 0.001$ SHR *vs*. WKY rats.

Table 5-2. Intensity of CEL and CML staining in the aorta from SHR and WKY at different ages (*n* = 3 for each group)

	Rats	5 wks	8 wks	13 wks	20 wks
CEL	WKY	5545 ± 528	8525 ± 664	8391 ± 664	6977 ± 320
	SHR	6849 ± 93	11316 ± 478*	14678 ± 521**	11175 ± 258**
CML	WKY	5589 ± 86	7519 ± 327	7768 ± 550	6938 ± 410
	SHR	6288 ± 339	11248 ± 690**	12065 ± 795**	10349 ± 987*

CEL, N^{ε}-carboxyethyl-lysine; CML, N^{ε}-carboxymethyl-lysine; WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats.

The intensity of staining was quantified using GeneTools image analysis software and expressed using arbitrary units.

*$P < 0.05$, **$P < 0.01$ SHR *vs*. WKY rats.

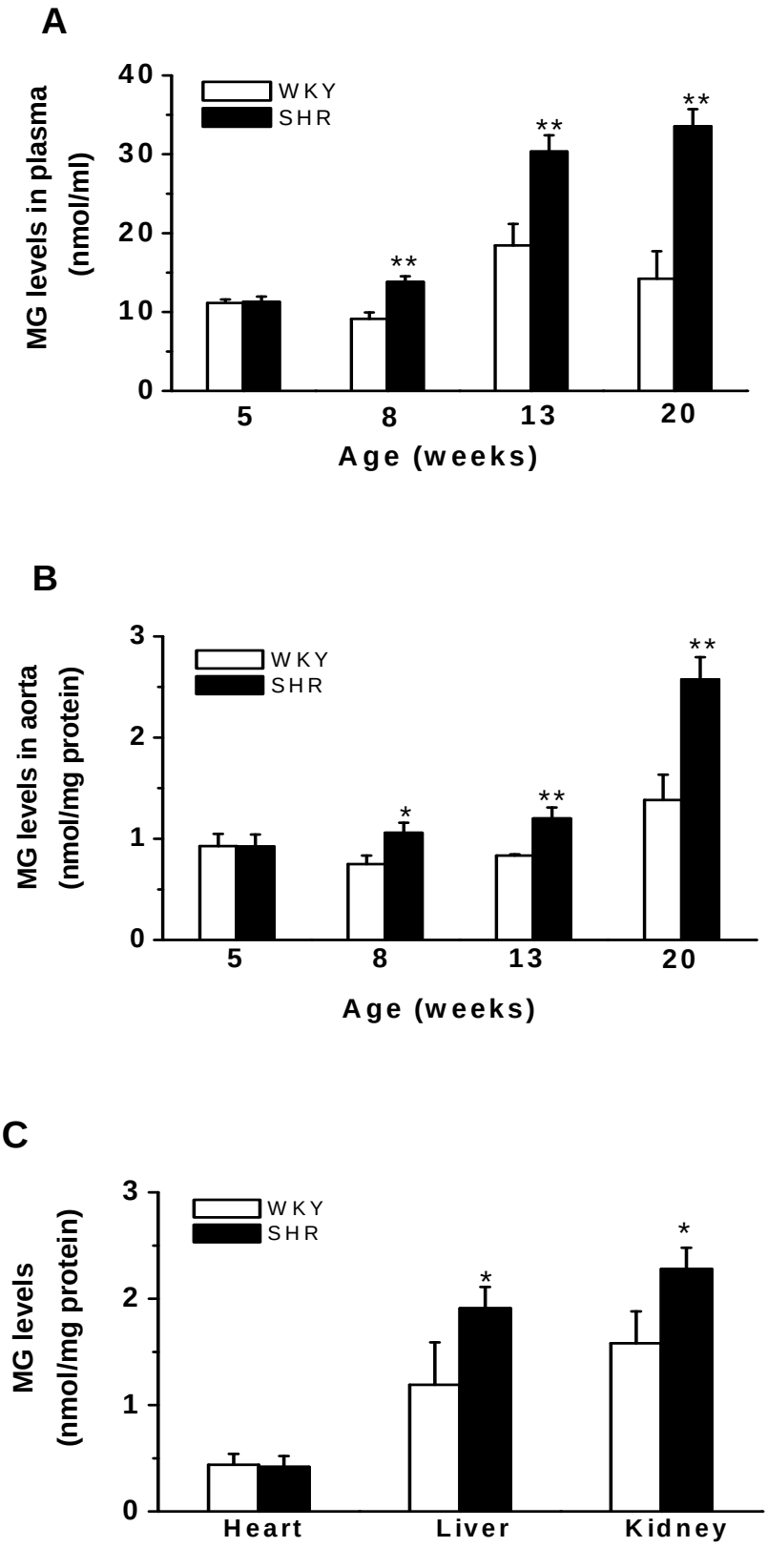
A
MG levels in plasma (nmol/ml)
WKY
SHR
40
30
20
10
0
**
**
**
5
8
13
20
Age (weeks)
B
MG levels in aorta (nmol/mg protein)
WKY
SHR
3
2
1
0
*
**
**
5
8
13
20
Age (weeks)
C
MG levels (nmol/mg protein)
WKY
SHR
3
2
1
0
*
*
Heart
Liver
Kidney

Figure 5- 1. Methylglyoxal (MG) levels in spontaneously hypertensive rats (SHR). MG levels were measured in plasma (A), and the aorta (B) from SHR and age-matched Wistar Kyoto (WKY) rats at 5 wks (n = 3), 8 wks (n = 4), 13 wks (n = 4-5) and 20 wks (n = 4) of age. MG levels in other tissues (C) were measured in SHR and WKY rats at 13 wks of age (n = 4-5). *P <0.05, **P < 0.01 SHR *vs*. WKY rats.

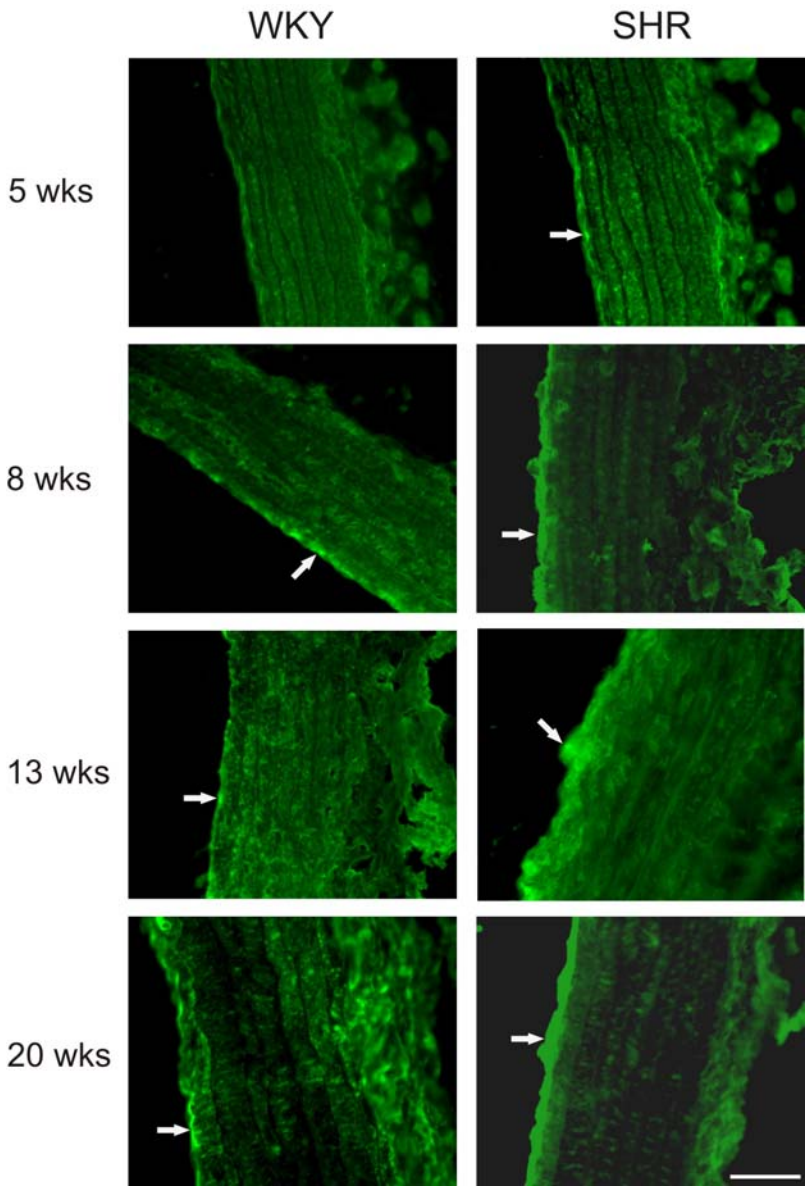

Figure 5-2. Immunohistochemical detection of N^{ε}-carboxyethyl-lysine (CEL) in rat aortic tissues. CEL staining was detected in the aorta from spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats. Aortic sections from SHR (right panel) show more intense staining than age-matched WKY rats (left panel) (n = 3 to each group). Arrows indicate the representative immunoreactivity in the endothelium. Scale bar: 25μm

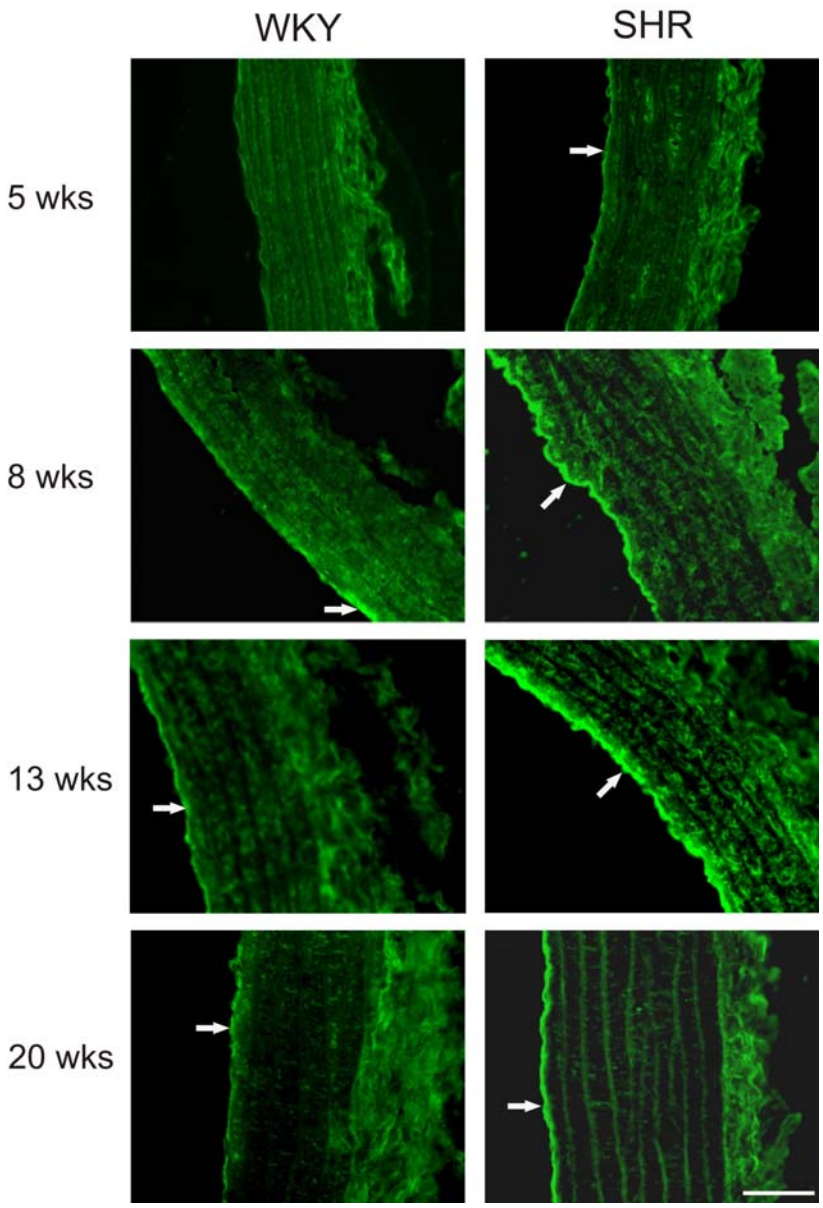

Figure 5-3. Immunohistochemical detection of N^{ε}-carboxymethyl-lysine (CML) in rat aortic tissues. CML staining was detected in the aorta from spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats. Aortic sections from SHR (right panel) show more intense staining than age-matched WKY rats (left panel) (n = 3 to each group). Arrows indicate the representative immunoreactivity in the endothelium. Scale bar: 25μm

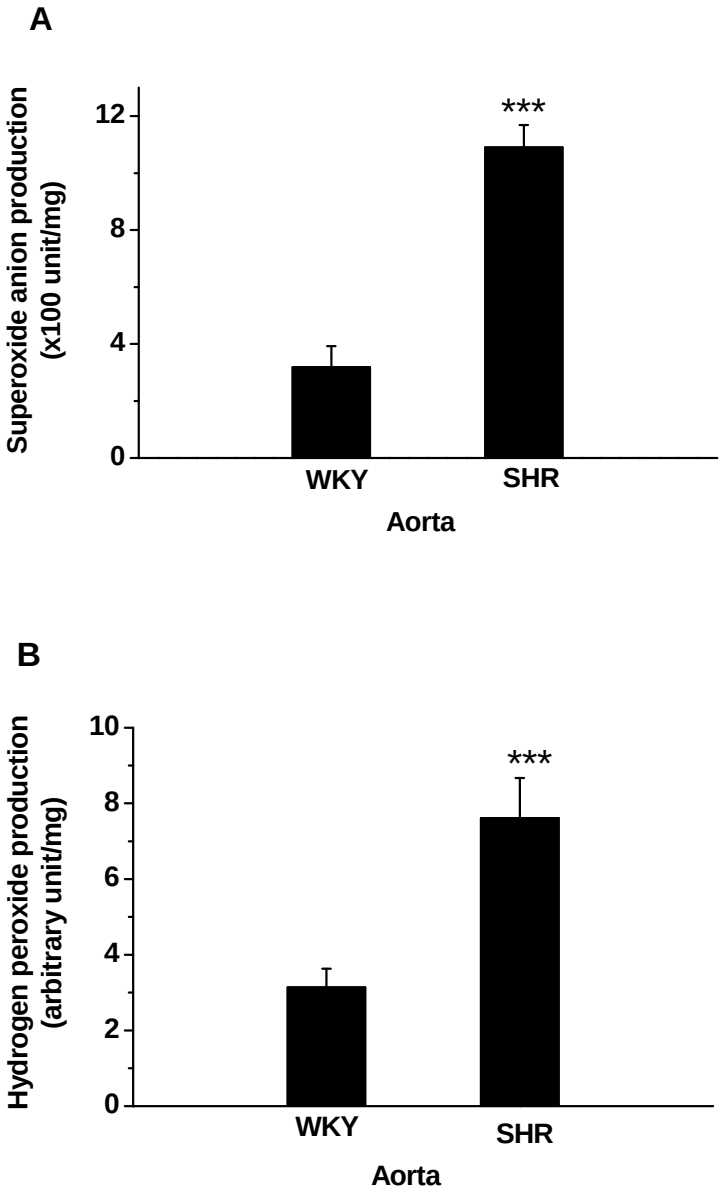

Figure 5-4. Oxidative stress levels in aorta of SHR. Superoxide anion ($O_2^{-\cdot}$) (A) and hydrogen peroxide (H_2O_2) (B) levels were measured in spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats at 13 wks of age. (n = 4). $O_2^{-\cdot}$ production was measured using the lucigenin-enhanced chemiluminescence method, and H_2O_2 production was determined by a DCFH-assay. *** $P < 0.001$ SHR *vs*. WKY rats.

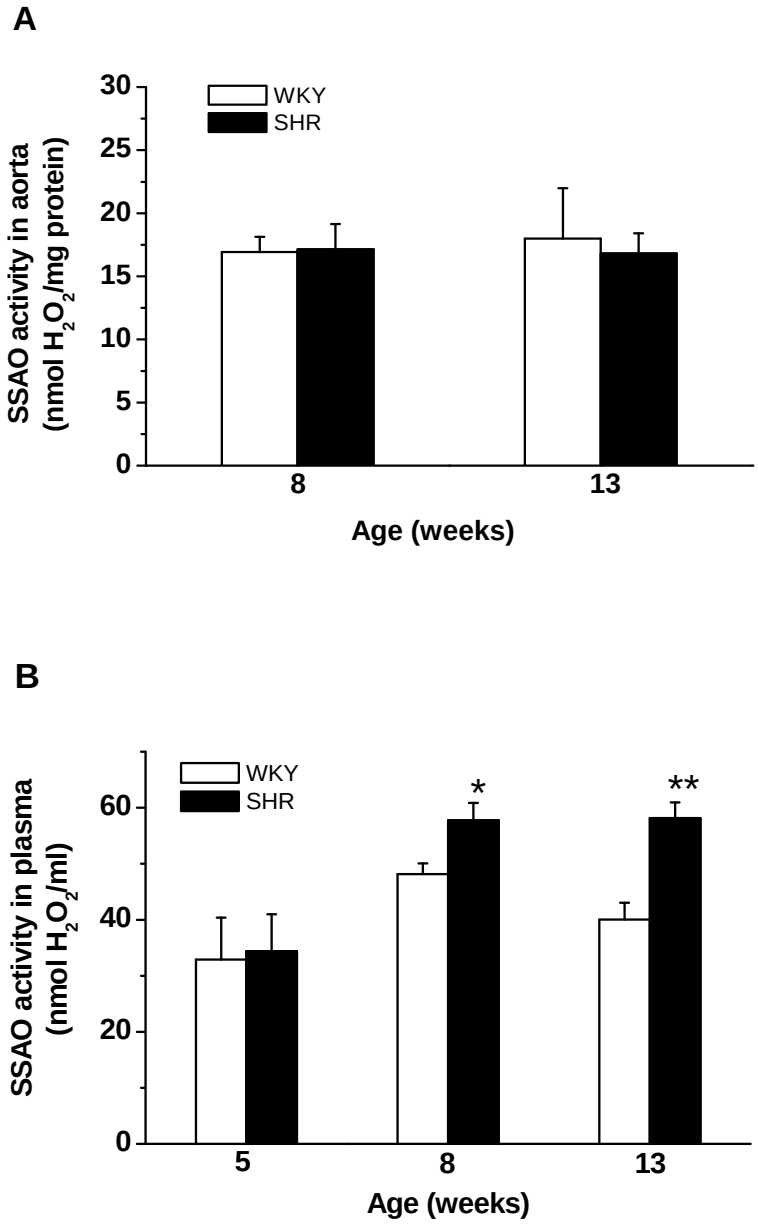

Figure 5-5. Increased semicarbazide-sensitive amine oxidase (SSAO) activity in spontaneously hypertensive rats (SHR). A. Aortic SSAO activity was measured from SHR and Wistar Kyoto (WKY) rats at 8 wks and 13 wks (n = 4 each group). B. SSAO activity was measured in plasma from SHR and WKY rats at 5 wks (n = 4), 8 wks (n = 5) and 13 wks (n = 4). *$P < 0.05$, **$P < 0.01$ SHR *vs*. WKY rats.

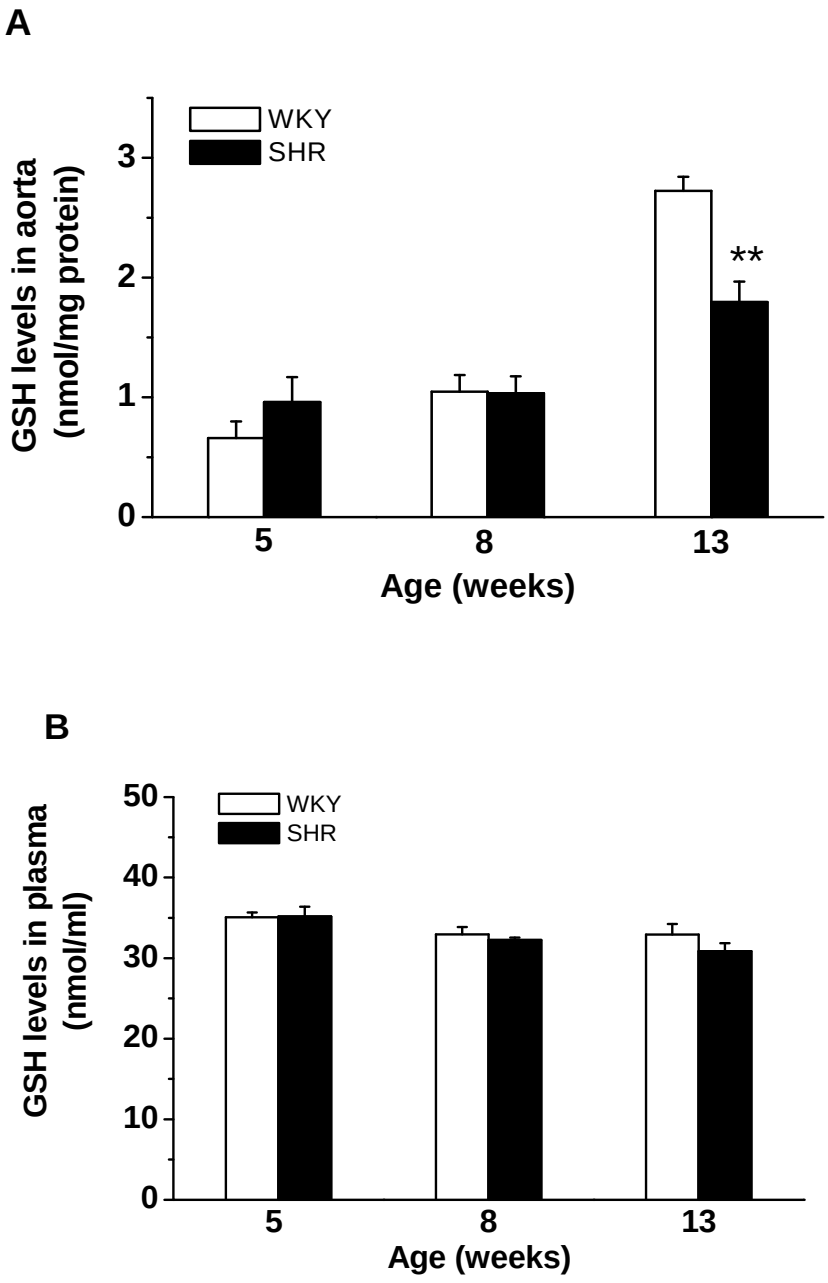

Figure 5-6. Reduced glutathione (GSH) levels in the aorta and plasma of spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats at different ages. GSH levels were measured in the aorta (A), and plasma (B) from SHR and WKY at 5 wks (n = 3), 8 wks (n = 4), and 13 wks (n = 4). *P < 0.05, **P < 0.01 SHR *vs*. WKY rats.

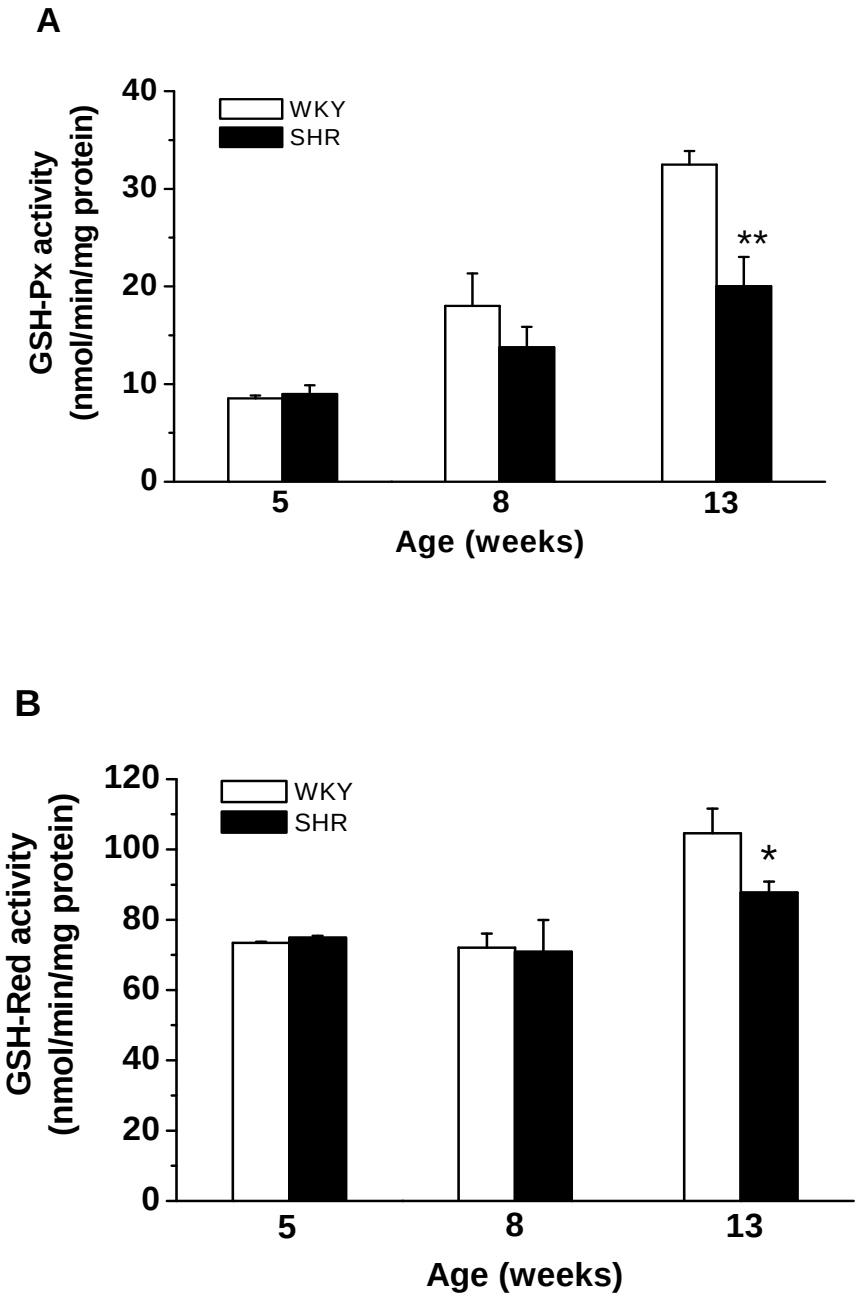

Figure 5-7. The activities of the enzymes in the aorta of spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats at different ages. Glutathione peroxidase (A) and reductase (B) activities in the aorta of SHR and WKY rats (n = 3-4 for each group). *$P < 0.05$, **$P < 0.01$ SHR *vs*. WKY rats.

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CHAPTER 6

ATTENUATION OF HYPERTENSION DEVELOPMENT BY AMINOGUANIDINE IN SPONTANEOUSLY HYPERTENSIVE RATS: ROLE OF METHYLGLYOXAL

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ABSTRACT

Objectives: Methylglyoxal (MG), a metabolite of glucose, and MG-induced advanced glycation endproducts (AGEs) are causatively associated with vascular complications of diabetes mellitus. We have previously reported elevated levels of MG and MG-induced AGEs in spontaneously hypertensive rats (SHR). The purpose of this study was to investigate the causative role of MG and MG-induced AGEs in the pathogenesis of hypertension in SHR.

Methods: Young SHR were treated with an AGE inhibitor; aminoguanidine, for 9 wks. HPLC was used to determine plasma and aortic MG and reduced glutathione levels. MG-induced AGEs, N^{ε}-carboxyethyl-lysine (CEL) and argpyrimidine, in the aorta were determined by immunohistochemistry. Vascular relaxation of small mesenteric arteries was measured using myograph.

Results: Chronic treatment with aminoguanidine attenuated age-dependent blood pressure increase in SHR. Plasma and aortic MG levels, and aortic levels of MG-induced AGEs were significantly reduced after aminoguanidine treatment, which were comparable to those from age-matched Wistar Kyoto rats. Free radical level was significantly lowered, while reduced glutathione level was significantly increased by aminoguanidine treatment in the aortic tissues from SHR. Moreover, aminoguanidine therapy prevented the morphological damage of vascular tissues in SHR, and restored the endothelium-dependent relaxation to acetylcholine. Chronic aminoguanidine treatment also increased aortic eNOS expression and reduced iNOS expression.

Conclusion: MG and MG-induced AGEs contribute to the pathogenesis of hypertension by altering the redox balance, causing vascular eutrophic inward remodeling, and inducing endothelial dysfunction in SHR.

Key Words: methylglyoxal ■ advanced glycation endproducts ■ hypertension

INTRODUCTION

Advanced glycation end products (AGEs) are a heterogeneous group of compounds formed by the non-enzymatic reaction of aldehydes and ketones with amino groups of proteins via the Maillard reaction[1]. AGEs are characterized by brown color and intra-or inter-molecular cross-linking. AGEs accumulate slowly in aging vascular and renal tissues, but this process accelerates with diabetes[2]. AGEs can be formed by glucose, reactive α-oxoaldehydes and other saccharide derivatives with methyglyoxal (MG) being the most reactive precursor for AGE formation[3]. MG is a highly reactive dicarbonyl compound that originates from dephosphorylation of the glycolytic intermediates[4]. MG reacts with arginine residues of proteins to form the major fluorescent product, argpyrimidine, and the irreversible reaction of MG with lysine residues of proteins forms N^{ε}-carboxyethyl-lysine (CEL) [5].

MG and the related AGEs have been recognized as indicators of carbonyl overload *in vivo*[1]. Numerous studies have shown that increased MG and AGE formation under conditions of hyperglycemia has a causative association with diabetes mellitus and its complications[6]. However, the role of MG and AGEs in hypertension with an apparently normoglycemic state has not been investigated systematically. An enhanced AGE formation was found in stroke-prone spontaneously hypertensive rats[7,8]. Our previous study[9] demonstrated increased MG and AGEs in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) in comparison with those from

Wistar Kyoto (WKY) rats. We have also shown that MG and MG-induced AGEs increase in an age-dependent manner associated with the development of hypertension in SHR[10,11].

The purpose of this study was to determine whether increased MG and MG-induced AGEs caused blood pressure increase in SHR. We chronically treated young SHR with aminoguanidine, an inhibitor of AGEs formation, before their blood pressure started to increase, and found that reduction of MG and MG-AGEs attenuated the development of hypertension in SHR. This finding has important implications for devising new preventive approaches for hypertension management.

MATERIALS AND METHODS

Animals

A total of 24 4-week old male rats, including 16 SHR and 8 WKY rats, were purchased from Charles River laboratories (St-Constant, Quebec, Canada). At this age the SHR are normotensive. In order to avoid inter-batch variability the rats were all ordered at the same time in a single batch. The rats were treated in accordance with the guidelines of the Canadian Council on Animal Care, and the experimental protocols were approved by the Animal Care Committee at the University of Saskatchewan. After one week of adaptation, SHR were divided into 2 groups: untreated group (n = 8) and aminoguanidine treated group (1 g/L in drinking water for 9 wks, n = 8). Systolic blood pressure was determined weekly using a standard tail cuff noninvasive blood pressure

(BP) measurement system (Model 29-SSP, Harvard Apparatus, St. Laurent, QC, Canada). At the end of the 9-wk treatment, rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight) given intraperitoneally. Blood was collected from the heart, and plasma was separated by centrifuging blood samples at 1,000 g for 10 min at 4ºC. Tissues cleaned in ice-cold phosphate buffer saline (PBS) were snap-frozen in liquid nitrogen immediately and stored at -80ºC until processing.

MG measurement

Aortic tissues were pulverized with a Mikro-Dismembrator (B. Braun Biotech. Int., Bethlehem, PA, USA). Samples prepared in 10 mmol/L PBS were sonicated on ice twice for 10 s each and thereafter, processed by the addition of 1 mol/L perchloric acid (PCA). Then the samples were incubated on ice for 10 min and centrifuged at 15,000 g to remove the PCA-precipitated material. The supernatants were used freshly for MG measurement. MG was quantitated by the o-phenylenediamine-based assay as described previously[10,12].

Measurement of reduced glutathione levels

The reduced glutathione (GSH) level in the aorta was determined by derivation with 5, 5'-dithio-bis (2-nitrobenzoic acid), and reverse-phase HPLC using ultra-violet detection as described in our previous study[13].

Measurement of oxidized 2,7-dichlorofluorescein

The formation of oxidized 2,7-dichlorofluorescein (DCF) was measured by a dichlorofluorescin (DCFH)-assay, as described previously[9]. The membrane permeable and nonfluorescent probe DCFH-DA was used to load aortic tissues. Oxidized-DCF was quantified by monitoring the DCF fluorescence intensity with excitation at 485 nm and emission at 527 nm with Fluoroskan Ascent plate-reader (Thermo Labsystem, Franklin, MA, USA), expressed in arbitrary units.

Measurement of superoxide level

$O_2^{-\cdot}$ production was measured using the lucigenin-enhanced chemiluminescence method as described previously[14]. Chemiluminescence intensity was detected with a luminometer (TD-20/20, Tunner Designs, CA, USA).

Measurement of nitric oxide production

Nitric oxide (NO) concentration was measured by a newly developed reagent, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM)[15]. To detect the production of NO, aortic tissues were preloaded with membrane permeable and nonfluoresecent DAF-FM (5 μmol/L) in Kreb's buffer for 2 h at 37°C. DAF-FM is deacetylated by intracellular esterases and further reacts with NO to form a fluorescent benzotriazole (DAF fluorescence). The intensity of DAF-fluorescence was determined by Fluoroskan Ascent plate-reader with excitation at 495 nm and emission at 515 nm.

Morphometric analyses

Morphological examinations were performed by the method described by DeBlois *et al.*[16] with some modifications. Briefly, aorta and mesenteric arteries from rats were dissected and fixed by immersion in 4% paraformaldehyde for 16-18 h. The samples were then incubated in 30% sucrose at 4ºC for 3 days. After embedding in O.C.T. compound (Somagen Diagnostics, AB, Canada), 8 μm thick sections were cut using a cryostat. After amplifying the images on the slides using a microscope (Olympus 1×70), the circumferences of the vessels were measured. Diameter (D) was calculated from the equation $C = \pi D$, where C is the circumference. Arterial cross-sectional area (CSA) was calculated from $CSA = \pi (r_o^2 - r_i^2)$, where r_o is the external radius of the media layer and r_i is the radius of the lumen.

Immunohistochemistry

Immunostaining was performed using the method described in our previous study[10]. In brief, paraformaldehyde-fixed and OCT-embedded 8 μm aorta and mesenteric artery sections were incubated overnight at 4°C with primary antibodies diluted 1:100 against the following proteins: CEL (Novo Nordisk, A/S, Denmark); argpyrimidine (Gift from Dr. Uchida, Japan); endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) (BD Bioscience, Mississauga, Canada). Anti-mouse and anti-rabbit IgG-FITC (Sigma, Oakville, ON, Canada) diluted 1:200 was the secondary antibody. The sections were quantified using GeneTools image analysis software (PerkinElmer®, Wellesley, MA, USA). For each animal thirty spots of each picture

with 10 pictures of each section and three sections from each animal were analyzed and the average value was used to express the intensity of staining.

Vascular contractility determination

Third-order branches of the superior mesenteric artery were isolated and transferred to cold physiological saline solution (PSS) containing (in mmol/L): 119 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4 \cdot 7H_2O$, 2.5 $CaCl_2 \cdot 2H_2O$, 25 NaHCO3, and 5.5 glucose (pH 7.4). Mesenteric arteries were mounted on a four-channel wire myograph (Multi myograph, Model 610M, Denmark) and equilibrated for 1 hour in PSS (bubbled with 95% air, 5% CO_2, pH 7.4) at 37°C. The passive tension-internal circumference was determined by stretching arteries to achieve an internal circumference equivalent to 90% of that of the blood vessel under a transmural pressure of 100 mmHg. Phenylephrine at a concentration of $3x10^{-6}$ mol/L that induced 60-70% of maximal contraction was used to pre-contract vessel. After that the endothelium-dependent vasodilator acetylcholine (10^{-10} to 3×10^{-6} mol/L) or the endothelium-independent vasodilator sodium nitroprusside (10^{-10} to 3×10^{-6} mol/L) were cumulatively added to generate dose-dependent relaxation responses. Myodaq-Myodata software (Multi myograph, Model 610M, Denmark) was used for collecting and analyzing data.

Data analysis

Data are expressed as mean ± SEM and analyzed using Student's *t*-test or one

way analysis of variance (ANOVA) in conjunction with the Newman-Keul's test where applicable. Significant difference between treatments was defined at a level of $P< 0.05$.

RESULTS

As shown in Table 1, the initial body weight was very comparable among the groups. At the end of the study, WKY rats weighed more than untreated SHR and aminoguanidine-treated SHR, while aminoguanidine treatment did not alter the body weight of SHR. No significant difference was found in the ratios of kidney and heart weight to body weight, respectively, among animal groups. Aortic cross-section area was comparable in all groups. But aortic media-to-lumen ratio was significantly increased in SHR compared with age-matched WKY rats.

Anti-hypertensive effects of aminoguanidine and changes in MG-induced AGEs in aorta of SHR

At the age of 5 wks, there was no difference in the blood pressure between WKY rats and SHR, which were 121± 2 and 123 ± 1, respectively. During the observation period, systolic blood pressure remained unchanged in WKY rats. But the blood pressure of untreated SHR increased gradually and significantly from age 5 to 14 wks (Fig. 1A). Aminoguanidine administration significantly attenuated the increase of blood pressure in the treated group. At the end of 9-wk treatment, systolic blood pressures of SHR were

175 ± 1 mmHg for aminoguanidine-treated (n = 8) SHR and 202 ± 1 mmHg for untreated SHR (n = 8, $P < 0.001$).

Plasma MG level significantly increased in untreated SHR from 11.3 ± 0.6 nmol/ml at 5 wks to 25.9 ± 4.1 nmol/ml at 14 wks. After chronic treatment of SHR with aminoguanidine, plasma MG level was significantly decreased to that of WKY rats at the same age, which were 16.6 ± 3.7 and 14.2 ± 3.5 nmol/ml, respectively (Fig. 1B).

MG level in the aorta of untreated SHR was increased 3 times at the age of 14 wks (2.71 ± 0.44 nmol/mg protein) compared with that at 5 wks (0.92 ± 0.12 nmol/mg protein). The MG level in the aorta from aminoguanidine-treated SHR was significantly reduced to 1.33 ± 0.14 nmol/mg protein at the age of 14 wks, which was comparable to that in WKY rats at the same age (Fig. 1C).

Using immunohistochemical staining method, we further investigated whether MG-induced AGEs were decreased in the aorta and mesenteric artery from SHR with aminoguanidine treatment. Figure 2 shows reduced intensity of argpyrimidine and CEL staining in the aorta from aminoguanidine-treated SHR, compared with untreated controls (n = 3-5 for each group). Chronic treatment with aminoguanidine also normalized the intensities of argpyrimidine and CEL in SHR to that of WKY rats (Fig. 2C & D). Table 2 shows that intensity of argpyrimidine and CEL staining in the mesenteric artery was also significantly decreased after aminoguanidine treatment. Most of the positive CEL and argpyrimidine staining was localized to the endothelial layer in all the animal groups. The negative (no primary antibody) and positive (control immune

globulin) control sections revealed no staining (not shown).

Decreased oxidative stress in the aorta of AG-treated SHR

Superoxide anion ($O_2^{\cdot-}$) level was significantly reduced in the aorta of aminoguanidine-treated SHR at the end of 9-wk treatment period ($P < 0.001$) (Table 3). Compared with untreated SHR, DCF-fluorescence intensity was also significantly reduced by aminoguanidine treatment (Table 3). Using a NO sensitive probe DAF-FM, NO production was directly measured. As shown in Table 3, DAF fluorescence was significantly decreased by chronic aminoguanidine treatment in comparison with untreated SHR. The GSH level in the aorta from aminoguanidine-treated SHR was significantly increased, as opposed to untreated SHR (Table 3).

Effects of chronic aminoguanidine treatment on nitric oxide synthase expression in aortic tissues

To investigate possible mechanisms for effect of aminoguanidine treatment on blood pressure *in vivo*, aortic iNOS and eNOS expressions were measured. As shown in Fig. 3A, compared with untreated SHR, the aorta of aminoguanidine-treated SHR had more eNOS staining. (Fig 3C). Immunofluorescence revealed more intense iNOS staining in the untreated SHR than in SHR treated with aminoguanidine and WKY rats (Fig. 3B & D). Both eNOS and iNOS levels in the aorta from aminoguanidine-treated SHR were comparable to those from WKY rats at the same age.

Morphological changes in mesenteric arteries of SHR

Morphological damage of vascular tissues was prevented in SHR treated with aminoguanidine (Table 4). The lumen diameter of the mesenteric arteries from aminoguanidine-treated SHR was significantly enlarged, the wall thickness was reduced, and the media/lumen ratio was decreased ($P < 0.05$) in comparison with those of untreated SHR (Table 4). On the other hand, there was no significant change in cross-section area of the mesenteric arteries (Table 4) (n = 3-4 for each group). These results suggest that long-term exposure to aminoguanidine prevents the development of eutrophic inward vascular remodeling in SHR.

Aminoguanidine treatment improves endothelium-dependent relaxation in mesenteric arteries of SHR

Acetylcholine (0.1 nmol/L to 3 μmol/L)-induced relaxations were significantly increased in small mesenteric arteries from adult SHR treated with aminoguanidine, which was very comparable to that from WKY rats of the same age (Fig. 4A). The sensitivity of small mesenteric arteries to acetylcholine was also significantly enhanced after chronic treatment with aminoguanidine compared with untreated SHR (EC_{50}, 1.80 ± 0.15 vs. 13.23 ± 1.67 nmol/L with or without aminoguanidine treatment). There was no difference in sodium nitroprusside mediated relaxation of vascular tissues from WKY rats and SHR treated with or without aminoguanidine (Fig. 4B), which indicates that acetylcholine-induced relaxation is endothelium-dependent.

DISCUSSION

Our previous studies[10,11] showed that an age-dependent increase in blood pressure was associated with a progressive increase in the aortic and kidney MG and MG-induced AGE levels in SHR. These earlier observations suggested that MG may play an important role in the development of hypertension. In the present study, we show that administration of aminoguanidine, a non-specific inhibitor of AGE formation, to male SHR from 5 to 14 weeks significantly reduced plasma and aortic MG levels, decreased the levels of MG-induced AGEs, CEL and argpyrimidine, in the aorta and prevented the morphologic damage in the SHR, leading to a significant attenuation of blood pressure increase. Thus, we provide evidence for the first time for a role of MG and MG-induced AGEs in the development and progression of hypertension in the SHR. Recently, it was shown that aminoguanidine[17], used as an inhibitor of collagen cross-linking, reduced increase in blood pressure and inflammatory changes in the left ventricle in DOCA-salt hypertensive rats. The role of MG was however, not investigated in this study.

Retarded development of diabetic complications by chronic aminoguanidine treatment for experimental diabetes was the initial evidence that AGE accumulation is a risk factor for diabetic complications[18,19]. Recent studies have also demonstrated that aminoguanidine treatment of rats decreased age-related cardiovascular damage[20]. Aminoguanidine, a nucleophilic hydrazine compound, is a prototype α,β-dicarbonyl scavenger that prevents the formation of AGEs from α,β-dicarbonyl precursors[18].

Aminoguanidine is mostly efficient in scavenging MG[21]. AG is also a potent and irreversible inhibitor of semicarbazide-sensitive amine oxidase (SSAO)[22]. Besides glycolysis, MG is also generated from primary amines and from acetone during lipolysis, catalyzed by SSAO or acetol mono-oxygenase[22] and increased activities of SSAO might be responsible for the enhanced circulating MG levels in diabetes[23] as well as in hypertension[10]. Thus, prevention of formation or scavenging of MG by aminoguanidine would decrease the formation of AGEs. Moreover, aminoguanidine also blocks the formation of a *de novo* crosslink by irreversibly binding to glycated proteins[24].

Oxidative stress is one of the pathogenic factors in the development of hypertension in experimental models[6,25]. Our previous study demonstrated that MG increased the generation of $O_2^{\cdot -}$ and NO in rat aortic SMCs, which in turn enhanced $ONOO^-$ formation[15]. As shown in Table 3, administration of aminoguanidine significantly reduced $O_2^{\cdot -}$, $ONOO^-$ and H_2O_2 generation in SHR. This effect of aminoguanidine could be an indirect one, through inhibition of AGE formation, as well as a direct one, through an effect on free radicals themselves. Moreover, GSH level was significantly higher after aminoguanidine treatment than that in the untreated SHR group. These antioxidant properties of aminoguanidine are in agreement with other studies[26]. Aminoguanidine is a known inhibitor of iNOS[27]. Our results confirm these findings and we show decreased NO production (Table 3), probably derived from iNOS, as well as reduced iNOS staining (Fig. 3) after aminoguanidine-treatment in SHR. Chronic aminoguanidine treatment in cirrhotic rats has been shown to reduce aortic iNOS mRNA

and protein while having no effect on eNOS[28].

NO is a primary vasodilator derived from the endothelium. Recently, Rojas *et al*.[29] found that AGEs can decrease eNOS expression by increasing the rate of eNOS mRNA degradation. This effect can contribute to reduced endothelium-dependent relaxation, as observed by us (Fig. 4). Our previous study[10] revealed most of the staining for MG-induced AGEs, CEL and CML, in the endothelium and very little in the smooth muscle of the aorta. After aminoguanidine therapy, the endothelial levels of MG-induced CEL and argpyrimidine were normalized to the levels found in WKY rats at the same age. The expression of eNOS was also increased by aminoguanidine treatment. Moreover, the small mesenteric arteries from aminoguanidine-treated SHR showed restored endothelium-dependent relaxation to acetylcholine and unaltered endothelium-independent relaxation to sodium nitroprusside (Fig. 4).

It is well established that essential hypertension is associated with structural changes in the resistance vessels, a process known as vascular remodeling. Mulvany[30] has proposed that essential hypertension is associated only with eutrophic remodeling in small arteries. In our study, chronic aminoguanidine administration prevented AGEs-induced morphological damage in mesenteric arteries from SHR, as evidenced by increased lumen diameter and decreased media/lumen ratio without changes of CSA, which indicate that AGEs-induced eutrophic inward remodeling. Aminoguanidine, a known inhibitor of collagen cross-linking[17], may be reducing aortic stiffness, like β-aminopropionitrile[31], although such an effect on resistance vessels that regulate blood

pressure needs to be proved in future studies.

In summary, plasma and aortic MG levels in SHR were significantly reduced by aminoguanidine treatment. Aortic levels of MG-induced AGEs in SHR after aminoguanidine treatment were normalized to the levels found in age-matched WKY rats. Free radical production was significantly lowered while reduced glutathione level was significantly increased after aminoguanidine treatment in the aortic tissues from SHR. Moreover, aminoguanidine treatment prevented the morphological damage of vascular tissues in SHR, and restored endothelium-dependent relaxation to acetylcholine. Chronic treatment of SHR with aminoguanidine significantly increased aortic eNOS levels and reduced iNOS expression. All these beneficial effects of aminoguanidine lead to reduced age-dependent development of hypertension in SHR. Although further studies are needed to substantiate the causative roles of MG and MG-mediated AGEs in the progression of hypertension, our study strongly suggests that MG and MG-induced AGEs are involved as a factor in the pathogenesis of hypertension by modulating the redox state and inducing endothelial dysfunction and vascular remodeling in the SHR.

Table 6-1. Gross indications of animal growth and morphology of the aorta

	WKY	SHR - Untreated	SHR -Aminoguanidine
Initial (5 wks) body weight (g)	107 ± 2	94 ± 2	92 ± 3
Final (14 wks) body weight (g)	335 ± 10	300 ± 10*	302 ± 4*
Kidney weight/ Body weight (%)	0.67 ± 0.02	0.77 ± 0.05	0.77 ± 0.03
Heart weight/ Body weight (%)	0.33 ± 0.005	0.4 ± 0.01	0.38 ± 0.01
Aorta CSA (mm^2)	0.402 ± 0.01	0.456 ± 0.08	0.393 ± 0.04
Aorta media/lumen ratio (%)	6.03 ± 0.12	6.73 ± 0.02*	6.12 ± 0.4

n = 4-8 for each group.
Values are mean ± SEM. CSA, cross-section area.
*P< 0.05, SHR *vs*. WKY rats of the same age group

Table 6-2. Intensity of advanced glycation endproducts immunofluorescence staining in mesenteric arteries.

	Argpyrimidine	CEL
WKY	498 ± 22	534 ± 51
SHR - Untreated	731 ± 25*	801 ± 19*
SHR -Aminoguanidine	508 ± 37$^{\#}$	544 ± 26$^{\#}$

Intensity of argpyrimidine and CEL staining was quantified using GeneTools image analysis software (n = 3-5 in each group). *$P < 0.05$, SHR *vs*. WKY rats of the same age group. $^{\#}P < 0.05$, aminoguanidine-treated *vs*. untreated SHR of the same age group.

Table 6-3. Free radical and reduced glutathione levels in aortic tissues of rats.

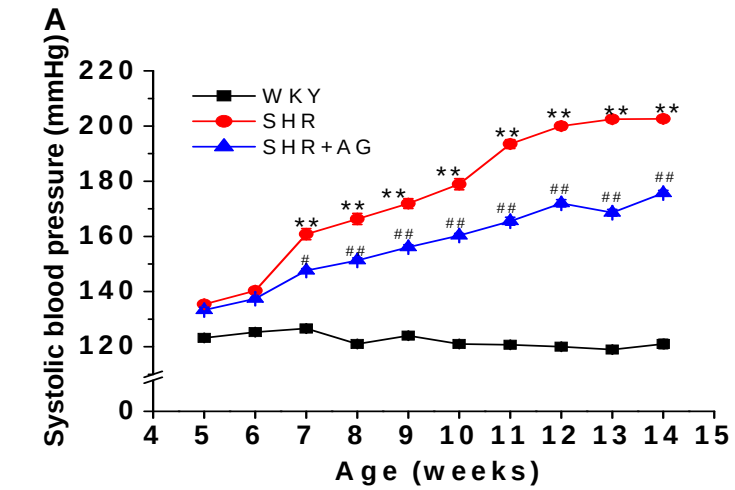
	WKY	SHR - Untreated	SHR -Aminoguanidine
Superoxide anion production (% of WKY rats)	100 ± 16	220 ± 9**	136 ± 8$^{\#\#}$
DCF-fluorescence intensity (% of WKY rats)	100 ± 4	122 ± 2 *	107 ± 4$^{\#}$
NO production (% of WKY rats)	100 ± 6	155 ± 6*	105 ± 5$^{\#}$
GSH levels in aorta (% of WKY rats)	100 ± 16	63.8 ± 5.9*	96.2 ± 8.6$^{\#}$

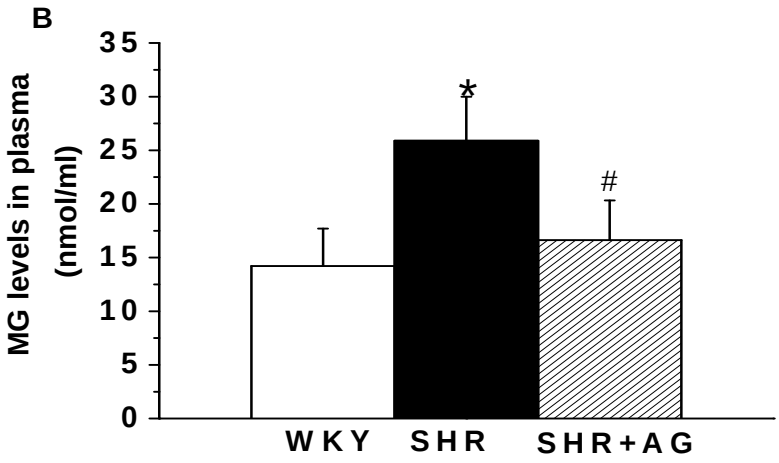
n = 5-8 in each group. $O_2^{-\cdot}$ production was measured using the lucigenin-enhanced chemiluminescence method, oxidized DCF production was determined by a DCFH-assay, NO concentration was measured by DAF-FM reagent, and reduced GSH was quantified by HPLC. *$P < 0.05$, **$P < 0.001$, SHR vs. WKY rats of the same age group. $^{\#}P < 0.05$, $^{\#\#} P < 0.001$, aminoguanidine -treated *vs*. untreated SHR of the same age group.

Table 6-4. Morphological characteristics of the mesenteric arteries of rats (14 wks)

	WKY	SHR- Untreated	SHR - Aminoguanidine
Lumen diameter (mm)	0.601 ±0.02	0.46 ± 0.01*	0.576 ± 0.03#
Wall thickness (μm)	42.39 ± 1.16	71.32 ± 2.26*	56.30 ± 4.25#
Media/lumen ratio (%)	7.09 ± 0.37	15.52 ± 0.7*	9.76 ± 0.45#
Cross-section area (mm^2)	0.085 ± 0.04	0.119 ± 0.04	0.112 ± 0.01

n = 3-4 for each group
**P*< 0.05, SHR *vs*. WKY rats of the same age group
#*P*<0.05, aminoguanidine-treated *vs*. untreated SHR of the same age group


A
Systolic blood pressure (mmHg)
220
200
180
160
140
120
0
WKY
SHR
SHR+AG
4 5 6 7 8 9 10 11 12 13 14 15
Age (weeks)


B
MG levels in plasma
(nmol/ml)
35
30
25
20
15
10
5
0
WKY
SHR
SHR+AG

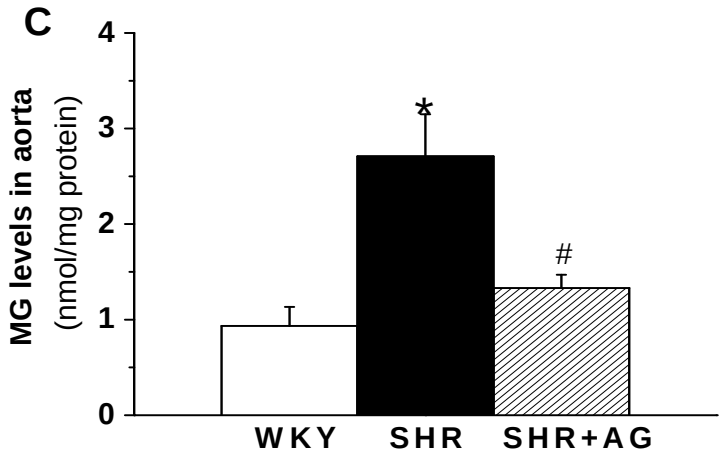

C
MG levels in aorta
(nmol/mg protein)
4
3
2
1
0
WKY
SHR
SHR+AG

Figure 6-1. Effect of aminoguanidine on systolic blood pressure and methylglyoxal (MG) levels in SHR. Systolic blood pressure was measured by tail-cuff (A). SHR were treated with aminoguanidine from 5 wks to 14 wks (n = 8). MG levels were measured in the plasma (B), and the aorta (C) from aminoguanidine-treated SHR, untreated SHR and WKY rats (n = 5-6). *P < 0.05, **P < 0.001, SHR *vs*. WKY rats of the same age group. $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.001, aminoguanidine-treated *vs*. untreated SHR of the same age group.

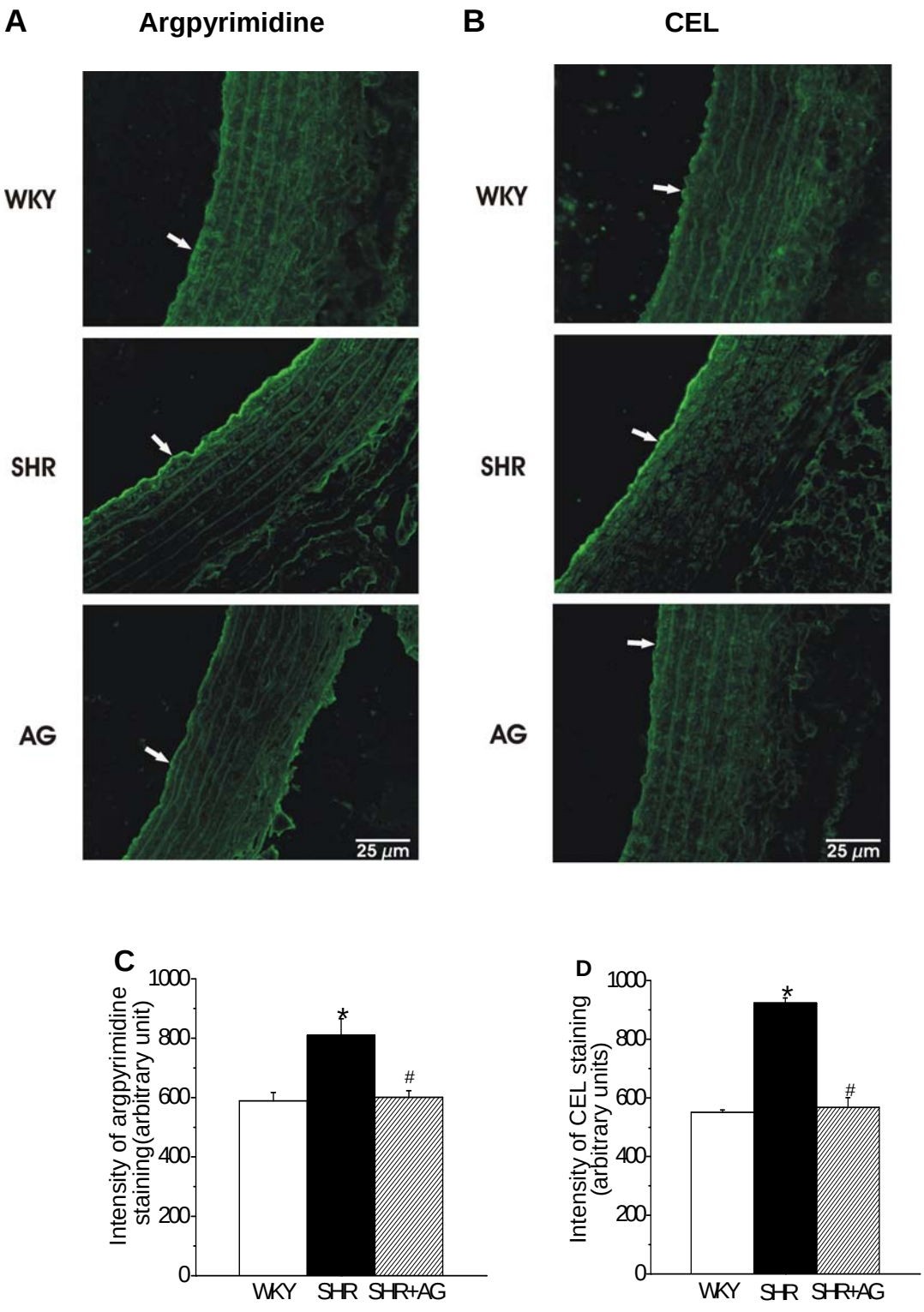
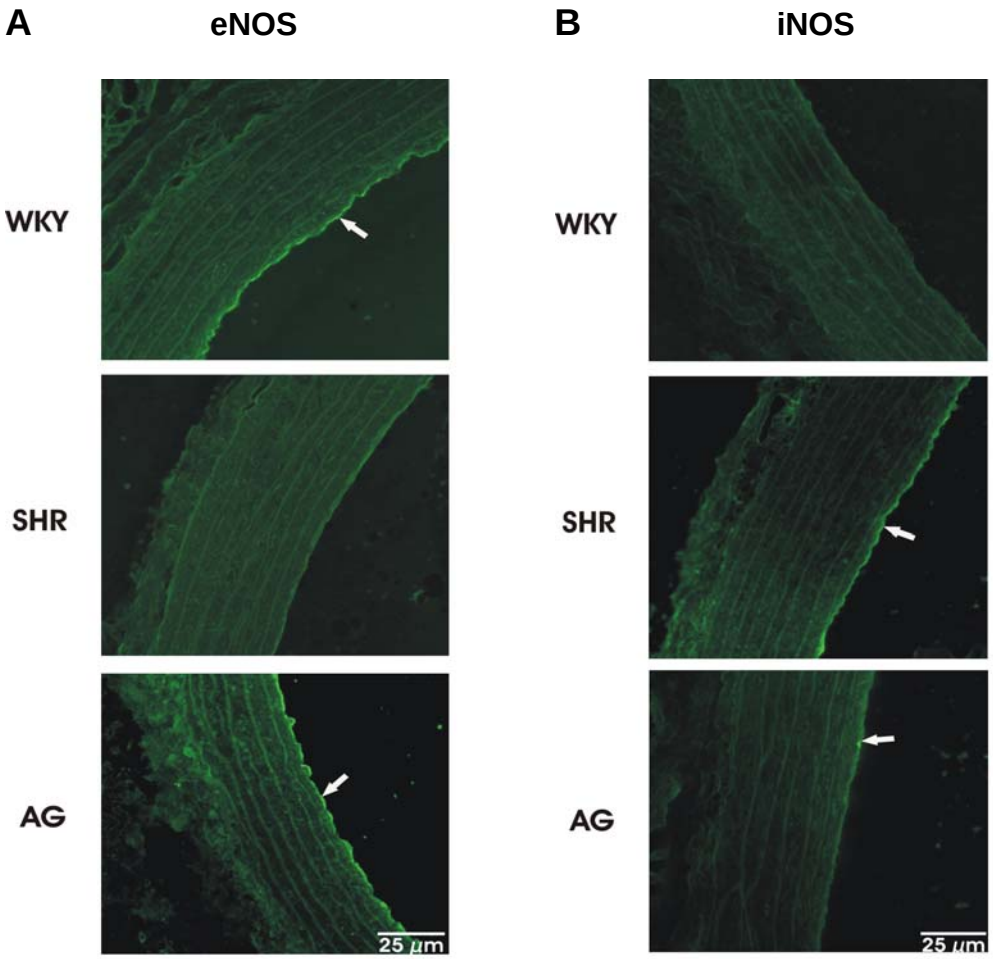
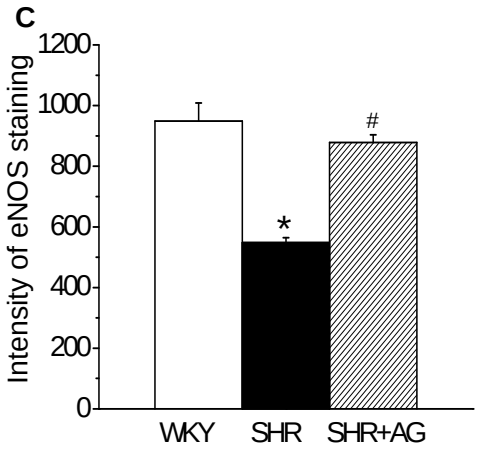

A
Argpyrimidine
B
CEL
WKY
SHR
AG
WKY
SHR
AG
25 μm
25 μm
C
Intensity of argpyrimidine staining(arbitrary unit)
1000
800
600
400
200
0
*
#
WKY
SHR
SHR+AG
D
Intensity of CEL staining (arbitrary units)
1000
800
600
400
200
0
*
#
WKY
SHR
SHR+AG

Figure 6-2. Immunohistochemical detection of AGEs in rat aortic tissues. Argpyrimidine (A) and N^{ε}-carboxyethyl-lysine (CEL) (B) staining was detected in the aorta from aminoguanidine-treated SHR, untreated SHR and WKY rats. Aortic sections from untreated SHR showed more intense argpyrimidine and CEL staining than age-matched SHR treated with aminoguanidine. Arrows indicate the representative immunoreactivity in the endothelium. Scale bar: 25μm. Intensity of argpyrimidine (C) and CEL (D) staining was quantified using GeneTools image analysis software (n = 3-5 in each group). *$P < 0.05$, SHR *vs*. WKY rats of the same age group. $^{\#}P < 0.05$, aminoguanidin -treated *vs*. untreated SHR of the same age group.


A
eNOS
B
iNOS
WKY
SHR
AG
WKY
SHR
AG
25 μm
25 μm


C
Intensity of eNOS staining
1200
1000
800
600
400
200
0
*
#
WKY
SHR
SHR+AG

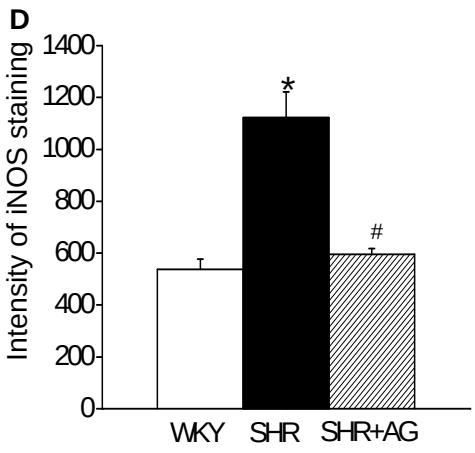
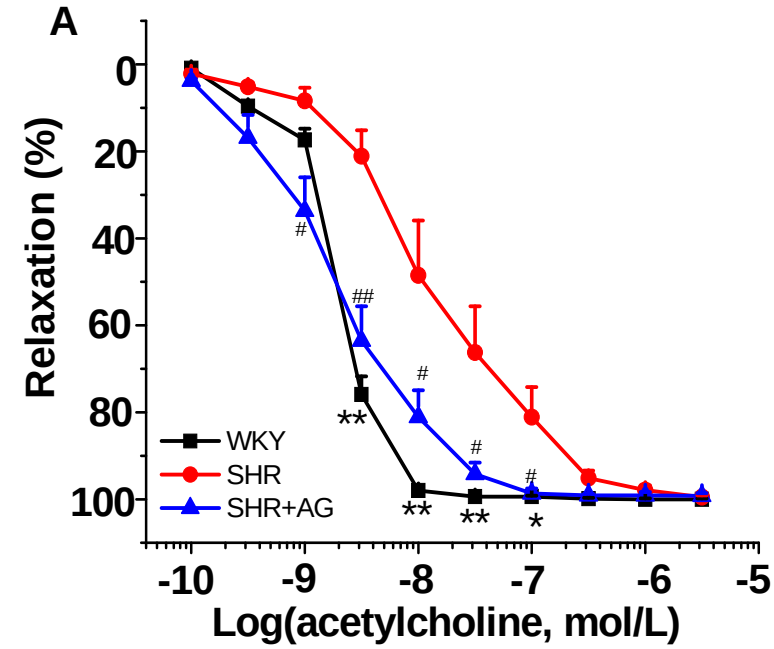

D
Intensity of iNOS staining
1400
1200
1000
800
600
400
200
0
*
#
WKY
SHR
SHR+AG

Figure 6-3. Immunoreactivities of eNOS and iNOS in aortic tissues of SHR. Staining of eNOS (A) and iNOS (B) were detected in the aorta from aminoguanidine-treated SHR, untreated SHR and WKY rats. Aminoguanidine-treated SHR and WKY rats show more positive eNOS staining than age-matched controls, while untreated SHR has more positive iNOS staining than WKY rats and aminoguanidine-treated SHR. The positive staining was only localized in the endothelium. Scale bar: 25μm. Intensity of eNOS (C) and iNOS (D) staining was quantified using GeneTools image analysis software. n = 3-5 for each group. $^{*}P < 0.05$, SHR *vs*. WKY rats of the same age group. $^{\#}P < 0.05$, aminoguanidine-treated *vs*. untreated SHR of the same age group.


A
Relaxation (%)
0
20
40
60
80
100
WKY
SHR
SHR+AG
**
**
**
*
#
##
#
#
#
-10
-9
-8
-7
-6
-5
Log(acetylcholine, mol/L)

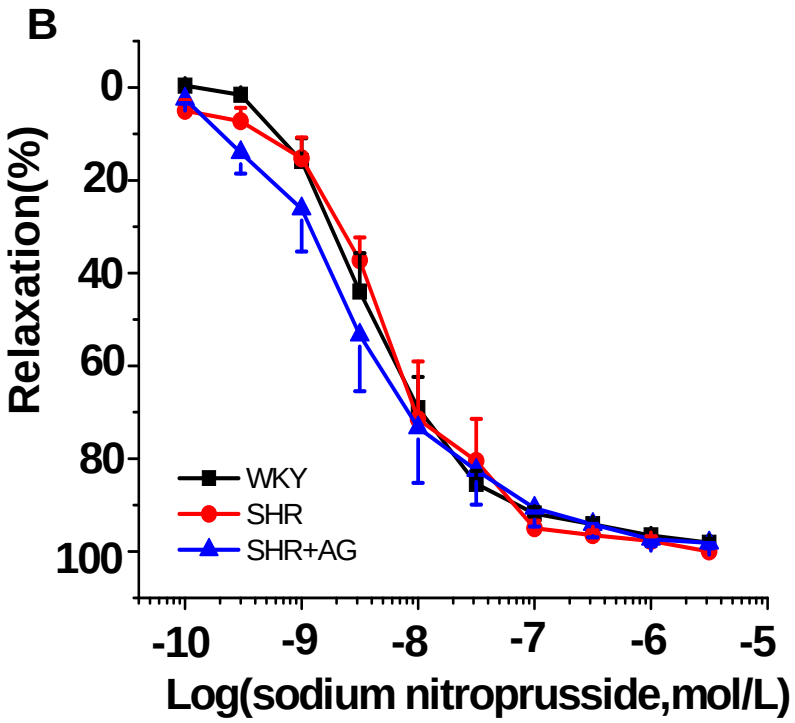

B
Relaxation(%)
0
20
40
60
80
100
WKY
SHR
SHR+AG
-10
-9
-8
-7
-6
-5
Log(sodium nitroprusside,mol/L)

Figure 6-4. Vasorelaxation of the third branches of the mesenteric arteries in SHR treated with aminoguanidine. A. The mesenteric arteries were precontracted with phenylephrine ($3x10^{-6}$ mol/L) followed by administration of acetylcholine and endothelium-dependent vasorelaxation was measured in aminoguanidine-treated SHR, untreated SHR and WKY rats. B. The mesenteric arteries were precontracted with phenylephrine ($3x10^{-6}$ mol/L) and endothelium-independent vasorelaxation was measured in the presence of various concentrations of sodium nitroprusside, a NO donor. n = 7-8 for each group. $^{*}P < 0.05$, $^{**}P < 0.001$, SHR *vs*. WKY rats of the same age group. $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, aminoguanidine-treated *vs*. untreated SHR of the same age group.

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CHAPTER 7

ATTENUATION OF HYPERTENSION DEVELOPMENT BY SCAVENGING METHYLGLYOXAL IN FRUCTOSE-TREATED RATS

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ABSTRACT

Objectives: Methylglyoxal (MG) is a reactive dicarbonyl intermediate of metabolism produced in the body. MG reacts with certain proteins and forms damaging advanced glycation endproducts (AGEs) such as N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-carboxymethyl-lysine (CML). Increased MG levels are found in diabetes mellitus and associated with hypertension development in the SHR. The purpose of this study was to investigate whether increased endogenous formation of MG and MG-induced AGEs caused hypertension development in normotensive Sprague Dawley (SD) rats.

Methods: The rats were fed chronically for 16 wks with fructose, a known precursor of MG formation. One group of rats was cotreated with fructose and metformin, an AGEs formation inhibitor. MG and reduced glutathione (GSH) were measured by HPLC, while hydrogen peroxide was measured by a dicholorofluorescin assay. Immunohistochemistry was performed for eNOS, CEL and CML.

Results: Fructose-fed rats had elevated blood pressure, serum MG, insulin and triglycerides, and reduced serum levels of GSH. MG, hydrogen peroxide and CEL were increased in the aorta while the endothelial eNOS was reduced. CEL and CML were also increased in the mesenteric artery endothelium along with media/lumen ratio, signifying structural remodelling. Fructose-fed rats also had reduced insulin-stimulated glucose uptake in adipose tissue. All of the harmful changes in fructose-fed rats were attenuated in metformin and fructose cotreated rats.

Conclusion: Increased MG, AGEs, oxidative stress and reduced endothelial nitric oxide synthase along with structural remodelling of the vessel wall in the aorta and mesenteric artery likely play a role in the pathogenesis of hypertension.

Key Words: methylglyoxal ■ advanced glycation endproducts ■ hypertension ■ oxidative stress

INTRODUCTION

Methylglyoxal (MG), a highly reactive dicarbonyl compound, is an intrinsic component of anaerobic glycolysis. It is also formed during other metabolic processes including the breakdown of threonine from protein catabolism and acetone from lipolysis [1]. MG is detoxified by the glyoxalase system that uses reduced glutathione (GSH) as a cofactor. An overproduction of MG may be caused by an increased availability of its precursors such as glucose and fructose; for example, in vascular smooth muscle cells, fructose increased MG formation in a concentration and time dependent manner [2]. MG can react with selective proteins to yield irreversible advanced glycation endproducts (AGEs), and it is one of the most reactive AGE precursors [3]. The irreversible reaction of MG with lysine residues of proteins forms N^{ε}-carboxymethyl-lysine (CML) and N^{ε}-carboxyethyl-lysine (CEL) [4].

Clinical and biochemical evidences suggest that increased formation of MG and its induced AGEs in diabetes mellitus is a causative factor for the development of diabetic complications such as retinopathy or nephropathy [5]. High MG levels may also play an important role in non-diabetic conditions such as hypertension. An enhanced AGE formation was found in stroke-prone spontaneously hypertensive rats (SHR) [6]. Our previous studies [7, 8] showed that an increase in blood pressure of SHR closely paralleled with an age-dependent increase in MG, CEL and CML levels in the kidney and aorta, which was not seen in age-matched Wistar Kyoto rats, despite similar blood

glucose levels between these two strains [9]. We also found that treatment of young SHR with an AGE inhibitor, aminoguanidine, attenuated the increase of blood pressure [10].

The aim of this study was to determine whether increased formations of MG and MG-induced AGEs caused blood pressure increase in young normotensive Sprague Dawley (SD) rats. We chronically treated young SD rats with fructose, a precursor of MG, and metformin, an inhibitor of AGEs. We found that fructose treatment significantly increased MG and MG induced AGEs formation. Metformin cotreatment attenuated the increase of blood pressure in fructose-fed SD rats by scavenging MG and AGEs. This finding implies that the increased level of MG may be a causative factor for hypertension development.

MATERIALS AND METHODS

Animals

A total of 79 4-week old male SD rats were purchased from Charles River Laboratories (St-Constant, Quebec, Canada). The rats were treated in accordance with the guidelines of the Canadian Council on Animal Care, and the experimental protocols were approved by the Animal Care Committee at the University of Saskatchewan. After one week adaptation, SD rats were divided into 4 groups: untreated control group (n = 17); 60% fructose (in chow) treated group (n = 25); metformin (500mg/kg/day in

drinking water) treated group (n = 18); and fructose plus metformin (60% fructose in chow, metformin 500mg/kg/day in drinking water) treated group (n = 19). Body weight, blood glucose levels, and systolic blood pressure were measured weekly. Blood glucose was determined using the OneTouch® blood glucose monitoring system (LifeScan Canada). Systolic blood pressure was determined weekly using a standard tail cuff noninvasive BP measurement system (Model 29-SSP, Harvard Apparatus, St. Laurent, QC, Canada). After 9 wks treatment, some rats were sacrificed to test the insulin resistance status. At the end of the 16-wk study, rats were anaesthetized with sodium pentobarbital (60 mg/kg body weight) given intraperitoneally. Blood was collected from the heart, and serum was separated by centrifuging blood samples at 1,000 g for 10 min at 4ºC. Tissues cleaned in ice-cold phosphate buffer saline (PBS) were snap-frozen in liquid nitrogen immediately and stored at -80ºC until processing.

Biochemical Measurements

The serum levels of hemoglobin A1C, triglycerides, total cholesterol and high density lipoprotein (HDL) cholesterol were assayed in the Laboratory of Biochemistry and Hematology, Royal University Hospital, University of Saskatchewan, Saskatoon, Canada. Serum insulin concentration was measured with a rat-specific insulin ELISA kit (Mercodia, Uppsala, Sweden).

Glucose tolerance test and the determination of insulin stimulated glucose uptake

An intraperitoneal glucose tolerance test (IPGTT) was carried out after 12-14 h fasting. After basal glucose level was measured, conscious rats were injected intraperitoneally with 1.5 g/kg body weight of 50% (w/v) glucose solution in 0.9% (w/v) saline. Blood was then collected from the tail vein and blood glucose was measured using OneTouch® blood glucose monitoring system (LifeScan, Canada) at 0, 10, 15, 30, 60, and 120 min after the glucose injection. [3H]-2-deoxyglucose ([^{3}H]-2-DOG, PerkinElmer) was used for glucose uptake experiments in abdominal adipose tissue as described previously [11].

MG measurement

Aortic and serum MG levels were quantified by the widely accepted o-phenylenediamine (o-PD)-based assay as described previously [7] with some modification. Briefly, the supernatant of the aortic homogenate or serum was incubated with 100 mmol/L o-PD (derivatizing agent) for 3 h at room temperature. The quinoxaline derivative of MG (2-methylquinoxaline) and the quinoxaline internal standard (5-methylquinoxaline) were measured using a Hitachi D-7000 HPLC system (Hitachi Ltd., Mississauga, ON, Canada). Samples were calibrated by comparison with 2-MQ standards.

Measurement of reduced glutathione levels

The GSH levels in the aorta and serum were determined by derivation with 5, 5'-dithio-bis (2-nitrobenzoic acid), and reverse-phase HPLC using ultra-violet detection as described in our previous study [12].

Measurement of hydrogen peroxide levels

The formation of H_2O_2 was measured by a dichlorofluorescin (DCFH)-assay, as described previously [13]. The membrane permeable and nonfluorescent probe DCFH-DA was used to load aortic tissues. Oxidized-DCF was quantified by monitoring the DCF fluorescence intensity with excitation at 485 nm and emission at 527 nm with Fluoroskan Ascent plate-reader (Thermo Labsystem, Franklin, MA, USA), expressed in arbitrary units.

Morphometric analyses

Morphological examinations were performed by the method described by DeBlois *et al.* [14] with some modifications. Briefly, aorta and mesenteric arteries from rats were dissected and fixed by immersion in 4% paraformaldehyde for 16-18 h. The samples were then incubated in 30% sucrose at 4ºC for 3 days. After embedding in O.C.T. compound (Somagen Diagnostics, AB, Canada), 8 μm thick sections were cut using a cryostat and picked up on poly-l-lysine coated slides. After amplifying the images on the slides using a microscope (Olympus 1×70), the circumferences of the vessels were measured. Diameter (D) was calculated from the equation $C = \pi D$, where C is the circumference. Arterial cross-sectional area (CSA) was calculated from CSA =

π (r_o^2-r_i^2), where r_o is the external radius of the media layer and r_i is the radius of the lumen.

Immunohistochemistry

Immunostaining was performed using the method described in our previous study [8]. In brief, paraformaldehyde-fixed and OCT-embedded 8 μm mesenteric artery sections were permeabilized and incubated overnight with primary antibodies against the following proteins: CEL and CML (Novo Nordisk, A/S, Denmark); diluted 1:100. Anti-mouse IgG-FITC (Sigma, Oakville, ON, Canada) diluted 1:200 was the secondary antibody.

For double immunofluorescence staining, aortic sections were incubated overnight in a cocktail solution containing mouse anti-CEL antibody (1:100) and rabbit anti-eNOS antibody (1:200; Chemicon International, Temecula, CA, USA). After multiple washes in PBS, sections were incubated for 2 h in goat anti-mouse IgG-FITC (1:200) and Alexa Fluor 568–conjugated anti-mouse secondary antibody (1:400; Invitrogen, Burlington, ON, Canada). Sections were mounted on gelatinized slides and coverslipped. Immunofluorescence images were obtained under a confocal laser-scanning microscope (LSM 510 META; Zeiss).

The sections were quantified using GeneTools image analysis software (PerkinElmer®, Wellesley, MA, USA). For each animal thirty spots of each picture,

with 10 pictures of each section and three sections from each animal were analyzed and the average value was used to express the intensity of staining.

Data analysis

Data are expressed as mean ± SEM and analyzed using Student's *t*-test or one way analysis of variance (ANOVA) and post hoc analysis (Tukey test) where applicable. Significant difference between treatments was defined at a level of $P< 0.05$.

RESULTS

The development of insulin resistance in fructose-fed rats

After 9 weeks' treatment with fructose, the rats showed a significant increase in serum insulin (Figure 1A), and enhanced serum triglyceride levels (Table 1). As shown in Figure 1A, the serum insulin level was enhanced significantly from 2.80±0.12 μg/L in control group to 4.87 ± 0.18 μg/L in fructose-fed group. Likewise, serum triglycerides increased from 0.35 ± 0.04 mM to 0.84 ± 0.05 mM by fructose feeding (Table 1). However, total cholesterol, HDL-cholesterol and HbA1c in serum were not changed (Table 1). In all groups of rats treated with or without MG and/or metformin, the fasting blood glucose level was maintained at 5 - 6 mM. At the end of the 9 wks treatment, IPGTT was performed and a notable difference was observed in metformin-treated rats,

compared with the control rats ($P<0.05$, $n = 4$ for metformin treated group, $n = 7$ for other groups; Figure 1B).

A decreased insulin-stimulated glucose uptake in insulin targeted tissues has been identified as a key indicator of insulin resistance. We observed that the insulin-induced glucose uptake by abdominal adipose tissue dropped dramatically in fructose-fed rats, in comparison with that from untreated control rats ($P<0.05$). With insulin (100 nM) stimulation, as shown in Figure 1C, glucose uptake by adipose tissue is 198% of the basal glucose uptake without insulin stimulation, in the control group ($n = 6$), and 117% of the basal uptake in fructose-fed rats ($n = 6$, $P<0.05$). The lowered glucose uptake in adipose tissue induced by fructose feeding was restored significantly by co-treatment with metformin (Figure 1C).

Increased endogenous accumulation of MG in fructose-fed rats

Blood pressure was significantly increased after only two weeks of the fructose feeding, while metformin cotreatment attenuated the increase of blood pressure in the treated group (Fig 2A). Metformin alone did not change the blood pressure compared to control SD rats.

As shown in Fig 2B, 9-week fructose treatment caused a significant increase in serum MG concentration in SD rats. Serum MG level in fructose-fed rats was increasing

with time. At the end of the study, serum MG level was significantly increased by 72.5% in fructose treated SD rats to 4.28 ± 0.5 μM compared to 2.48 ± 0.3 μM in control SD rats (n = 5-6). MG level in aorta was also significantly increased after fructose treatment (Fig 2C). This increased endogenous MG accumulations in aorta as well as in serum were significantly reduced by metformin co-treatment.

Effects of chronic fructose treatment on CEL and eNOS expression in aortic tissues

Using immunohistochemical double-staining method, we further investigated the effect of fructose on CEL and eNOS in the aorta from SD rats. Figure 3 shows increased intensity of CEL staining in the aorta from fructose-treated SD rats, compared with untreated controls (n = 3-5 for each group). Chronic co-treatment with metformin normalized the intensities of CEL in fructose-fed SD rats to that of control SD rats (Fig. 3B). Aortic eNOS expression was measured in order to investigate possible mechanisms for the effect of fructose treatment on blood pressure *in vivo*. As shown in Fig. 3A, compared with untreated SD rats, the aorta of fructose-treated SD rats had less eNOS staining. The intensity of eNOS staining was significantly increased by chronic treatment with metformin in fructose-fed rats (Fig 3C). Metformin alone had no effect on CEL and eNOS expression compared to control SD rats. Most of the positive CEL and eNOS staining was localized to the endothelial layer in all the animal groups. The negative (no primary antibody) and positive (control immune globulin) control sections revealed no staining (not shown).

Increased oxidative stress in fructose-treated SD rats

Compared with untreated SD rats, hydrogen peroxide production in aorta measured with DCF-fluorescence intensity was significantly increased more than two times by fructose treatment, from 8.7 ± 1.3 to 21.6 ± 4.3 (arbitrary units) respectively (Fig. 4A). DCF-fluorescence was significantly reduced in fructose plus metformin treated group compared to fructose alone treated group. The reduced GSH level in the serum from fructose-fed rats was significantly decreased, as opposed to untreated SD rats (Fig. 4B). In the fructose plus metformin treated group, serum GSH level was very comparable to that in control group. There was no significant difference in aortic GSH levels among all groups (data not shown).

Morphological changes in mesenteric arteries of SD rats

The lumen diameter of the mesenteric arteries from fructose-treated SD rats was significantly decreased, the wall thickness was enlarged, and the media/lumen ratio was increased ($P < 0.05$) in comparison with those of untreated SD rats (Fig. 5A). Morphological damage of vascular tissues was prevented by co-treatment with metformin. On the other hand, there was no significant change in cross-section area of the mesenteric arteries among the groups (Fig. 5B) (n = 3-4 for each group). These results suggest that long-term exposure to fructose induces the development of eutrophic inward vascular remodeling in SD rats.

Enhanced expression of AGEs in mesenteric arteries by fructose treatment

Figure 6 shows more intense or positive staining for CEL in the mesenteric artery from fructose-fed SD rats, compared to control SD rats. The intensity of CEL staining was significantly higher in fructose-treated rats than in age-matched control rats (Fig 6 A&B). Most of the positive staining was localized to the endothelial layer in both strains. Staining for CML was also more intense in the mesenteric artery from fructose-treated rats as opposed to control rats (n = 3 for each group) (Fig. 6 A&C). CEL and CML expression was significantly decreased by chronic metformin cotreatment.

DISCUSSION

In the present study we show that chronic administration of fructose to SD rats significantly increased serum and aortic MG levels, enhanced the MG-induced AGEs formation in the aorta and the mesenteric artery, and caused morphological damage in the arterial wall, leading to a significant increase in blood pressure. Our previous study [8] showed that an age-dependent increase in blood pressure was associated with a progressive increase in the aortic MG and MG-induced AGE levels in SHR. More importantly, chronic treatment with aminoguanidine, a non-specific inhibitor of AGEs formation, significantly reduced MG and MG-induced AGEs in the aorta and attenuated the blood pressure increase in SHR [10]. The SHR is a genetic model of hypertension with multiple pathogenetic factors which have not been clearly defined. To define the role of MG and MG-induced AGEs more clearly in the pathogenesis of hypertension we

used fructose, a precursor of MG formation [15], to treat SD rats for 4 months and follow changes in blood pressure, MG, AGEs and other parameters.

During the last two decades, the consumption of fructose has increased considerably. Excessive fructose consumption has been shown to cause obesity, diabetes mellitus and hypertension [16]. Endogenous fructose is metabolized by hexokinase or ketohexokinase to dihydroxyacetone phosphate (DHAP) and glyceraldehydes-3-phosphate (G3P), both of which can be further metabolized in the glycolytic pathway. Although the circulation level of fructose is lower than that of glucose, fructose is much more reactive in glycating proteins. It is reported that fructose was 7.5 times more reactive than glucose in causing glycation of hemoglobin, and 10 times more reactive in the rate of protein cross-linking [17, 18]. Fructose feeding did not impair the glucose tolerance significantly but the serum insulin levels were elevated indicating development of insulin resistance. Metformin reversed insulin levels back to normal values and improved insulin-stimulated glucose uptake in fructose treated rats.

As mentioned earlier, the main source for MG formation is anaerobic glycolysis. Both DHAP and G3P can be converted to MG. Increased intracellular level of fructose, which gives rise to G3P and DHAP levels, increases intracellular MG concentration. In vascular smooth muscle cells, formation of MG induced by fructose occurred in a concentration and time dependent manner [2]. Our results showed that serum and aortic MG levels were significantly increased after chronic treatment with fructose.

Metformin is widely used as an oral antihyperglycaemic agent for the management

of type 2 diabetes mellitus. It is a guanidine compound with some structural similarity to aminoguanidine, one of the best characterized and commonly used AGEs inhibitor [19], which suggests that metformin may also inhibit the glycation processes. Recently, a number of studies [20, 21] have shown that metformin is an anti-glycation agent. It was found that metformin can reduce tissue MG levels in diabetic patients [22]. Tanaka et al. [21] showed that chronic treatment with metformin can reduce AGE levels in lens, kidney and nerves in diabetic animals. Several mechanisms by which metformin can inhibit glycation processes have been proposed. One mechanism suggests that metformin traps reactive carbonyl species like MG and glyoxal. An *in vitro* study [20] showed that metformin directly reacts with MG to form stable triazepinone derivatives. Another mechanism proposes that metformin reduces MG level via enhancement of MG detoxification through the glyoxalase pathway [22]. Metformin may also react with post-Amadori products [23]. Our present study demonstrated that metformin significantly reduced aortic and serum MG levels and dramatically decreased MG-induced AGEs in vascular tissues.

Our present study showed that the increase of blood pressure induced by chronic administration of fructose was attenuated by metformin, which implies that fructose induces blood pressure elevation partially via the enhanced formation of MG and MG-induced AGEs. Increased MG and MG-induced AGEs in fructose-fed rats can cause the increase of blood pressure mainly by two mechanisms. One is oxidative stress and the other is increased vascular contractility. From literature, a number of studies [24-26]

have shown that there is a synergistic relationship between MG, AGEs and oxidative stress. Oxidative stress is one of the pathogenic factors in the development of hypertension in experimental models [27]. Our previous study showed that MG increased the formation of superoxide, H_2O_2 and peroxynitrite in rat aortic SMCs [13]. As shown in figure 4, administration of metformin significantly attenuated the increase in H_2O_2 in fructose-fed SD rats. Moreover, the reduced serum level of the antioxidant GSH in fructose-fed rats was significantly increased after cotreatment with metformin. Regarding increased vascular contractility, a number of studies [28, 29] have shown that the endothelium-dependent vascular relaxation is impaired in fructose-fed rats while the mechanisms are not clear. In the present study, we found not only increased formation of MG-induced AGE, CEL, and decreased level of eNOS in the aorta, but also increased CEL and CML, another AGE product of MG, in the mesenteric arteries where they were mainly localized in the endothelium (Fig. 6). These changes possibly cause endothelial dysfunction observed in fructose-fed rats. Our results showed that chronic fructose treatment causes a eutrophic inward remodeling in mesenteric arteries from SD rats, as evidenced by decreased lumen diameter and increased media/lumen ratio without changes of cross-sectional area. This remodeling was restored by metformin which implies that this vascular morphological damage was likely caused by MG-induced AGEs. Moreover, it was reported that AGEs can not only reduce eNOS activity [30] but also deactivate this enzyme [31].

In summary, our present study provides further evidence for MG as one of the

causative factors in the pathogenesis of hypertension. The study shows that chronic fructose treatment in normotensive SD rats significantly enhanced MG and MG-induced AGEs formation. An overproduction of MG and MG-induced AGEs induced an elevation of blood pressure most likely by increasing oxidative stress and vascular contractility. Increasing consumption of fructose in the Western diet is a health concern.

Table 7-1. Biochemical parameters of serum from rats with different treatment.

Test	Control (n=7)	Fructose (n=10)	Fruc +Met (n=8)	Metformin (n=4)
Total cholesterol (mM)	0.85 ± 0.09	0.88 ± 0.07	0.77 ± 0.1	0.70 ± 0.07
Triglyceride (mM)	0.35 ± 0.04	0.84 ± 0.05*	0.72 ± 0.06	0.16 ±0.03*#
HDL-cholesterol (mM)	0.77 ± 0.07	0.66 ± 0.06	0.63 ± 0.04	0.61 ± 0.08
Hemoglobin A1C(mM)	4.3 ± 0.21	4.4 ± 0.07	4.41 ±0.16	4.6 ±0.16

Fruc: fructose, Met: metformin, *P<0.05 vs. control, $^{\#}$ P<0.05 vs. fructose-treated group.

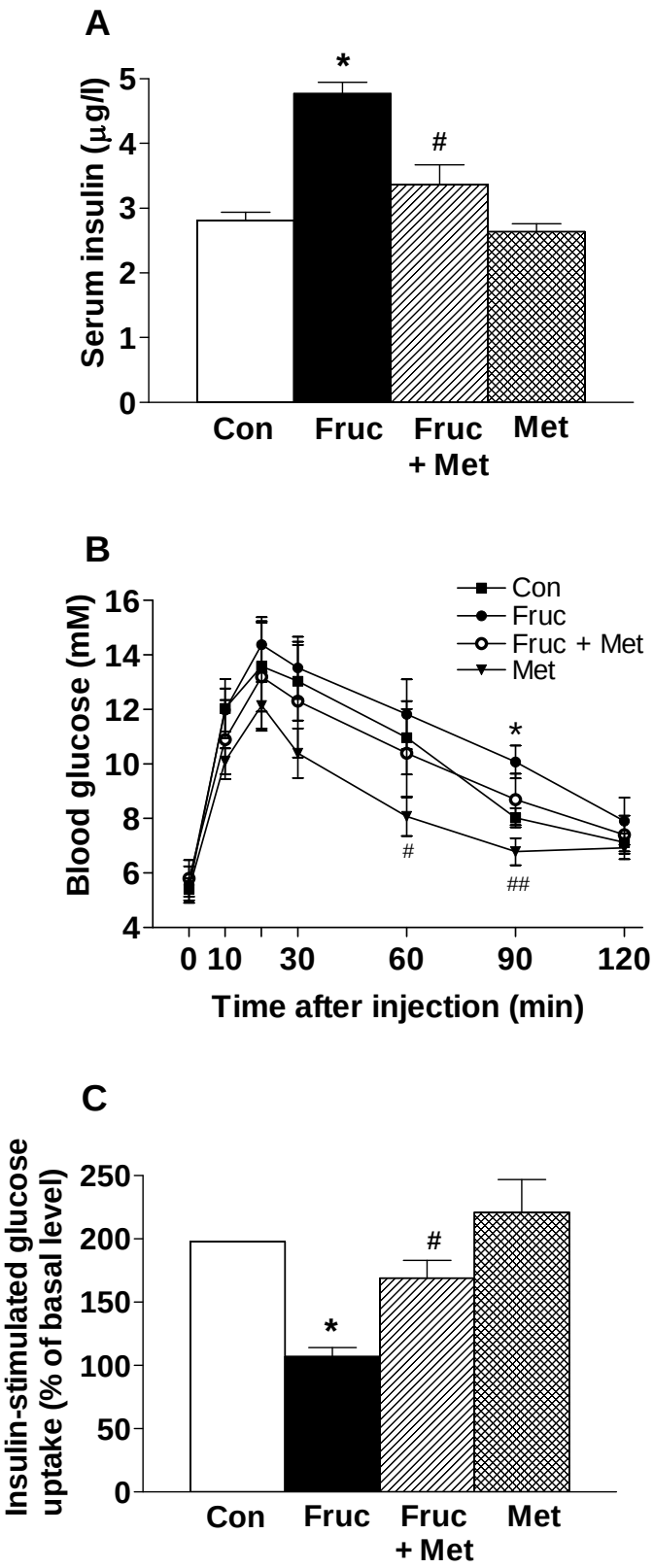
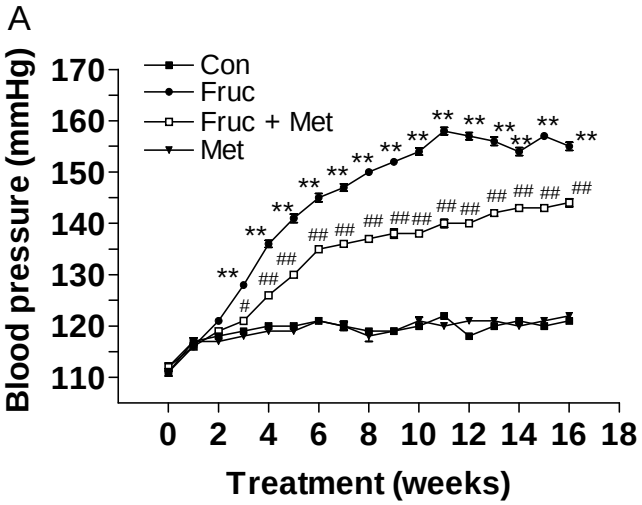
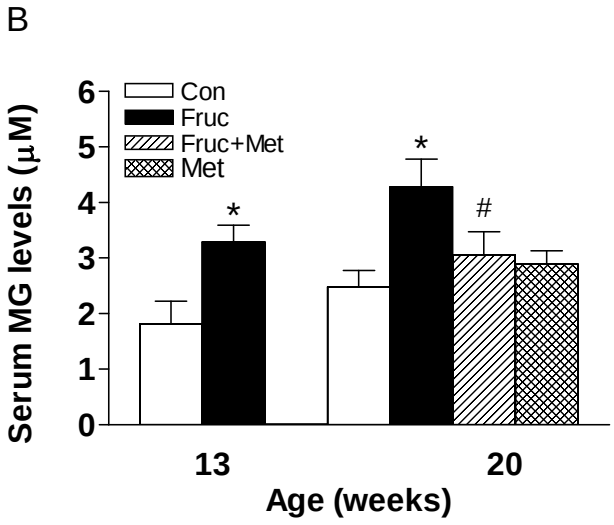

A
Serum insulin (μg/l)
0
1
2
3
4
5
*
#
Con
Fruc
Fruc + Met
Met
B
Blood glucose (mM)
4
6
8
10
12
14
16
Con
Fruc
Fruc + Met
Met
*
#
##
0
10
30
60
90
120
Time after injection (min)
C
Insulin-stimulated glucose uptake (% of basal level)
0
50
100
150
200
250
*
#
Con
Fruc
Fruc + Met
Met

Figure 7-1. Effect of fructose, methylglyoxal (MG) and metformin on the development of insulin resistance. **A.** Serum insulin levels in different treated rats. Blood samples from rats were collected after 9 weeks of treatment with fructose (n=10), fructose plus metformin (n=8), or metformin (n=4), respectively. **B.** Intraperitoneal glucose tolerance test. The blood glucose was determined using OneTouch® blood glucose monitoring system at 0, 10, 15, 30, 60, and 120 min after the glucose injection. C. Insulin-stimulated glucose uptake by adipose tissues from different treated rats. The glucose uptake in insulin-treated cells was presented as % of the basal glucose uptake level in untreated cells (n= 4-5 in each group). $^{*}P<0.05$ vs. control rats; $^{\#}P<0.05$ vs. fructose-treated rats.


A
Blood pressure (mmHg)
170
160
150
140
130
120
110
Con
Fruc
Fruc + Met
Met
0 2 4 6 8 10 12 14 16 18
Treatment (weeks)


B
Serum MG levels (μM)
6
5
4
3
2
1
0
Con
Fruc
Fruc+Met
Met
13
20
Age (weeks)

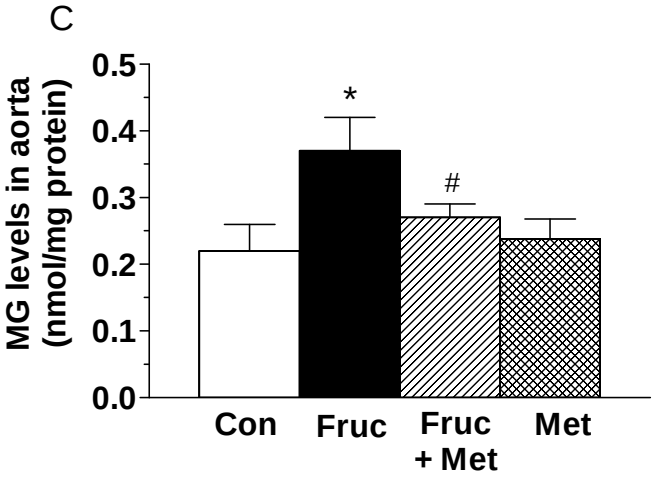

C
MG levels in aorta (nmol/mg protein)
0.5
0.4
0.3
0.2
0.1
0.0
Con
Fruc
Fruc + Met
Met

Figure 7-2. Effects of fructose on systolic blood pressure and methylglyoxal (MG) levels in SD rats. Systolic blood pressure was measured by tail-cuff noninvasive BP measurement system (**A**). MG levels were measured in the serum at 13 and 20 weeks of age (**B**). At the end of the study, MG levels were measured in the aorta (**C**) from SD rats with different treatments (n = 5-6). *P<0.05 vs. control rats; $^{\#}P$<0.05 vs. fructose-treated rats.

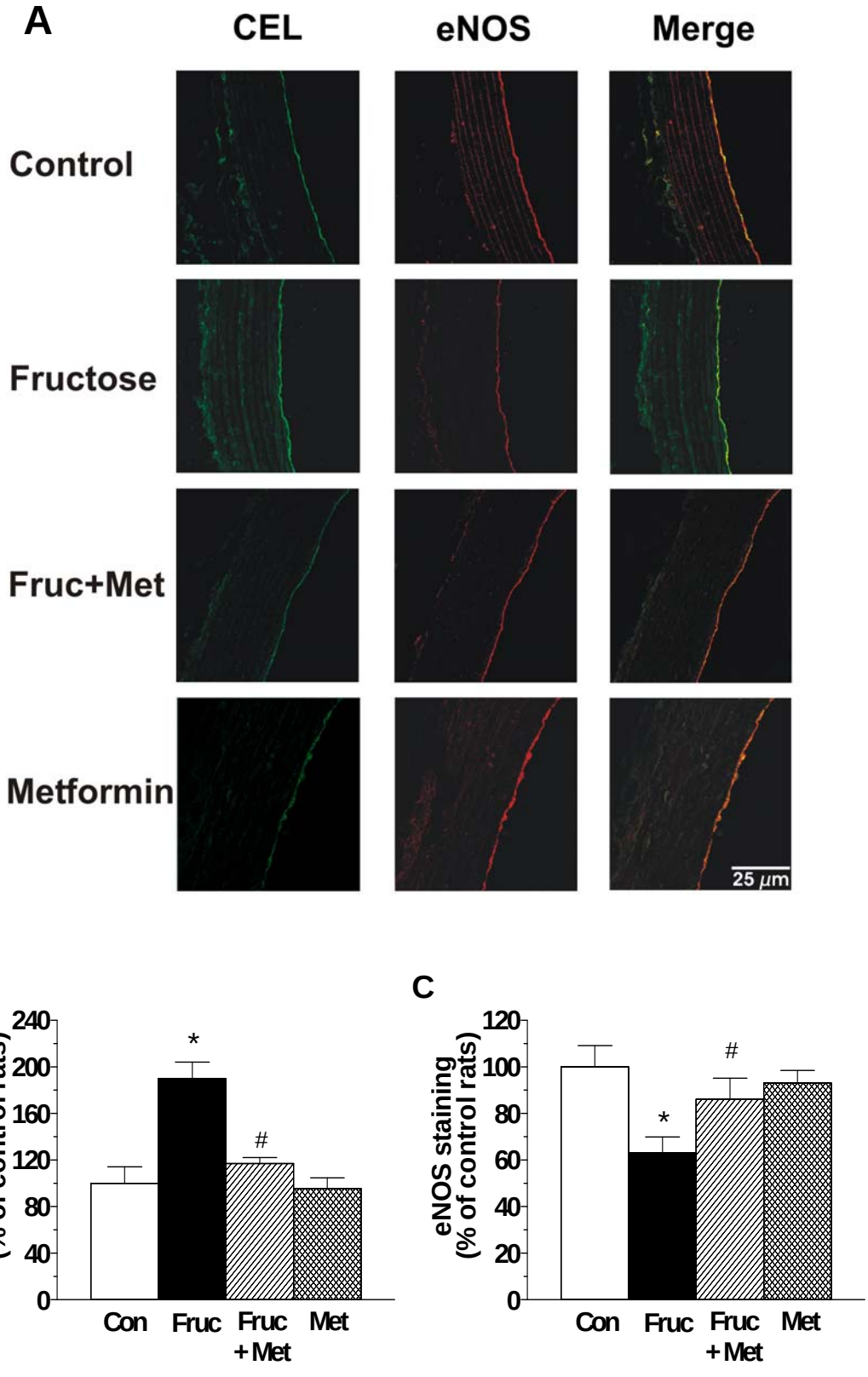

A
CEL
eNOS
Merge
Control
Fructose
Fruc+Met
Metformin
25 μm
B
CEL staining
(% of control rats)
240
200
160
120
80
40
0
*
#
Con
Fruc
Fruc
+Met
Met
C
eNOS staining
(% of control rats)
120
100
80
60
40
20
0
*
#
Con
Fruc
Fruc
+Met
Met

Figure 7-3. Immunohistochemical detection of CEL and eNOS in the aorta from SD rats. **A.** Double immunofluorescence staining was used to detect CEL (green color) and eNOS (red color). Both CEL and eNOS staining were mainly localized in the endothelium of aorta. Intensity of CEL (**B**) and eNOS (**C**) staining was quantified using GeneTools image analysis software (n = 3-5 in each group). *P<0.05 vs. control rats; $^{\#}P$<0.05 vs. fructose-treated rats.

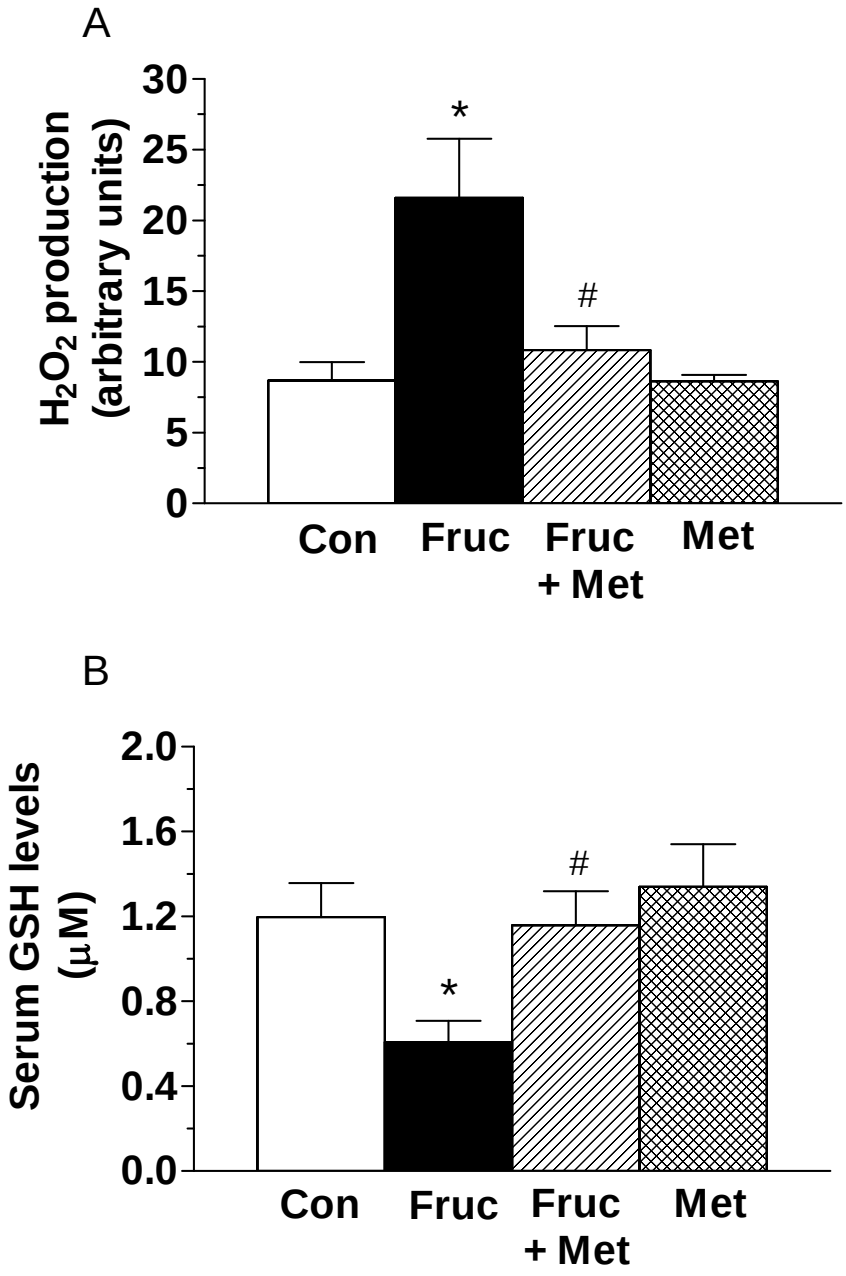


Figure 7-4. Oxidative stress levels in SD rats with different treatments. Hydrogen peroxide formation was measured in the aorta at the end of the study (**A**). Hydrogen peroxide (H_2O_2) production was determined by a DCFH-assay. Reduced glutathione (GSH) level was detected in the serum by HPLC method (**B**). *$P < 0.05$, *vs*. control rats. #$P < 0.05$, *vs*. fructose-treated rats.

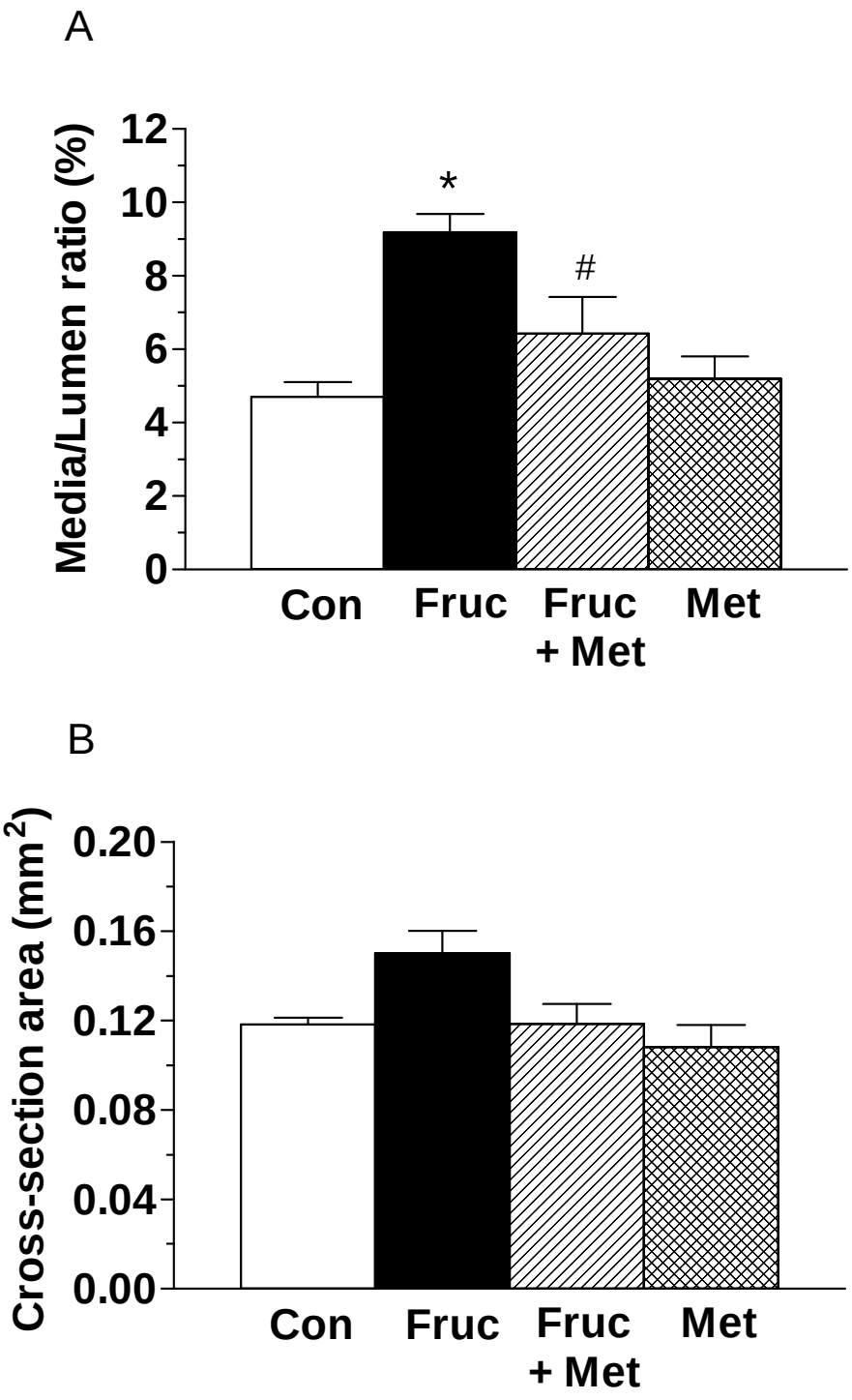


Figure 7-5. Morphological characteristics of the mesenteric arteries of rats. *P < 0.05, *vs*. control rats. #P < 0.05, *vs*. fructose-treated rats.

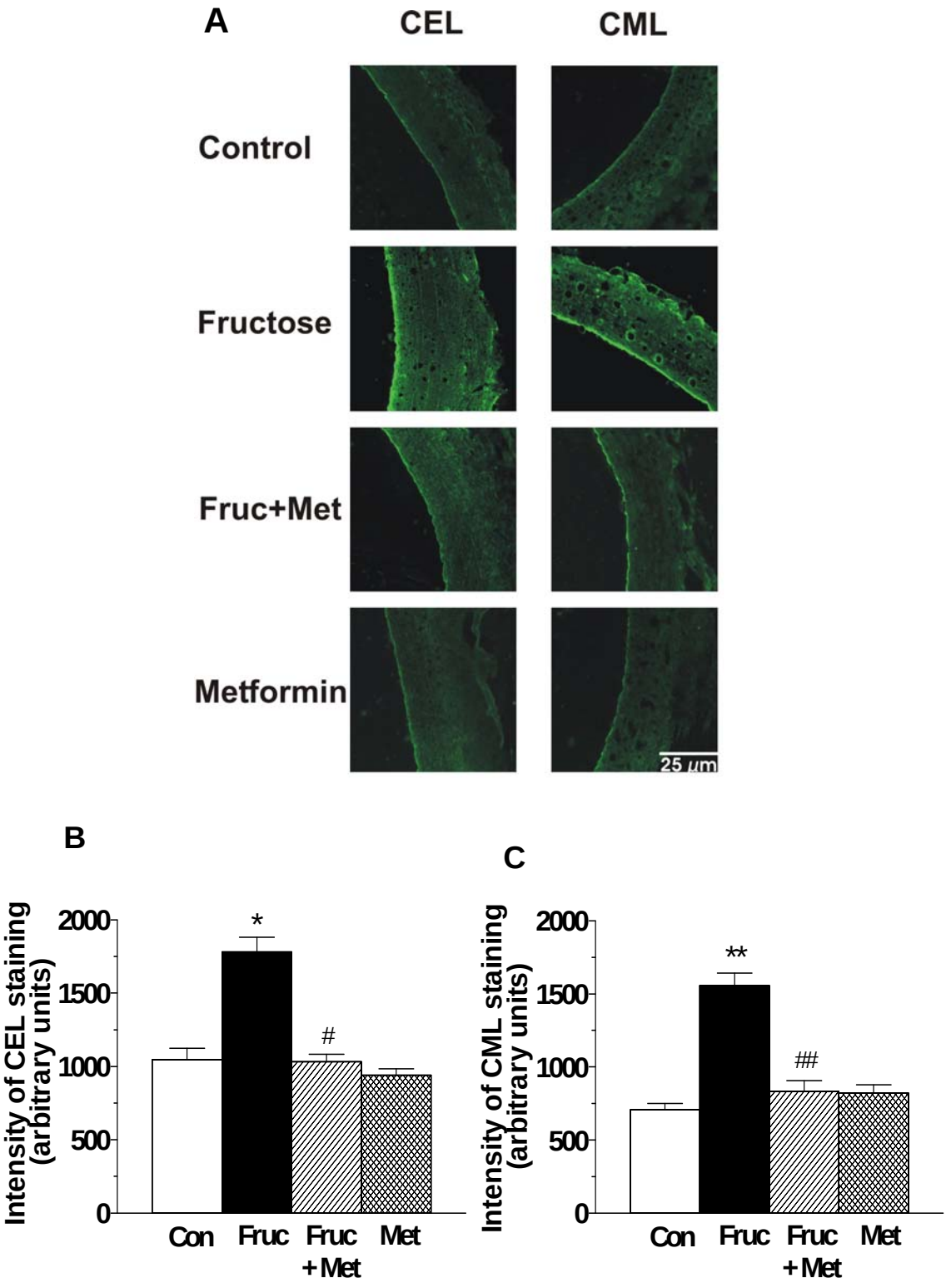

A
CEL
CML
Control
Fructose
Fruc+Met
Metformin
25 μm
B
Intensity of CEL staining (arbitrary units)
2000
1500
1000
500
0
*
#
Con
Fruc
Fruc +Met
Met
C
Intensity of CML staining (arbitrary units)
2000
1500
1000
500
0
**
##
Con
Fruc
Fruc +Met
Met

Figure 7-6. Immunohistochemical detection of AGEs in mesenteric arteries. CEL and CML staining was detected in the mesenteric arteries from control and treated SD rats (**A**). Scale bar: 25μm. Intensity of CEL (**B**) and CML (**C**) staining was quantified using GeneTools image analysis software (n = 3-5 in each group). *$P < 0.05$, *vs*. control rats. $^{\#}P < 0.05$, *vs*. fructose-treated rats.

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CHAPTER 8

DISCUSSION AND CONCLUSIONS

General discussion

Since the discussions related to specific results have been given in chapter 3-7, the general association of the results, therefore, is only discussed in this chapter.

Elevated MG and AGEs are commonly associated with hyperglycemic conditions such as diabetes mellitus. The role of MG and AGEs in hypertension has not been investigated widely. Our present studies show a strong association of MG and its AGE products, CML and CEL, with hypertension in SHR. The blood pressure of SHR was not different from that of WKY rats at 5 wks of age. From 8 wks onwards, it was significantly elevated compared to age-matched WKY rats. MG levels were increased in plasma and aorta, but not in kidney or heart, in SHR at an early age of 8 weeks. Importantly, the blood pressure increase was associated with a progressive increase of MG levels in plasma and aorta of SHR. MG level in SHR kidney was also significantly higher compared to WKY rats from 13 wks onwards. Moreover, the levels of MG-induced AGEs, CML and CEL, in the aorta and kidney were significantly higher in SHR than in WKY from 8 wks onwards, in parallel with the increased blood pressure in SHR. These changes in MG and AGEs in SHR are clearly not due to increased glucose metabolism since the latter shows no hyperglycemia (Natalucci et al. 2000; Wang et al. 2005). Our studies suggest that in addition to diabetes/hyperglycemic or hyperlipidemic conditions (Alderson et al. 2003), MG may be one of the causative factors for the development of hypertension or its complications in this non-diabetic model.

To determine whether decreased MG and MG-induced AGEs could prevent vascular damage/remodeling and the development of hypertension, we chronically treated young SHR at the age of 5 weeks with aminoguanidine, an inhibitor of AGE formation. We found that administration of aminoguanidine to male SHR from 5 to 14 wks significantly reduced plasma and aortic MG levels, decreased the levels of MG-induced AGEs (CEL and argpyrimidine) in the aorta, and prevented the morphologic damage in mesenteric artery, thus leading to a significant attenuation of blood pressure increase. Therefore, we provide evidence, for the first time, for a role of MG and MG-induced AGEs in the development of hypertension in SHR.

SHR is a genetic hypertension model with multiple pathogenetic factors which have not been clearly defined. To further determine the role of MG and MG-induced AGEs in the pathogenesis of hypertension, we used fructose, a precursor of MG (Thornalley 2002), to treat normotensive SD rats for 4 months. Chronic administration of fructose to SD rats significantly increased serum and aortic MG levels, enhanced MG-induced AGE formation in the aorta and the mesenteric artery, and caused morphological damage in the arterial wall, leading to a significant increase in blood pressure. Metformin cotreatment attenuated the increase of blood pressure in fructose-fed SD rats by scavenging MG and AGEs. This finding further confirmed that the increased level of MG may be one of the causative factors for the development of hypertension.

Possible mechanisms for MG-induced hypertension

Our present studies demonstrated, for the first time, that MG plays a very important role in the development of hypertension. The following mechanisms by which MG induces blood pressure increase are proposed.

1. MG and oxidative stress

Oxidative stress is a situation of imbalance between the production of reactive oxygen species (ROS) and antioxidant defense mechanisms. ROS are highly reactive byproducts of various metabolic processes and they are associated with cumulative damage and many disease conditions (Davies 1995; Finkel and Holbrook 2000). The primary sources of ROS include the mitochondrial electron transport chain and oxidase enzymes such as NADPH oxidase and xanthine oxidase. Vascular ROS are produced in endothelial, adventitial, and VSMCs. Under physiological condition, the generation of ROS is proportional to the rate of cellular metabolism and ROS function as signaling molecules (Finkel and Holbrook 2000). However, over generation of ROS may lead to endothelial dysfunction, VSMC remodeling and changes in vascular contractility, which are important factors in vascular dysfunction and hypertension (Chang and Wu 2006; de Champlain et al. 2004).

MG has been shown to increase production of ROS. The reaction of MG with alanine produces three free radical species: the cross-linked methylglyoxal dialkylimine radical cation, the enediol radical anion of methylglyoxal and the superoxide radical

anion (Yim et al. 1995). MG induces hydrogen peroxide production in platelets (Leoncini and Poggi 1996). A recent study has shown that MG enhanced the production of hydrogen peroxide and superoxide anion in neutrophils through a process involving p38 MAPK-dependent exocytosis of intracellular storage granules (Ward and McLeish 2004). Our previous study (Wu and Juurlink 2002) showed that increased MG level in VSMCs from SHR was correlated with elevated oxidative stress which was demonstrated by an enhanced ability to oxidize DCFH and decreased GSH/GSSG ratio, compared with that from WKY rats. Our recent study (Chang et al. 2005) found that MG induced NO and O_2^- production in a concentration and time-dependent manner which was inhibited by *N*-acetyl-L-cysteine, superoxide dismutase and NAD(P)H oxidase inhibitor in rat VSMCs. In addition, oxidized DCF, reflecting H_2O_2 and $ONOO^-$ production, was also significantly increased by MG. Our present study showed that administration of aminoguanidine significantly reduced the generation of $O_2^{\cdot -}$, $ONOO^-$ and H_2O_2 in SHR. The GSH level in the aorta from aminoguanidine-treated SHR was significantly increased, as opposed to untreated SHR. In normotensive SD rats, administration of fructose significantly increased H_2O_2 production, which was reduced by metformin cotreatment.

In addition to directly increasing ROS production, MG can also increase oxidative stress by inducing AGE formation. In diabetic animals, oxidative stress is elevated in proportion to the accumulation of AGEs (Forbes et al. 2002). On one hand, AGEs increase ROS production by enhancing the expression and activity of NADPH oxidase

(Christ et al. 2002). On the other hand, AGEs are able to inactivate antioxidant enzymes such as glutathione reductase (Blakytny and Harding 1992; Morgan et al. 2002) and glutathione peroxidase (Paget et al. 1998). In addition, AGEs enhance ROS generation by binding to AGE receptors, therefore, inducing receptor-mediated production of ROS (Brownlee 2001). A recent study reported that MG modifies Cu, Zn-superoxide dismutase by covalent crosslinking, which leads to the loss of enzymatic activity and release of copper ions from the protein (Kang 2003). Aminoguanidine, an AGE inhibitor by scavenging MG, can increase the activities of catalase, gluthathione reductase and gluthathione peroxidase in insulin-dependent diabetic rats (Stoppa et al. 2006). The previous study from our laboratory demonstrates that MG decreases activities of glutathione peroxidase and reductase (Wu and Juurlink 2002). Our present study further confirms that increased MG level in aortic tissues is accompanied with decreased activities of these two antioxidant enzymes.

2. MG and vascular contractility

The impairment of vascular relaxation was observed in MG-treated rats (Berlanga et al. 2005). After rats were treated with MG for 7 wks, photoplethysmography study showed that there was completely no vasodilatory response to nitroglycerine, while in normal control rats, the nitroglycerine increased the response more than 2-fold of the basal level. MG can also decrease vasodilation via AGEs. Increased MG level gives rise to elevated formation of AGEs, contributing to vascular dysfunction. It has been

suggested that the formation of cross links increases the accumulation of vascular collagen, and reduces the elastic properties of the arterial wall, contributing to the development of hypertension (Dart and Kingwell 2001).

Large amount of evidence from clinical and animal studies indicates that increased vascular contractility or impaired endothelial-dependent vascular relaxation is associated with progressive elevation of blood pressure (Koller 2002; Oparil et al. 2003). NO is a primary vasodilator derived from the endothelium. Bucala R et al. (Bucala et al. 1991) first reported that acceleration of the advanced glycation process impaired endothelium-dependent relaxation by inactivating NO in diabetic rats. This observation was further confirmed by Sanchez-Ferrer's group. They (Angulo et al. 1996) reported that an impairment of endothelium-dependent vasoregulation correlated with the presence of nonenzymatic glycation of hemoglobin, and this effect was caused by interference with NO by means of superoxide anion production. We observed reduced acetylcholine-induced relaxation in mesenteric arteries of SHR was significantly restored after 9 wks treatment with aminoguanidine, which indicates that reduced endothelium-dependent relaxation in SHR is linked to the AGE formation.

AGEs impair endothelial-dependent vascular relaxation mainly by reducing the bioavilability and activity of NO. Several mechanisms by which AGEs block or reduce NO activity have been proposed. The first mechanism is that AGEs quench and inactivate NO via a rapid chemical reaction (Bucala et al. 1991). The second mechanism proposes that AGEs reduce not only eNOS protein expression but also the half-life of

eNOS mRNA through an increased rate of mRNA degradation (Rashid et al. 2004; Rojas et al. 2000). The third mechanism suggests that AGEs impair NO production by binding to endothelial AGE receptors, which causes a decrease in serine phosphorylation of eNOS, resulting in deactivation of this enzyme (Xu et al. 2003). In addition, other mechanisms may be also involved since it has been observed that AGEs decreased vasodilator PGI_2 production (Yamagishi et al. 1998) and increased vasoconstrictor endothelin-1 expression (Quehenberger et al. 2000) in endothelial cells.

Our data revealed most of the staining for MG-induced AGEs was localized in the endothelium of vessel wall. After aminoguanidine therapy, the MG-induced CEL and argpyrimidine in endothelium were normalized in comparation with that from age matched WKY rats. The expression of eNOS was also significantly recovered by aminoguanidine treatment. Moreover, the small mesenteric arteries from aminoguanidine-treated SHR showed restored endothelium-dependent relaxation to acetylcholine. A number of studies (Takagawa et al. 2001; Verma et al. 1996) have shown that the endothelium-dependent vascular relaxation is impaired in fructose-fed rats with unclear mechanisms. In the present study, we demonstrated an increase in MG and MG-induced CEL with a decreased level of eNOS in the aorta of fructose-fed rats. We also found increased AGEs (CEL and CML) in the mesenteric arteries, especially localized in the endothelium (Fig. 7-6). Therefore, these changes possibly cause endothelial dysfunction observed in fructose-fed rats.

3. MG and vascular remodeling

It is well established that essential hypertension is associated with structural changes in the resistance vessels, a process known as vascular remodeling (Baumbach and Heistad 1989). At the cellular level, vascular remodeling involves changes in VSMC growth, cell migration, inflammation and fibrosis. Vascular remodeling in hypertension involves two processes. One is hypertrophic remodeling, in which media cross section is increased and encroaches on the lumen, indicating the presence of growth (Dao et al. 2001; Zhou et al. 2005). The other one is eutrophic remodeling characterized by rearrangement of wall material around a reduced lumen without evidence of net growth (Intengan et al. 1999). Mulvany M (Mulvany 2002) suggests that essential hypertension is associated only with eutrophic remodeling in small arteries. Chronic aminoguanidine administration restored AGEs associated morphological changes in mesenteric arteries from SHR, as evidenced by increased lumen diameter and decreased media/lumen ratio without changes of cross-sectional area. These data indicate that AGEs may play an important role in eutrophic inward remodeling in SHR. Our results also showed that chronic fructose treatment caused a eutrophic inward remodeling in mesenteric arteries of SD rats. This vascular remodeling was restored by metformin, suggesting the vascular morphological change was likely caused by MG-induced AGEs. The mechanisms by which AGEs induce vascular remodeling are unclear. The possible mechanisms are the following; (1) Increased proliferation and fibronectin production of VSMCs (Sakata et al. 2000). (2) Promotion of endothelial cell adhesion and

transendothelial migration (Thomas et al. 2005). (3) Enhanced synthesis of extracellular matrix components (Sims et al. 1996). (4) Synthesis and release of cytokines, in particular IL-1β and TNF-α, and growth factors like insulin-like growth factor I (Kirstein et al. 1992), which are involved in SMC growth, migration and apoptosis.

4. MG and vascular stiffening

Systolic blood pressure increases with age mainly because of increased stiffness of the large conduit arteries. The stiffness of the arteries primarily involves two major structural proteins, collagen and elastin. Because collagen contains large amounts of lysine and arginine residues, it is very vulnerable to glycation. Once glycation occurs, collagen loses its normal elasticity, strength and flexibility in the vessel walls (Bailey 2001). It has been found that glycated collagen is directly increased by AGEs in a concentration- and time-dependent manner, both in the presence (Basta et al. 2004) or absence (Kim et al. 2001) of hyperglycemia. This increase in glycated collagen is associated with a thicker and less distensible vessel wall matrix (Kim et al. 2001). AGE-collagen crosslinking is accelerated in response to increased mechanical stress in hypertension (Bishop and Lindahl 1999). In addition to glycated collagen, elastin fibrils in the vascular wall matrix may be also vulnerable to glycation, therefore, further reduces arterial distensibility (Bailey 2001). Large artery stiffness was reversed by AGE breaker ALT-711 in diabetic rats (Wolffenbuttel et al. 1998). In old SHR, ALT-711 treatment not only reduced aortic mass, but also improved systolic pressure (Susic et al.

2004). A recent study (McNulty et al. 2007) reported that AGEs contribute to aortic stiffness independent of age and blood pressure in untreated hypertensive patients.

An increased AGE formation was found in the aorta of stroke-prone spontaneously hypertensive rats (Mizutani et al. 1999). Our results also showed that MG-induced AGEs, CEL and argpyrimidine levels in the aorta from SHR were significantly higher compared to age-matched WKY rats, which were reduced by AGE inhibitor aminoguanidine. Moreover, the CEL level was significantly increased in the aorta from SD rats after chronic fructose treatment. Increased AGE levels in aorta may contribute to the decreased aortic distensibility and mechanical strength in rats.

5. MG and inflammation

Inflammation is one of the causative factors in the pathogenesis of hypertension (Vaziri and Rodriguez-Iturbe 2006). AGEs trigger the inflammatory effectors like cytokines and chemokines by multiple intracellular signal transduction pathways, including MAP kinase, p21ras and NAD(P)H oxidase (Goldin et al. 2006). Activated macrophage can be induced by the interaction of AGEs with mononuclear phagocytes, manifested by the induction of insulin-like growth factor-1 and proinflammatory cytokines (Basta et al. 2004). AGE-RAGE interaction causes activation and translocation of NF-κB, leading to a transcriptional activation of many genes. Indeed, many of those genes are highly relevant to inflammation such as IL-1α, IL-6 and TNF-α.

It was found that MG treated rats had enhanced expression of TNF-α and IL-1β,

two pro-inflammatory cytokines, in granulation tissue cells (Berlanga et al. 2005). Our previous studies have shown that MG treatment activated NF-κB in cultured VSMCs from aorta (Wu and Juurlink 2002) and mesenteric artery (Wu 2005). MG increased activation of NF-κB, indicated by an enhanced nuclear localized NF-κB p65 (Wu and Juurlink 2002). Recently, we found that MG significantly increased the generation of cytokines such as TNF-α, IL-6 and IL-8, in neutrophils from non-diabetic subjects (Wang et al. 2007). Moreover, our present study showed increased expression of NF-κB and activated macrophages, together with enhanced AGEs, CML and CEL, in kidney of stroke-prone SHR.

6. MG and the kidney

The kidney plays an important role in blood pressure homeostasis (Cowley and Roman 1996) and possibly the pathogenesis of hypertension in SHR (Roman 1987) and other models of hypertension such as DOCA-salt sensitive hypertension (Hall et al. 1984). A number of enzymes including the nitric oxide synthase (NOS) isoforms, cytochrome P450s and various ion pumps are involved in the modulation of renin secretion and secretion/absorption of ions. An oxidative stress-induced alteration in the function of one or more of these enzymes/proteins can readily disturb the homeostatic mechanisms operating within the kidney and lead to increased blood pressure.

AGE-induced renal injury was demonstrated *in vivo* when diabetic mice were randomized to receive either regular diet or diet containing low AGEs. Mice on the

regular diet, but not those on the low AGE diet, developed typical diabetic nephropathy (Zheng et al. 2002). Immunohistochemical data showed an accumulation of AGEs in glomerular basement membrane, mesangium, podocytes, and tubular cells of kidney from diabetic rats. Ultrastructural studies have indicated that AGE peptides may be reabsorbed by the renal proximal tubular cells (Bendayan 1998; Gugliucci and Bendayan 1995). AGE deposition can lead to glomerulosclerosis, which is independent of diabetes (Vlassara et al. 1994). Our immunohistochemical studies revealed positive staining for CML and CEL in the renal tubules and the glomerular vessels of SHR compared to WKY rats from 8 wks onwards. This suggests that an increased MG level and MG-glycated proteins in kidney may induce local micro-vascular and tubular damage, consequently impairing renal function, thus contributing to the development of hypertension in SHR.

7. MG and renin-angiotensin system

Renin-angiotensin-aldosterone is primarily responsible for the long-term control of blood pressure. Angiotensin II (Ang II) increases blood pressure by various mechanisms, including stimulating aldosterone synthesis and release, enhancing renal tubular sodium reabsorption, constricting resistance vessels, and stimulating antidiuretic hormone release. AGEs have been shown to interact with the renin-angiotensin system. It was observed that AGEs increased Ang II production in a time- and concentration-dependent manner in rat mesangial cells (Fukami et al. 2004). Inhibition of angiotensin converting enzyme (ACE) also attenuates the formation and accumulation of AGEs in experimental

diabetes. ACE inhibitor captopril was found to reverse AGE-induced collagen production, probably by attenuating RAGE expression and JAK2-STAT1/STAT3 activities (Huang et al. 2001). Ramipril, another ACE inhibitor, was reported to reduce renal and serum AGEs in STZ-induced type 1 diabetic animals (Forbes et al. 2002). Even though further studies are needed, it is possible that AGEs increase blood pressure partially by stimulating renin-Ang II system.

CONCLUSIONS

Our present studies show a strong association of MG and its AGE products with hypertension in SHR. The blood pressure of SHR was not different from that of WKY rats at 5 wks of age. From 8 wks onwards, blood pressure was significantly elevated compared to age-matched WKY rats. Importantly, this increase in blood pressure was associated with an elevated MG levels in plasma and aorta of SHR in an age-dependent fashion compared to age-matched WKY rats. Moreover, immunohistochemistry revealed more intense staining of MG-induced AGEs (CML and CEL), in the aorta from SHR compared to WKY rats from 8 wks onwards, in parallel with blood pressure increase in SHR. These changes in MG and AGEs in SHR are not due to increased glucose metabolism since the glucose levels and insulin sensitivity in SHR and WKY rats are comparable. In addition, we observed that both male and female stroke-prone SHR at the age of 24 weeks had more positive CEL and CML staining in kidney compared to age-matched male and female SD rats. Our data suggest that in addition to

hyperglycemic or hyperlipidemic conditions, the accumulation of MG and MG-induced AGEs in blood vessel walls plays an important role in the development of hypertension or its complications in SHR.

We also observed that the blood pressure increase in SHR was attenuated and the mesenteric artery remodeling was reversed after inhibiting MG and its AGE formation by aminoguanidine. Moreover, the blood pressure of normotensive SD rats was increased, along with vascular remodeling, after chronic treatment with fructose to increase endogenous MG and its related AGE generation.

In conclusion, our studies strongly indicate that increased MG and AGE formation may be a causative factor in the development of hypertension. MG and MG-induced AGEs may increase blood pressure by modulating the redox state, inducing endothelial dysfunction, and causing vascular remodeling and arterial stiffening.

SIGNIFICANCE OF THE STUDY

Despite some prevention and treatment successes over the past twenty-five years, the largest mortality rate in Canada is still linked to cardiovascular diseases. According to the latest statistics, 37% of all deaths are due to cardiovascular diseases. The annual economic burden of hypertensive conditions in Canada has been estimated at $20 billion. Each year, 80,000 Canadians die from hypertension and related cardiovascular diseases. Moreover the situation appears to be worsening with earlier onsets of hypertensive states

and rising incidences.

The importance of MG in regulating cellular function has only been recognized in diabetes and a few other rare diseases with abnormal glucose metabolisms. Clarifying the role of MG in the development of essential hypertension has fundamental significance and may lead to the discoveries of new mechanism and methods for the management and prevention of hypertension as well as its complications. The derived novel discoveries can be directly transformed to pharmaceutical anti-hypertension drugs and to new diagnostic method and standard for early detection and follow-up the progression/regression of hypertension.

FUTURE DIRECTIONS

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

1. To further investigate the mechanism for increased MG production in SHR. The activities and expression levels (mRNA and protein) of different enzymes that are involved in MG generation and catabolism, such as glyoxalase I and II, will be determined and quantified using Real-Time PCR and Western blot analysis.

2. To investigate the mechanisms for MG-induced changes in vascular functions, including enhanced vascular contractility or reduced endothelium-dependent relaxation. These mechanisms include altered calcium levels and ion channel functions, and altered signal transduction pathways such as cAMP and cGMP.

3. To examine the effect of MG on vascular remodeling. We will test whether MG can directly induce vascular smooth muscle cell proliferation. The mRNA (Real time RT-PCR) and protein (Western blot) levels of mitogen-activated protein kinases (MAPK, including ERK1/2, p38, JNK), activator protein-1 and NF-κB in vascular tissues will be investigated for their involvement in cell proliferation process.

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