METHYLGLYOXAL-INDUCED INCREASE IN PEROXYNITRITE AND INFLAMMATION RELATED TO DIABETES

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ABSTRACT

Methylglyoxal (MG) is a reactive α-oxoaldehyde and a glucose metabolite. Previous studies in our laboratory have shown that MG induces the production of reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot -}$), nitric oxide (NO) and peroxynitrite ($ONOO^-$), in vascular smooth muscle cells (VSMCs, A-10 cells). However, the effect of endogenous MG and mechanisms of MG-induced oxidative stress have not been thoroughly explored. The present study investigated fructose (a precursor of MG)-induced $ONOO^-$ formation in A-10 cells and whether this process was mediated via endogenous MG formation; roles of MG in regulating mitochondrial ROS (mtROS) production and mitochondrial functions in A-10 cells; and effect of MG on neutrophils in patients with type 2 diabetes mellitus (T2DM). Fructose induced intracellular production of MG in a concentration- and time- dependent manner. A significant increase in the production of NO, $O_2^{\cdot -}$, and $ONOO^-$ was observed in the cells exposed to fructose or MG. Fructose- or MG-induced $ONOO^-$ generation was significantly inhibited by MG scavengers and by $O_2^{\cdot -}$ or NO inhibitors. The data showed that fructose treatment increased the formation of $ONOO^-$ via increased NO and $O_2^{\cdot -}$ production in A-10 cells, and this effect was directly mediated by an elevated intracellular concentration of MG. By inhibiting complex III and manganese superoxide dismutase activities, MG induced mitochondrial overproduction of $O_2^{\cdot -}$, and mitochondrial $ONOO^-$ further. MG also reduced mitochondrial ATP synthesis, indicating the dysfunction of mitochondria. In addition, MG increased plasma NO levels in patients with T2DM, which reflected the oxidative status in those patients. MG-induced oxidative stress in patients with T2DM significantly enhanced levels of cytokines released from neutrophils. Moreover, the

neutrophils from T2DM patients showed a greater proclivity for apoptosis, which was further increased by *in vitro* MG treatment. Our data demonstrate that MG-induced oxidative damage, particularly $ONOO^-$ production, contributes to the pathogenesis of T2DM and its vascular complications.

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DEDICATION

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

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AGEs	Advanced glycation endproducts
AMO	Acetol monooxygenase
ANOVA	One-way analysis of variance
CEL	N-ε-carboxyethyl-lysine
CML	N-ε-carboxymethyl-lysine
CVD	Cardiovascualr disease
DCF	2,7-dichlorofluoresein
DCPIP	2,6-dichlorophenolindophenol
DHAP	Dihydroxyacetone phosphate
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DPI	Diphenylene iodonium
ETC	Electron transport chain
F-1-P	Fructose-1-phosphate
F-1, 6-P	Fructose- 1, 6-biphosphate
G-3-P	Glyceraldehyde-3-phosphate
G-6-P	Glucose-6-phosphate
GLUT	Glucose transporter
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione

GSSG	Oxidized glutathione
HbA1c	Glycated hemoglobin A1c
HDL	High density lipoprotein
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
IL-6	Interleukin-6
IL-8	Interleukin-8
LDL	Low density lipoprotein
L-NAME	N (G)-nitro-L- arginine methyl ester
MAPK	Mitogen-activated protein kinase
MG	Methylglyoxal
MnSOD	Manganese superoxide dismutase
2-MQ	2-methylquinoxaline
5-MQ	5-methylquinoxaline
mtNOS	Mitochondrial nitric oxide synthase
mtROS	Mitochondrial reactive oxygen species
NAC	*N*-acetyl-L-cysteine
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\cdot-}$	Superoxide anions
OGTT	Oral glucose tolerance test
$ONOO^-$	Peroxynitrite

o-PD	*o*-phenylenediamine
PBS	Phosphate buffered saline
PCA	Perchloric acid
PKC	Protein Kinase C
ROS	Reactive oxygen species
SMCs	Smooth muscle cells
SOD	Superoxide dismutase
SSAO	Semicarbazide-sensitive amine oxidase
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TNF-α	Tumor necrosis factor alpha
TTFA	Thenoyltrifluoroacetone
VCAM-1	Vascular cell adhesion molecule-1
VSMCs	Vascular smooth muscle cells
UACRs	Urine albumin / creatinine ratios
UKPDS	United Kingdom Prospective Diabetes Study

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Methylglyoxal (MG), a metabolite of sugar, is a highly reactive dicarbonyl molecule. It is formed mainly from the spontaneous transformation of triose phosphates. Therefore, MG is an intrinsic component of the glycolytic pathway in mammalian cells, including vascular smooth muscle cells (VSMCs) (Ekblom, 1998). An increased MG formation may occur because of an increased availability of precursors such as increased plasma glucose or administration of ethanol or threonine (Thornalley, 1988). On the other hand, MG is detoxified by the glyoxalase system that highly relies on the cellular level of reduced glutathione (GSH). Reduced availability of GSH also contributes to the increased levels of MG. Our previous study showed that MG induced a time- and concentration- dependent increase of oxidized 2,7-dichlorofluoresein (DCF) fluorescence intensity, which indicates the formation of peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2), in VSMCs (Chang, et al., 2005). Administration of MG for 3 hours also increased the production of $ONOO^-$ in cultured mesenteric artery smooth muscle cells from Sprague-Dawley (SD) rats (Wu, 2005). Numerous studies showed that levels of MG were elevated in patients with type 2 diabetes mellitus (T2DM) (Beisswenger, et al., 1999). MG levels were correlated with the glycated hemoglobin (HbA1c) (Thornalley, et al., 1989) and reflected glycemic fluctuation (Nemet, et al., 2005) in diabetic patients. In addition, increased $ONOO^-$ formation was observed in patients with T2DM (El-Remessy, et al., 2003b). $ONOO^-$ damages DNA, lipids and proteins, contributing to the development of T2DM and its complications (Rosen, et al., 2001). To date, the effect of endogenous MG on $ONOO^-$ production, the underlying mechanism and the role of MG-induced oxidative stress in T2DM have been unclear.

1. Methylglyoxal (MG)

Methylglyoxal (MG) is a reactive α-oxoaldehyde and a metabolite of glucose. It is a small molecule with molecular weight of 72. As shown in figure 1-1, MG has a ketone group and an aldehyde moiety. The aldehyde group is more reactive than the ketone. MG is a yellow liquid with characteristic pungent odor. It has 3 forms in aqueous solution: unhydrated (1%), monohydrate (71%) and dehydrate (28%), which are in rapid equilibrium (Rae, et al., 1990).

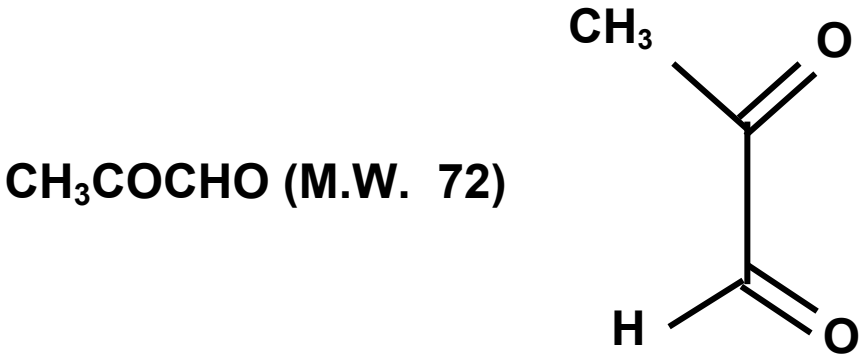

Figure 1-1. Structure of MG

1.1 Formation of MG

1.1.1 Endogenous MG formation

MG is produced during the metabolism of carbohydrates, lipids and proteins. Several enzymatic or non-enzymatic pathways are involved in the endogenous formation of MG (Figure 1-2).

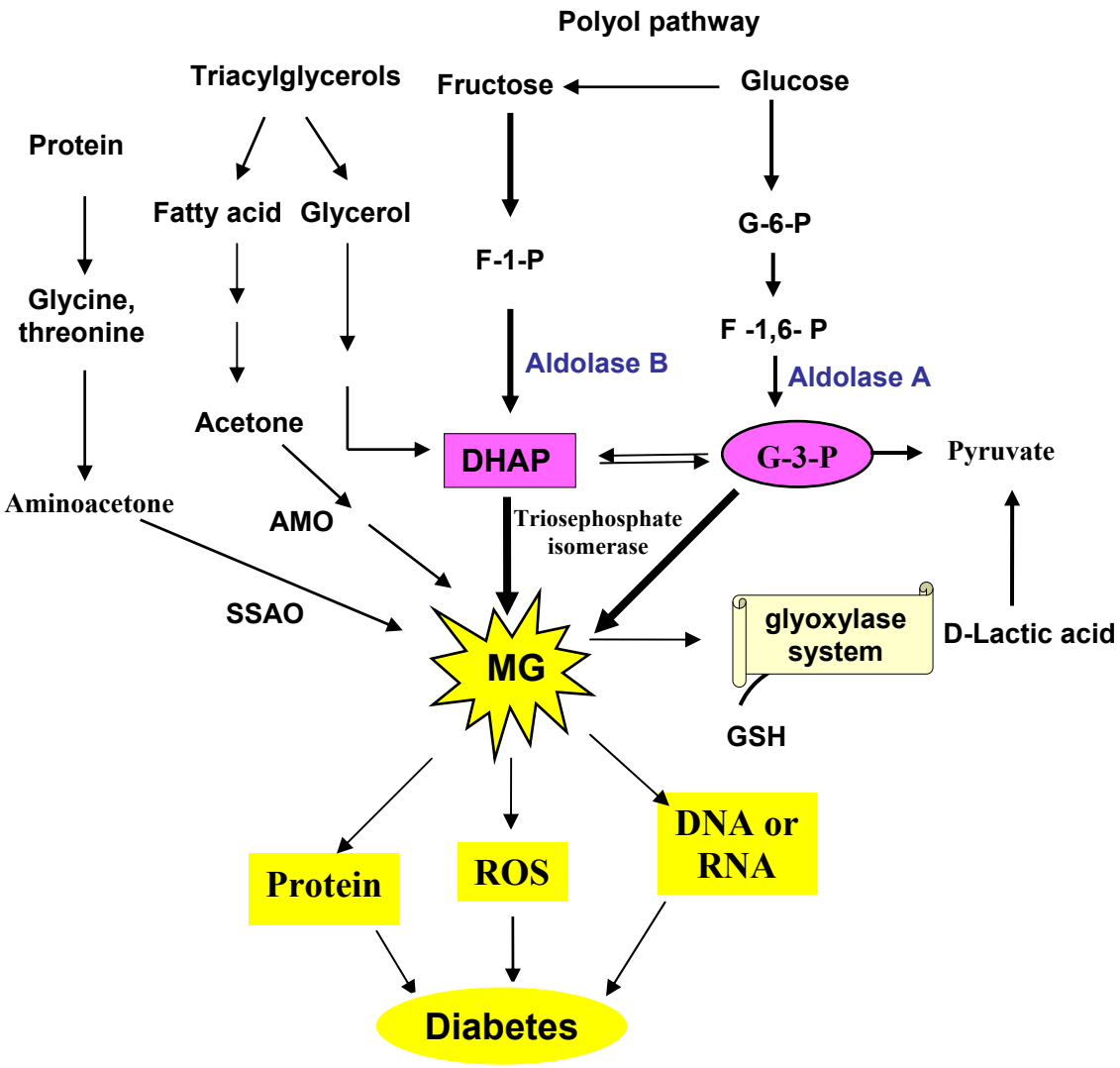

Figure 1-2. Formation, metabolism and toxicity of MG

AMO: Acetol monooxygenase
DHAP: Dihydroxyacetone phosphate
F-1-P: Fructose-1-phosphate
F-1, 6-P: Fructose 1, 6-biphosphate
G-6-P: Glucose-6-phosphate
G-3-P: Glyceraldehyde-3-phosphate
GSH: Reduced glutathione
MG: Methylglyoxal
ROS: Reactive oxygen species
SSAO: Semicarbadize-sensitive amine oxidase

1.1.1.1 Major pathways

Endogenous MG is formed from metabolic intermediates of carbohydrates, proteins and fatty acids (Figure 1-2). The majority of MG is derived from the metabolites of carbohydrate, such as glucose and fructose.

MG is formed during glycolysis. Glucose is phosphorylated by glucokinase to form glucose-6-phosphate (G-6-P). This reaction decreases the intracellular glucose levels and promotes continuous transportation of glucose into the cell through the glucose transporter on the cell membrane. G-6-P is then converted to fructose-6-phosphate (F-6-P) via glucose phosphate isomerase. This step is reversible but easily driven to F-6-P due to the lower levels of F-6-P. Subsequently, fructose-1, 6- biphosphate (F-1, 6-P) is irreversibly formed from F-6-P / G-6-P due to catalysis by phosphofructokinase-1 (PFK-1). This reaction is the key point in the glycolytic process. F-1, 6-P, then, is split by aldolase into two triose sugars, dihydroxyacetone phosphate (DHAP), a ketone, and glyceraldehyde-3-phosphate (G-3-P), an aldehyde. DHAP and G-3-P can spontaneously convert to MG (Phillips and Thornalley, 1993).

MG is mainly formed nonenzymatically from DHAP and G-3-P, and the non-enzymatic formation of MG occurs in all cells and organisms. For example, MG formation in human red blood cells *in vitro* under normal glycemic conditions is due to nonenzymatic fragmentation of triosephosphates (Phillips and Thornalley, 1993). Indeed, the instability of G-3-P at physiological pH was observed in 1969 (Mel'nichenko, et al., 1969). Two products, inorganic phosphate and MG, were found from non-enzymatic G-3-P reaction in the presence of lysine (Bonsignore, et al., 1973). Moreover, it was

observed that deprotonation of G-3-P or DHAP to an enediolate phosphate followed by cleavage of phosphate group from the carbon skeleton yielded to the formation of MG (Richard, 1993). The formation of MG in human red blood cells was increased by the addition of metabolites stimulating the flux of triosephosphates, like glucose, fructose, dihydroxyacetone and D-glyceraldehyde.

The formation of MG in early glycation was investigated by Thornalley *et al*. (Thornalley, et al., 1999). Glucose (50 mM) degraded slowly at 37 ºC to form MG throughout a period of 3 weeks. Therefore, a short period of hyperglycemia may be sufficient to induce MG formation *in vivo* (Thornalley, et al., 1999). Bovine retinal endothelial cells exposed to D-glucose (30 mM) for 7 days produced significantly higher levels of MG than cells cultured with L-glucose or control cells (Padayatti, et al., 2001). In addition, high glucose caused increased MG formation and MG modification of the corepressor mSin3A in mouse kidney endothelial cells. Consequently, MG-modified mSin3A mediated high glucose-induced expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in cells and high sensitivity of endothelial cells to tumour necrosis factor α (TNF-α). It was shown that glucose induced vascular inflammation and disease via the formation of MG (Yao, et al., 2006).

Fructose is also a monosaccharide and a component of sucrose. It is phosphorylated by fructokinase to form fructose-1-phosphate (F-1-P) and enters the process of glycolysis. Fructose and glucose share the same pathway to produce MG. Beverages and processed food are rich in fructose, and the consumption of fructose has dramatically increased in the past decades. Dietary fructose is closely linked to the pathophysiology of the metabolic syndrome, which includes insulin resistance,

hyperlipidemia, hypertension and obesity, although the mechanism is unclear (Miller and Adeli, 2008; Hallfrisch, et al., 1983). It was reported that fructose was more reactive than glucose to cause hemoglobin glycation and protein cross-links (McPherson, et al., 1988; Bunn and Higgins, 1981). Cytotoxicity of fructose on hepatocytes has been observed. Fructose (7.5 mM) caused a rapid 50% ATP depletion (5 min) in isolated hepatocytes. ATP depletion did not occur with glucose because fructokinase that catalyzes fructose to F-1-P is more reactive than glucokinase that catalyzes glucose to G-6-P. It is suggested that metabolites of fructose caused ATP depletion (Latta, et al., 2000). Fructose caused cell death at a high concentration of 1.5 M, but even 12 mM fructose caused death in 50% of hepatocytes in the presence of a non-cytotoxic dose of H_2O_2. Fructose / H_2O_2 cytotoxity was prevented by ROS scavengers and by the MG scavenger aminoguanidine. MG was proposed to be the most likely endogenous toxin generated from fructose (Lee, et al., 2009; Lee, et al., 1993).

Fructose as a precursor for MG has been investigated *in vivo* (Wang, et al., 2008). Increased serum and aortic levels of MG have been observed in fructose-fed rats (Wang, et al., 2008). MG induced structural remodeling in mesenteric artery and ROS production in aorta of fructose-fed rats. MG is also responsible for the high blood pressure and hypertriglyceridemia seen in those rats. In addition, MG impairs insulin signaling in adipose tissue of fructose-fed rats through decreasing insulin-induced insulin-receptor substrate-1 (IRS) tyrosine phosphorylation and reducing the activity of phosphatidylinositol (PI) 3-kinase (Jia and Wu, 2007). The endogenous MG formation from fructose in VSMCs is not yet reported.

MG can also be formed enzymatically from G-3-P and DHAP. Triosephosphate isomerase (EC.5.3.1.1) hydrolyses G-3-P and DHAP and removes phosphate to yield MG (Pompliano, et al., 1990). Triosephosphate isomerase is a very effective enzyme in the glycolytic pathway, and its cellular levels are very high (Albery and Knowles, 1976).

1.1.1.2 Minor pathways

MG is a byproduct of acetone metabolism. Acetone monooxygenase catalyzes acetone to acetol, and acetol monooxygenase (AMO) converts acetol to MG (Casazza, et al., 1984). The two enzymes belong to cytochrome 450, and the consequent steps need NADPH and O_2. This pathway is inducible. Its capacity can be induced by several agents (acetone, ethanol, pyrazole, imidazole, etc.) or under different physiological / pathological circumstances (e.g. in fasting or in diabetes) (Gonzalez, 1988).

In addition, formation of MG is also found during the metabolism of aminoacetone, which is a metabolite of proteins. Semicarbazide-sensitive amine oxidase (SSAO) is able to convert aminoacetone into MG (Lyles, 1996). SSAO is localized on the surface of endothelial cells, VSMCs and adipocytes. It exists in two forms, in a soluble form in plasma and in a tissue-bound form in the plasma membrane. Increased serum SSAO activities have been found in patients with diabetes and vascular disorders, and treatment with selective SSAO inhibitors reduced atherogenesis in diabetic mice fed a high-cholesterol diet (Yu, et al., 2003). Also, increased activity of AMO and SSAO in plasma may be responsible for the increased circulating MG levels in diabetic mice (Yu, et al., 2003). Furthermore, levels of MG and activities of SSAO were significantly

elevated in lenses from streptozotocin-induced diabetic rats (Hamada, et al., 2005). SSAO induced AGEs synthesis in aortic smooth muscle from diabetic rats by the mediation of MG (Mathys, et al., 2002).

1.1.2 MG in food and beverages

MG is formed in food and beverages during the processing, cooking and prolonged storage (Nemet, et al., 2006). MG can be formed from carbohydrates by fragmentation of the sugar moiety during retro-aldol condensation and auto-oxidation. In addition, the formation of MG was observed during the heating process of glucose, fructose, and maltose, where the amount of MG obtained from monosaccharides was markedly higher than that from disaccharides (Nemet, et al., 2006). Moreover, decomposition of different lipids, caused by storage and processing, can also affect the accumulation of MG in food. A broad range of MG levels was obtained during accelerated storage (60ºC for 3 and 7 days) or cooking (200ºC for 1 h) of oil, depending on oil origin (salmon, cod liver, soybean, olive, and corn oils) (Fujioka and Shibamoto, 2004). For instance, the formation of MG ranged from 2.03 ppm in cod liver oil to 2.89 ppm in tuna oil heated at 60 ºC for 7 days. However, olive oil is the only vegetable oil that yields MG under the accelerated storage conditions.

Coffee is a wildely consumed beverage. It is interesting to know whether MG is present in green and roasted coffee beans. The amount of MG is small in green coffee beans, but increases in the early phases of the roasting process and then declines. Thus, mild or medium toasted coffee beans have the highest amount of MG content (Daglia, et

al., 2007). It has been determined that 1 gram of coffee powder contains about 100 μg of MG, and MG owns the strongest mutagenicity in dicarboyls in coffee (Nagao, et al., 1986).

Besides food and beverages, drinking water can also be an exogenous source of MG. Ozonation and chlorination of natural water, the applied process in the treatment of drinking water, can lead to the formation of MG (Matsuda, et al., 1992a; Matsuda, et al., 1992b).

1.2 Detoxification of MG

Several pathways are involved in the metabolism of MG: glyoxalase enzyme system, aldose reductase, aldehyde dehydrogenase and 2-oxoaldehyde dehydrogenase (2-ODH).

1.2.1 Glyoxalase enzyme system

The glyoxalase enzyme system is the major pathway catalyzing the detoxification of MG (Figure 1-2). This metabolic system is present in the cytosol of all mammalian cells and most micro-organisms (Rhee, et al., 1987; Thornalley, 1990). Glyoxalase I and glyoxalase II are two major enzymes in this system. GSH is a cofactor. The nonenzymatic reaction between GSH and MG forms hemithioacetal. Glyoxalase I catalyzes hemithioacetal to S-D-lactoylglutathione. Glyoxalase II then hydrolyzes S-D-lactoylglutathione to D-lactate. MG detoxification is, therefore, strongly dependent on

the availability of GSH. Deficiency of GSH limits the production of hemithioacetal, leading to the accumulation of MG (Abordo, et al., 1999). The reactions catalyzed by the glyoxalase system are irreversible. Both glyoxalase I and glyoxalase II are metalloproteins and dependent on zinc in their active sites (Cameron, et al., 1999). Glyoxalase I inhibitor S-p-bromobenzylglutathione prevented the metabolism of exogenous MG in cell extracts (Phillips and Thornalley, 1993a).

1.2.2 Aldose reductase

Aldose reductase catalyzes the NADPH-dependent reduction of MG into lactaldehyde and then to propanediol in the presence of GSH (Nemet, et al., 2006; Vander Jagt, et al., 2001). However, at low concentrations of GSH, MG is converted to acetol, and acetol accumulates finally. Acetol has been reported to accumulate to millimolar levels in some diabetic patients (Reichard, et al., 1986). Acetol can also be converted back to MG either by oxidation mediated by CYP2E1 or by undergoing disproportionation in the presence of copper ions without the catalysis of any enzymes (Vander Jagt, et al., 2001).

In liver, where GSH levels are the highest and aldose reductase is almost absent, the glyoxalase system is the key metabolic system to detoxify MG (Vander Jagt, et al., 2001). In tissues with high levels of aldose reductase, such as eyes, nerves, kidneys and the vasculature, aldose reductase contributes to MG degradation (Kador and Kinoshita, 1985).

1.2.3 Aldehyde dehydrogenase

Aldehyde dehydrogenase catalyzes NAD-dependent oxidation of MG into pyruvate (Nemet, et al., 2006). The family of aldehyde dehydrogenase consists of 3 isoforms, names of which are dependent on the intracellular locations (Izaguirre, et al., 1998). Aldehyde dehydrogenase 1 (cytosolic) and aldehyde dehydrogenase 2 (mitochondrial) are the predominant isozymes (Hsu, et al., 1985), while aldehyde dehydrogenase 3 (cytosolic) is the least abundant (Kurys, et al., 1989). MG is reported to be a substrate of, and can be completely hydrated by these 3 isoforms of aldehyde dehydrogenase (Izaguirre, et al., 1998).

1.2.4 2-oxoaldehyde dehydrogenase (2-ODH)

2-ODH also catalyzes the oxidation of MG to pyruvate (Nemet, et al., 2006). This enzyme was purified from sheep liver (Monder, 1967). It is specific for the metabolism of α-oxoaldehydes. It needs NAD or NADPH as a cofactor.

1.3 Toxicity of MG

1.3.1 Modification of protein

Under physiological conditions, more than 90% of MG is bound reversibly with cellular proteins (Lo, et al., 1994). Addition of 1 μM [^{14}C] MG to human plasma and

incubation at 37ºC lead to complete and irreversible binding of MG to plasma protein within 24 hours (Thornalley, 2005). MG reacts with arginine, lysine and cysteine residues of proteins to form advanced glycation endproducts (AGEs). Arginine-derived hydroimidazolone and lysine-derived N-ε-carboxyethyl-lysine (CEL) and N-ε-carboxymethyl-lysine (CML) are products of irreversible reactions of protein residues with MG (Lo, et al., 1994). The concentration of AGEs in mammalian tissues, plasma and extracellular matrix *in vivo* depends on the protein substrate, tissue location and type of AGEs. For instance, the highest concentration of hydroimidazolone was found in the lens of older individuals, and CML accumulates on lens, skin and cartilage (Ahmed, et al., 1997; Verzijl, et al., 2000).

MG-induced AGEs are involved in the pathogenesis of many diseases, such as diabetes, hypertension and neurodegenerative diseases (Desai and Wu, 2007; Münch, et al., 2003). AGEs induce cross-linkage of proteins to decrease arterial and myocardial compliance and promote vascular stiffness, leading to the alteration of vascular structure and function, which contributes to the development of hypertension and diabetic vascular complications (Goh and Cooper, 2008). AGEs also have been seen accumulated in diabetic kidney, retina and atherosclerotic plaques (Hammes, et al., 1999; Bucala and Vlassara, 1995; Makita, et al., 1994), and are closely linked to the development of diabetic complications. In addition, AGEs interact with some receptors, like the receptor for AGEs (RAGE), where they interfere with cell signaling and nuclear factor-κB (NF-κB) mediated pathway, leading to enhanced oxidative stress and generation of proinflammatory cytokines (Goh et al., 2008).

Previous work in our lab has shown that MG-induced AGEs formation is a causative factor for the pathogeneis of hypertention. Levels of MG-induced CEL and CML were higher in kidneys and aortas from spontaneously hypertensive rats (SHR) compared to Wistar Kyoto (WKY) rats from 8 weeks onward. Immumohistochemistry staining revealed that most of the staining was localized to renal tubules and aortic endothelium (Wang, et al., 2004; Wang, et al., 2005). In addition, MG-induced CEL and CML formation was observed in mesenteric artery of fructose-fed rats (Wang, et al., 2008). The accumulation of AGEs in endothelium, artery and kidney may lead to endothelial dysdunction, vascular and tubular damage, which contribute to the development of hypertension and its complications.

MG-induced AGEs formation impairs anti-oxidant enzymes, leading to the excessive accumulation of reactive oxygen species (ROS). Arginine, lysine and cysteine are residues involved in the active sites of enzymes, and the irreversible reaction of MG with residues may alter the activity of those enzymes. For example, activities of glutathione reductase and glutathione peroxidase were reduced significantly, accompanied by the increased MG-induced AGEs formation in aorta from adult SHR (Wang, et al., 2005). MG also modifies Cu / Zn SOD by covalent cross-linking of the proteins, leading to the release of copper ions from the enzyme and the inactivity of the enzyme (Kang, 2003). Furthermore, decreased extracellular SOD activity was due to excessive glycation, not to the impaired synthesis of this enzyme in patients with diabetes (Ciechanowski, et al., 2005). Aminoguanidine, a scavenger of MG and AGEs, increased the activities of catalase, glutathione reductase and glutathione peroxidase in insulin-

dependent diabetic rats and prevented the impairment of blood antioxidant systems (Stoppa, et al., 2006).

1.3.2 Modification of nucleic acid

MG can be a mutagen since it modifies nucleotides poly A, poly G and poly C, but not poly-U (Krymkiewicz, 1973). MG inhibited skin cell proliferation and caused extensive DNA strand cleavage by the extensive formation of DNA-protein cross-links (Roberts, et al., 2003). MG-induced cytotoxicity and mutation were concentration dependent. Multi-base deletions were predominant (50%) in MG-induced mutations, followed by base-pair substitutions (35%), in which G:C-->C:G and G:C-->T:A transversions were predominant (Murata-Kamiya, et al., 2000). Furthermore, MG increased point mutations in *Salmonella typhimurium* (Migliore, et al., 1990), and the occurrence of point mutations correlated with the glycation rate of DNA (Pischetsrieder, et al., 1999).

The cross-link formation of protein with DNA by glycation with MG has been investigated. A protein-DNA cross-link was observed after 90 min exposure to MG (1.5 mM) in Chinese hamster ovary cells (Brambilla, et al., 1985). In addition, MG (1 mM) cross-linked a guanine residue of the substrate DNA and lysine and cysteine residues near the binding site of the DNA polymerase during DNA synthesis, and that DNA replication was severely inhibited by the MG-induced DNA-DNA polymerase cross-link in *E-coli* (Murata-Kamiya and Kamiya, 2001).

1.4 Scavengers of MG

To date, specific MG scavengers are not available in the market, but some agents like aminoguanidine, metformin and alagebrium are capable of reducing MG levels and are currently used in different studies, although the mechanism is unclear.

1.4.1 Aminoguanidine

Aminoguanidine (Pimagedine) is an agent that prevents the formation of AGEs from α, β-dicarbonyl precursors. It is a derivative of guanidine and has two reaction centers: the nucleophilic hydrazine group and the dicarbonyl-directing guanidine group (Ahmed, et al., 2002). The guanidine part of aminoguanidine is the key site of glycation by α, β-dicarbonyl compounds (Ahmed and Thornalley, 2002). The two groups together make a reactive scavenger of α, β-dicarbonyl glycating agents, particularly α-oxoaldehydes, such as MG (Thornalley, et al., 2000). MG, otherwise, would react with lysine, arginine and cysteine residues of proteins to form AGEs. Therefore, aminoguanidine prevents the formation of AGEs from MG. Aminoguanidine is also a potent and irreversible inhibitor of human and rat SSAO (Yu and Zuo, 1997). Activity of SSAO in rat kidney and aorta was significantly inhibited by aminoguanidine at 3 hours after injection. Aminoguanidine rapidly inhibits the activity of SSAO. For instance,

aminoguanidine inhibited 90% of SSAO activity without preincubation, and 15 minutes preincubation of aminoguanidine with SSAO caused the complete inhibition of SSAO acitivity (Yu and Zuo, 1997). SSAO is an enzyme catalyzing aminoacetone to MG, although this pathway is considered as a minor source of MG formation.

Aminoguanidine is not a specific MG scavenger since it also reacts with other carbonyl metabolites, such as 3-deoxyglucosone, malondialdehyde and 4-hydroxy-2-nonenal and formaldehyde (Brownlee, et al., 1986; Kazachkov, et al., 2007). In addition, aminoguanidine is a well known nitric oxide synthase (NOS) inhibitor. NOS catalyzes the production of nitric oxide (NO) from L-arginine (Corbett, et al., 1992). Thus, aminoguanidine would inhibit NO formation when used to scavenge MG.

1.4.2 Metformin

Metformin has a similar structure to aminoguanidine. It is a biguanide compound generally used to control blood glucose levels in T2DM. Administration of metformin in patients with T2DM significantly reduced MG production and increased MG degradation (Beisswenger, et al., 1999). The effect of metformin to reduce MG formation was concentration dependent. Metformin also reduced MG levels in serum and aorta of fructose-fed rats (Wang, et al., 2008). Metformin was observed to trap reactive carbonyls like MG and glyoxal (Ruggiero-Lopez, et al., 1999). Therefore, metformin is not a specific scavenger of MG. A high dose of metformin, 2.5 g/day, was used to reduce MG levels in patients with T2DM (Beisswenger, et al., 1999). Stable triazepinone derivatives were found following the reaction of MG and metformin *in vitro* (Ruggiero-Lopez, et al.,

1999). Furthermore, triazepinone was identified in plasma and urine of type 2 diabetic patients treated with metformin (Beisswenger and Ruggiero-Lopez, 2003). Those studies clearly indicate that metformin directly reacts with MG.

1.4.3 Alagebrium

Alagebrium is an AGEs cross-link breaker. It reduces collagen cross-linking in diabetic animals by cleaving the bonds between adjacent carbonyl groups of cross-linked proteins (Vasan, et al., 1996). Alagebrium is the only AGE cross-link breaker which is being tested in human clinical trials (Zieman, et al., 2007). Clinical studies demonstrated that alagebrium improved arterial compliance and cardiac function and attenuated diabetic nephropathy and atherosclerosis. In addition, alagebrium was reported as safe and well-tolerated by patients in clinical trials (Kass, et al., 2001; Zieman, et al., 2007). Another clinical trial in 23 patients with diastolic heart failure showed that alagebrium significantly decreased left ventricular mass and improved left ventricular diastolic filling (Little, et al., 2005). Therefore, alagebrium is a promising drug to treat MG and AGEs associated diseases, such as diabetes, hypertension and aging.

Moreover, evidence suggests that alagebrium directly scavenges MG. In a study conducted by Nobecourt *et al.* (Nobécourt, et al., 2008), alagebrium did not reverse MG-mediated cross-linking of apolipoprotein (Apo) A-I, the main apolipoprotein of HDL. However, alagebrium prevented MG-mediated modification of ApoA-I. This result indicates that alagebrium is a MG scavenger, although the mechanism is still unknown (Nobécourt, et al., 2008).

1.5 Physiological and pathological levels of MG

The levels of MG in plasma of normal human subjects vary from 123 nM to 650 nM depending on different studies (Beisswenger, et al., 1999; Nemet, et al., 2005; Odani, et al., 1999; Lapolla, et al., 2005). The inconsistency of those values seems dependent on different methods used to test the compound.

Elevated levels of MG have been observed in different kinds of diseases, such as hypertension, diabetes and renal failure. Previous work in our laboratory showed that the plasma MG level was progressively increased with age in SHR. Compared to age-matched WKY rats, the plasma levels of MG were significantly increased in SHR at 8 weeks (13.8 ± 0.72 vs. 9.1 ± 0.8 μM), 13 weeks (30.3 ± 2.05 vs. 18.5 ± 2.71 μM), and 20 weeks (33.6 ± 2.16 vs. 14.2 ± 3.48 μM) (Wang, et al., 2004). MG was significantly elevated in patients with T2DM versus normal subjects (189.3 ± 38.7 vs. 123.0 ± 37 nM, P = 0.0001) (Beisswenger, et al., 1999). The data from another laboratory indicated that plasma MG levels were significantly higher in patients with T2DM (158 ± 46 ng/ml) and patients with chronic renal failure (110 ± 18 ng/ml) than those from normal subjects (47 ± 12 ng/ml) (Odani et al., 1999). In addition, MG levels were significantly elevated in patients with end-stage renal disease versus normal controls (17.5 ± 6.9 vs. 8.5 ± 0.5 μg/ml) (Lapolla, et al., 2005). However, MG levels were reduced with aging in the liver (24.7 ± 3.6 vs. 88.8 ± 10.6 pmol/mg protein) and skeletal muscle (12.7 ± 2.4 vs. 27.5 ± 4.6 pmol/mg protein) between 30-month old aging rats and 5-month old rats. Decreased

MG levels in liver and skeletal muscles might contribute to the low levels of D-lactate in those aging rats (Kawase, et al., 1995).

2. Peroxynitrite ($ONOO^-$)

Peroxynitrite ($ONOO^-$) is a highly reactive free radical and an extremely toxic oxidant. Free radicals are atoms or molecules with an unpaired electron and are more reactive than other atoms or molecules. Two free radicals can share their unpaired electrons by forming a covalent bond to form a stable compound. On the other hand, a free radical can give the unpaired electron to a molecule or get an electron from a molecule and the latter one then becomes a free radical (Desai and Wu, 2008). The reaction chain continues further. Reactive oxygen species (ROS) includes oxygen-derived free radicals, such as superoxide anion ($O_2^{\cdot-}$), and highly reactive non-radicals which do not have an unpaired electron, such as hydrogen peroxide (H_2O_2) (Desai and Wu, 2008). Oxidative stress generally describes a condition in which cellular antioxidant defenses are insufficient to completely detoxify free radicals that have been generated. Oxidative stress results from either excessive production of ROS, or loss of antioxidant defenses, or both (Giugliano, et al., 1996).

2.1 Formation of $ONOO^-$

The major sources of intracellualr $ONOO^-$ are mitochondria and cytosol (Figure 1-3). $ONOO^-$ is formed by the reaction of two free radicals $O_2^{\cdot-}$ and nitric oxide (NO).

The rate of this reaction is so fast that almost every collision between NO and $O_2^{\cdot -}$ results in the formation of $ONOO^-$ (Padmaja and Huie, 1993).

$O_2^{\cdot -}$ is an oxygen molecule with an extra electron. It is mainly produced nonenzymatically in mitochondria during the electron transportation. Under physiological conditions, electrons carried by the electron transportation chain leak out of the pathway and are passed to oxygen directly, leading to the formation of $O_2^{\cdot -}$. The production of $O_2^{\cdot -}$ in the cytosol is mediated by enzymes NADPH oxidase, xanthine oxidase, and cytochrome P450, which are present in the plasma membrane, cytosol and endoplasmic reticulum, respectively (Curtin, et al., 2002). $O_2^{\cdot -}$ is one of the main causes of oxidative stress (Figure 1-3).

NO is produced endogenously from the oxidation of L-arginine to L-citrulline by catalysis by a member of nitric oxide synthase (NOS) family, such as neuronal nitric oxide synthase (nNOS; type 1), inducible nitric oxide synthase (iNOS; type 2), and endothelial nitric oxide synthase (eNOS; type 3). nNOS and eNOS are constitutively expressed, but their activities are calcium concentration dependent. iNOS is inductively expressed and its activity is calcium concentration independent (Alderton, et al., 2001). iNOS and nNOS are localized in the cytoplasma, whereas eNOS is membrane-bound. Upregulation of iNOS expression leads to the production of large amount of NO. NO is also produced in mitochondria via the mediation of mitochondrial NOS (mtNOS). Co-stimulation of mitochondrial $O_2^{\cdot -}$ formation and mtNOS causes the overproduction of $ONOO^-$ in mitochondria (Figure 1-3).

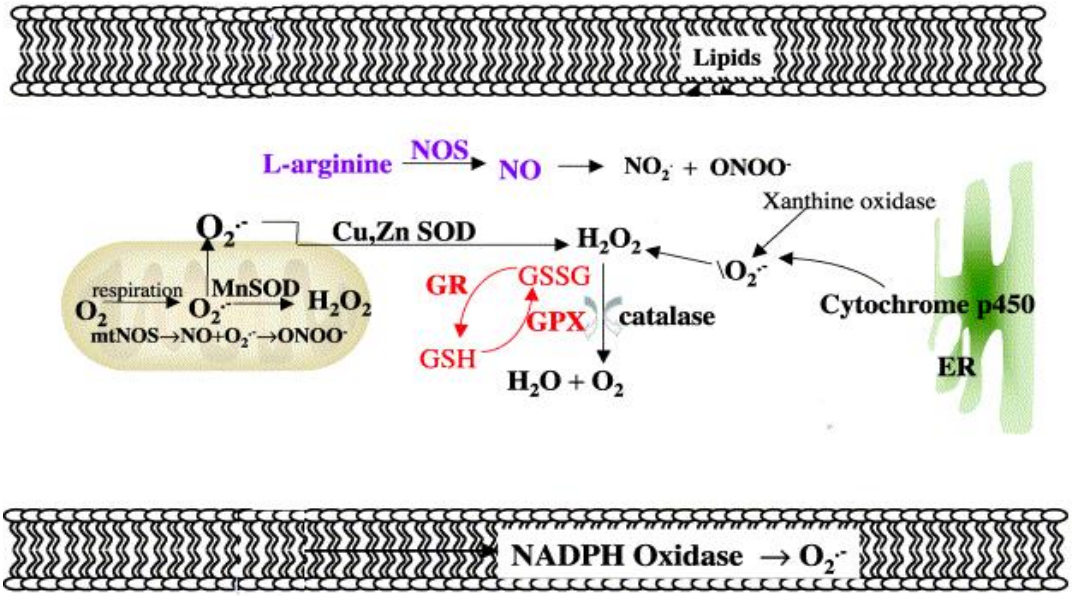

Figure 1-3. Intracellular sources of $ONOO^-$ (Adapted from *J Immunol Methods. 2002; 265(1-2):49-72*).

ER: Endoplasmic reticulum
GSH: Reduced glutathione
GSSG: Oxidized glutathione
GPX: Glutathione peroxidase
GR: Glutathione reductase

2.2 Toxicity of $ONOO^-$

Excessive production of $ONOO^-$ has been reported in many conditions, such as cardiovascular disease (Pacher, et al., 2007). $ONOO^-$ diffuses intra- and intercellularly and modifies DNA, proteins and lipids, contributing to the pathogenesis of diabetes and hypertension. Modification of DNA includes oxidation of nucleotide bases and formation of cross-links (Piconi, et al., 2003). Guanine is the most reactive nucleotide base with $ONOO^-$ due to its low reduction potential. Ultimately, reaction of guanine with $ONOO^-$ results in guanine fragmentation, which contributes to mutagenesis and carcinogenesis (Niles, et al., 2006). DNA alteration caused by $ONOO^-$ will result in mutations, cellular aging and death. Mutagenic potential is closely linked with the number of non-repaired DNA lesions (Valko, et al., 2007).

$ONOO^-$ modifies protein structure and function by reacting with various amino acids. The most common reactions are with cysteine and tyrosine residues of proteins. The thiol oxidation of cysteine residues by $ONOO^-$ results in the inactivation of many enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Buchczyk, et al., 2003). Inactivation of GAPDH interrupts glycolytic pathways and leads to ATP depletion. On the other hand, GAPDH is an abundant protein, and its inactivation may cause the formation of insoluble protein aggregates, which promotes cell death (Buchczyk, et al., 2003). $ONOO^-$ can also nitrate tyrosine residues of proteins to form nitrotyrosine. Nitrotyrosine has been used as a marker of nitrosative damage to protein. Tyrosine nitration interferes with phosphorylation / dephosphorylation cell signaling pathways and alters cellular function (Minetti, et al., 2002). Lipid peroxidation induced

by $ONOO^-$ mostly occurs at cell membranes which contain large amount of lipids. Many membrane phospholipids are unsaturated, containing a methylene group between two double bonds that makes the fatty acid more sensitive to oxidation. The initial products of unsaturated fatty acid oxidation are short-lived lipid hydroperoxides. Hydroperoxides readily react with metals to produce a series of products, such as aldehydes and epoxides. Malondialdehyde, one of the major aldehyde products of lipid peroxidation (Zollner, et al., 1975), is mutagenic and carcinogenic (Basu and Marnett, 1983).

2.3 Antioxidant enzymes

Cells require antioxidant enzymes to neutralize ROS (Figure 1-3). $O_2^{\cdot -}$ is enzymatically degraded to H_2O_2 by superoxide dismutase (SOD) (Desai and Wu, 2008). SODs are metal-containing enzymes that depend on a bound manganese, copper or zinc for their antioxidant activity. In mammals, the manganese-containing enzyme (MnSOD) is most abundant in mitochondria, while the zinc or copper forms (Cu / Zn SOD) are predominant in cytosol (Fridovich, 1995).

H_2O_2 is also highly reactive although it is less toxic than $O_2^{\cdot -}$. Catalase is the major enzyme to remove H_2O_2 (Muzykantov, 2001). It is found in peroxisomes in eukaryotic cells. Catalase degrades H_2O_2 to water and oxygen, and hence finishes the detoxification reaction started by SOD.

Glutathione peroxidase (GPX) converts H_2O_2 to water with the addition of reduced glutathione (GSH), which is catalyzed to oxidized glutathione (GSSG). Besides protein-bound thiol, $ONOO^-$ directly reacts with low-molecular-weight thiol, like GSH

(Pacher, et al., 2007). Thus, GSH acts as an endogenous scavenger of $ONOO^-$ and defends cells against oxidative stress (Arteel, et al., 1999). The depletion of GSH enhances toxicity of $ONOO^-$, leading to cellular damage.

In addition to these enzymes, some compounds like Vitamin E, Vitamin C and uric acid may participate in the elimination of oxygen radicals.

2.4 MG and $ONOO^-$

2.4.1 MG and $ONOO^-$ production

Numerous studies demonstrated that MG induced $O_2^{\cdot -}$, NO, and further $ONOO^-$ formation, *in vitro* and *in vivo*. Increased generation of $ONOO^-$ is closely linked with MG treatment. Treatment of MG induced a time- and concentration- dependent increase of oxidized DCF fluorescence intensity, which indicates the formation of $ONOO^-$ and H_2O_2, in aortic VSMCs (A-10 cells). MG-induced oxidized DCF was inhibited by both GSH and anti-oxidant *N*-acetyl-L-cysteine (NAC). MG also increased the production of $O_2^{\cdot -}$ which was prevented by SOD and NADPH oxidase inhibitor diphenylene iodonium, and NO which was inhibited by N(G)-nitro-L-arginine methyl ester (L-NAME), an NOS inhibitor (Chang, et al., 2005).

Also in cultured mesenteric artery smooth muscle cells (SMCs) from SD rats, incubation of MG for 3 hours increased oxidized DCF fluorescence (Wu, 2005). Moreover, significantly increased levels of $O_2^{\cdot -}$ and H_2O_2 in the aorta of 13 week old SHR were associated with elevated plasma and aortic MG levels, compared with those in age-

matched WKY rats (Wang, et al., 2005). MG increased the production of $O_2^{\cdot -}$ in human platelets, and the oxidation effect of MG was significantly potentiated by thrombin (Leoncini, et al., 1989).

2.4.2 Mechanisms of MG induced $ONOO^-$ production

To date, the mechanism of MG-induced $ONOO^-$ is unclear. Effect of MG on mitochondrial proteins, cell signaling pathways and anti-oxidant enzymes is associated with MG-induced oxidative damage.

2.4.2.1 MG and mitochondrial proteins

Rosca *et al.* investigated the relationship between MG-modified mitochondrial proteins and mitochondrial oxidative stress. In mitochondrial suspension from streptozotocin-induced diabetic rat kidney cortex, MG attached to mitochondrial proteins to form MG-derived imidazole AGEs. MG-modified mitochondrial proteins significantly increased mitochondrial $O_2^{\cdot -}$ production. Administration of aminoguanidine improved mitochondrial respiration and decreased oxidative damage to mitochondrial proteins (Rosca, et al., 2005). This study indicates that glycation of mitochondrial proteins by MG is accounted for MG-induced mitochondrial oxidative stress. This study echoes the discovery made by Rabbani (Rabbani and Thornalley, 2008). Furthermore, overexpression of glyoxalase I, an enzyme degrading MG, in the Caenorhabditis elegans significantly decreased MG modification of mitochondrial proteins, reduced

mitochondrial oxidative stress and prolonged life span of those elegans (Morcos, et al., 2008).

2.4.2.2 MG and p38-mitogen-activated protein kinase (p38 MAPK)

MG (1 μM to 1 mM) increased production of $O_2^{\cdot-}$ and H_2O_2 in neutrophils in a concentration-dependent manner (Ward and McLeish, 2004). MG-induced production of $O_2^{\cdot-}$ and H_2O_2 was independent of the presence of plasma proteins and blocked by a MG scavenger aminoguanidine. MG activated p38 MAPK-dependent exocytosis of granules to provide cytochrome b588 for NADPH oxidase, which mediates $O_2^{\cdot-}$ generation (Ward and McLeish, 2004). MG may directly enter the cells and stimulate p38 MAPK signaling pathway. MG also modifies plasma protein, such as albumin, which then activates p38 MAPK (Fan, et al., 2003). Furthermore, reduced availability of GSH can activate p38 MAPK (Haddad, 2002). MG (200 μM, 0.5 h or 1 h)-induced phosphorylation and activation of p38 MAPK was also observed in Schwann cells, accompanied by the depletion of intracellular GSH and cell apoptosis (Fukunaga, et al., 2005).

2.4.2.3 MG and nuclear factor-κB (NF- κB)

Previous work in our laboratory tested the effect of MG on NF-κB (Wu and Juurlink, 2002). MG (50 to 500 μM, 24 h) significantly induced oxidized DCF intensity, indicating the generation of $ONOO^-$, and lowered GSH levels in VSMCs from SHR, compared to the VSMCs from WKY rats. In addition, MG (300 μM, 3 h) induced

activity of NF-κB and decreased cytoplasmic levels of IκBα unit (inhibitory protein for NF-κB) in VSMCs from SHR. These results suggest that MG may induce ROS generation via the activation of NF-κB. NF-κB is present in many cell types and controls numerous gene products. NF-κB, in turn, enhances the expression of iNOS (Spitaler and Graier, 2002) and further increases NO production. However, high levels of $O_2^{\cdot -}$ lead the reaction with NO to form $ONOO^-$ (Beckman and Koppenol, 1996). $ONOO^-$ causes the cellular damage by lipid peroxidation, nitration of amino acids and oxidation of tyrosine residues of protein to form nitrotyrosine.

2.4.2.4 MG and anti-oxidant enzymes

Because MG is degraded mainly by the glyoxalase system which depends on the availability of GSH, GSH depletion by MG has been shown (Kikuchi, et al., 1999). As mentioned earlier, GSH is a low-molecular-weight thiol which scavenges intracellular $ONOO^-$. Reduced GSH, therefore, increases cell susceptibility to $ONOO^-$-induced damage. Decreased GSH content was detected in VSMCs from SHR and WKY rats exposed to MG (0.5 mM). MG (0.3 mM) also increased the level of GSSG, which was inhibited by NAC (Wu and Juurlink, 2002). In addition, MG reduced GSH content in platelets (Leoncini, et al., 1989) and hepatocytes (Kalapos, et al., 1991). MG also affects the activity of other anti-oxidant enzymes. For instance, SOD activity was inhibited by MG (5 mM for 5 days) in a concentration- and time- dependent manner. The inactivation of SOD was more pronounced with 30 mM MG treatment for 24 hours (Jabeen, et al.,

2006). Moreover, incubation of human Cu / Zn SOD with MG (30 mM, 24 hours) led to the loss of enzymatic activity and release of copper ions from the protein (Kang, 2003).

3. Mitochondria

3.1 Structure of mitochondria

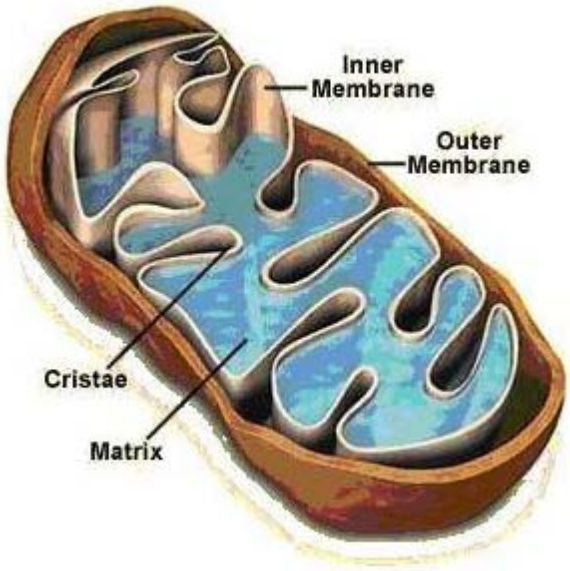

Figure 1-4. Structure of mitochondria (Adapted from *http://giantshoulders.wordpress.com/2007/10/21/the-mitochondrion-pt-1-structure-and-layout/*).

Mitochondria generate ATP and are powerhouses of cells. They are composed of an outer membrane and an inner membrane, which have different functions (Figure 1-4). The outer membrane contains integral proteins named porins, which form channels to allow proteins weighing 5,000 Daltons or less to diffuse through. The inner membrane is

folded inward many times to form cristae and is highly impermeable. Almost all molecules and ions need special membrane transporters to cross the inner membrane. Enzymes on the inner membrane have different functions: (1) conduct oxidative phosphorylation on the electron transport chain (ETC), which is also named respiratory chain; (2) produce ATP in the matrix (space enclosed by the inner membrane); (3) transport proteins into and out of the matrix. The two membranes together create the intermembrane space. Small molecules such as ions and sugars in the intermembrane space are the same as those in the cytosol. In addition, the intermembrane space contains other large proteins, which are transported across the outer membrane, like cytochrome C. The matrix, on the other hand, contains a highly selected set of proteins and is the location of ATP production. Many important biochemical reactions take place in the mitochondria, including electron transport, oxidative phosphorylation, and ATP generation (Alberts, et al., 1994; Gao, et al., 2008).

3.2 Electron transport chain (ETC) and oxidative phosphorylation

ETC is a series of protein complexes embedded in the inner mitochondrial membrane (Figure 1-5). There are five complexes involved: (1) NADH dehydrogenase (complex I); (2) Succinate dehydrogenase (complex II); (3) Cytochrome c reductase (complex III); (4) Cytochrome c oxidase (complex IV); (5) ATP synthase (ATPase, complex V) (Mandelker, 2008). Electrons flow from NAD^+ / NADH to O_2 / $2H_2O$, through complex I, which passes electrons on to ubiquinone; complex III, which transfers electrons from ubiquinone to cytochrome C; and finally complex IV, which carries

electrons to O_2, resulting in the reduction to H_2O. In addition, ubiquinone takes electrons through the conversion of succinate to fumarate at the site of complex II. The transportation of electrons through complex I, II and III results in the pumping of protons from the matrix to the intermembrane space across the inner membrane to create a hydrogen gradient. The potential energy in the hydrogen gradient drives the membrane-located ATP synthase, which catalyzes ATP production in the presence of ADP and P_i. The process of electron transportation through ETC to generate ATP is called oxidative phosphorylation (Leverve, 2007).

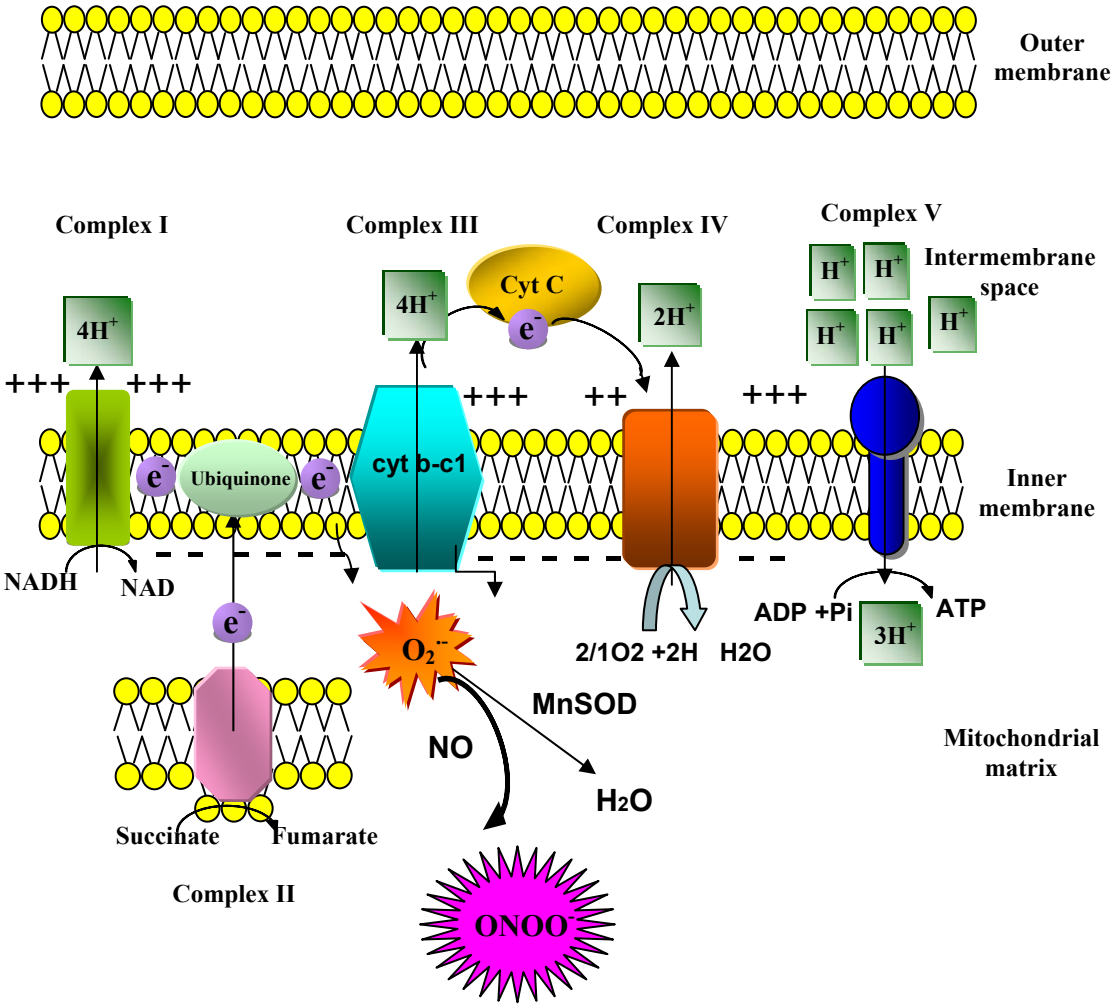

Figure 1-5. Electron transport chain, oxidative phosphorylation and mitochondrial oxidative stress

Complex I: NADH dehydrogenase
Complex II: Succinate dehydrogenase
Complex III: Cytochrome c reductase
Complex IV: Cytochrome c oxidase
Complex V: ATP synthase
Cyt C: Cytochrome C

3.3 Mitochondria and $ONOO^-$

Mitochondrial respiration, which is performed on the ETC, generates about 85% of total intracellular $O_2^{\cdot -}$ during the process of energy production (Chance, et al., 1973). Under physiological condition, 2-5% of electrons leak out from the ETC and interact with oxygen to form $O_2^{\cdot -}$ (Droge, 2002). Complex I and complex III are main sites where electrons leak out to oxygen (Turrens and Boveris, 1980) (Figure 1-5). Evidence suggest that $O_2^{\cdot -}$ is formed in the inner side of the inner membrane (Turrens, 1997).

mtNOS is a constitutive and Ca^{2+} dependent enzyme, which is located in the inner mitochondrial membrane and catalyzes the generation of mitochondrial NO (Dedkova, et al., 2004). The absence of mtNOS activity in mouse heart was observed in $nNOS^{-/-}$ mice but not in $eNOS^{-/-}$ or $iNOS^{-/-}$ mice, which identifies that mtNOS is nNOS (Kanai, et al., 2001). It is further proved that mtNOS is the alpha isoform of nNOS (Carreras, et al., 2002; Elfering, et al., 2002). In mitochondria, NO reacts with $O_2^{\cdot -}$ to form $ONOO^-$, which leaks out of mitochondria and accumulates in cytosol. MnSOD is the primary enzyme in mitochondria to convert $O_2^{\cdot -}$ to H_2O_2. It protects mitochondria against oxidative stress. The down regulation of MnSOD was found in the pathogenesis of diabetic complications and cancer (Shen, et al., 2006; Hu, et al., 2005).

Rotenone, thenoyltrifluoroacetone (TTFA), antimycin A and cyanide are inhibitors of complex I, II, III and IV, respectively (Zhang, et al., 2001). Application of mitochondrial inhibitors significantly increased mitochondrial ROS production (Turrens, 1997). The presence of mitochondrial inhibitors stimulated the production of $O_2^{\cdot -}$ (Li, et al., 2006; Chen, et al., 2007) (Figure 1-6).

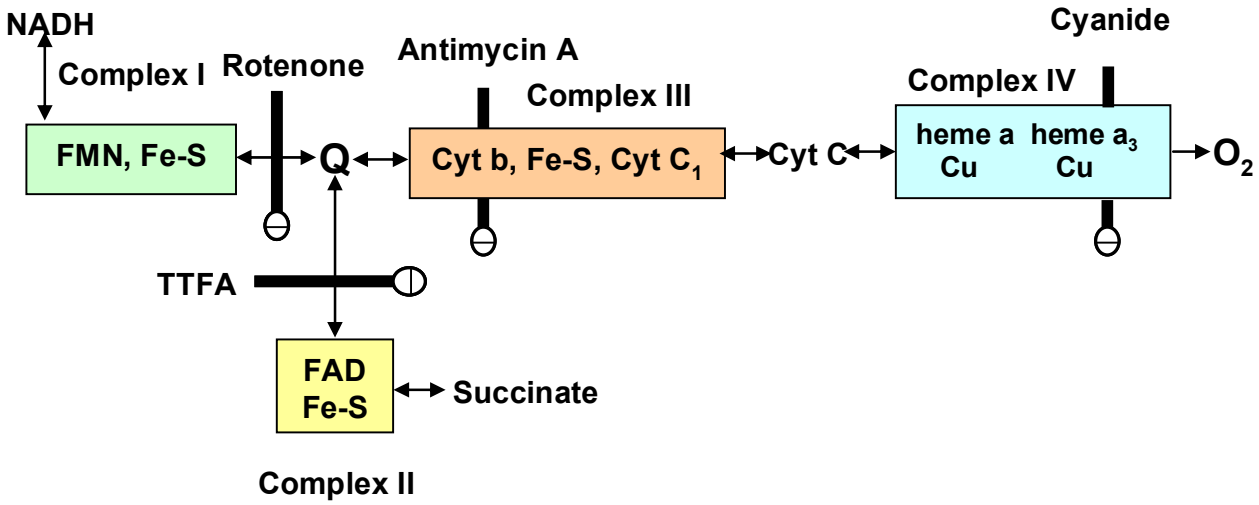

Figure 1-6. Sites of ETC inhibited by blockers.

Q: Ubiquinone
Fe-S: Iron-sulfur proteins
FMN or FAD: Flavin nucleotide;
Cyt: Cytochrome
TTFA: Thenoyltrifluoroacetone

4. Type 2 diabetes mellitus (T2DM)

4.1 Introduction of T2DM

Diabetes mellitus (DM) is characterized as recurrent or persistent hyperglycemia (high blood glucose levels). It is diagnosed as fasting glucose higher than 125 mg/dl (7.0 mM), and / or oral glucose tolerance test higher than 200 mg/dl (11.1 mM), and / or random glucose higher than 200 mg/dl (11.1 mM) with syndromes of diabetes, such as polyuria, polydispia and unexplained weight loss (2008 Clinial Practice Guidelines, Canadian Diabetes Association, http://www.diabetes.ca/files/cpg2008/cpg-2008.pdf). DM is classified into type 1 diabetes mellitus (T1DM) / insulin dependent diabetes mellitus and type 2 diabetes mellitus (T2DM) / non-insulin-dependent diabetes mellitus. T1DM results from the loss of the insulin-producing β cells in the pancreas, leading to a deficiency of insulin. T2DM is attributed to reduced insulin secretion and insulin resistance (Diabetes care, 2007).

Global expenditure in prevention and treatment of diabetes and related complications totalled 232 billion (USD) in 2007 (Diabetes atlas, 2007). Data from World Health Organization (WHO) indicate that T2DM accounts for about 90% of diabetic patients and T2DM prevalence is estimated to grow from approximately 162 million to 329 million by 2030 (WHO website: http://www.who.int/mediacentre/factsheets/fs312/en/index.html). Therefore, T2DM is a huge financial and social burden that affects both families and nations. It is of crucial importance to find the cause and to develop strategic therapies for T2DM.

Pathogenesis of T2DM is not completely known. Overeating and lifestyle are considered as trigger factors. Heredity is also involved. People with a positive family history have a 2.4-fold increased risk for T2DM compared to the general population (Pierce, et al., 1995). The prevalence of T2DM in the offspring is about 38% if one parent has T2DM, and the risk rises to 60% if both parents have T2DM (Pierce, et al., 1995; Tattersal and Fajans, 1975). In the diabetes research field, the inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilization in an individual as much as it does in a normal population is definied as insulin resistance, which is commonly used to explain the mechanism of T2DM (Lebovitz, 2001). The cause for insulin resistance is, however, largely unsettled.

Prolonged hyperglycemia causes a series of pathological and metabolic changes which contribute to the development of diabetic complications. The devastating complications reduce the quality of life and life expectancy and increase the morbidity and motility.

Some patients with T2DM are undiagnosed at early stage and only see their doctors at check up. T2DM affects many major organs, such as heart and kidney. Generally, diabetic complications can be divided into three categories: neuropathy; microvascular complications, which include retinopathy and nephropathy; and macrovascular complications, such as cardiovascular disease (CVD), stroke and peripheral vascular disease. CVD, especially heart failure and myocardial infarction, is the major cause of hospital admission and death in diabetic patients (Stuckey, et al., 2005). Statistical data from the American Diabetes Association demonstrate that more than 65% of deaths in diabetic patients are attributed to heart and vascular diseases

(American Diabetes Association, http://www.diabetes.org/diabetes-heart-disease-stroke.jsp). Hyperglycemia is a definite factor causing the occurrence of diabetic complications (Laakso, 1999). Intensive glucose control, i.e. keeping blood glycated hemoglobin (HbA1c) to less than 7%, reduces the incidence of diabetic complications. The United Kingdom Prospective Diabetes Study (UKPDS) demonstrates a reduction in microvascular complications: retinopathy by 25%, erectile dysfunction by 20% and macrovascular disease by 40% by controlling hyperglycemia in patients with T2DM (UKPDS, 1998).

4.2 MG and T2DM

4.2.1 MG levels in T2DM

Numerous studies demonstrated that MG played an important role in the pathogenesis of diabetes and diabetic complications. Nemet *et al.* observed that the plasma levels of MG in diabetic patients were significantly increased compared with those in normal controls (742 ± 141 vs. 520 ± 42 nM, $P = 0.000016$). MG was also a parameter reflecting glycemic fluctuation (Nemet, et al., 2005). For instance, the *M* value is a quantitative index of the deviations of diurnal blood glucose from an arbitrarily selected standard (5 mM), and provides a single numerical expression of glycemic control with a normal range of 0–20. Increased MG levels were observed in both whole blood and plasma samples in patients with *M* values > 20 compared to the same parameters obtained in patients with *M* values <20 (range 0.4–19.1). MG levels also

significantly correlated with the individual *M* values (Nemet, et al., 2005). Data from another lab showed that MG was significantly elevated in patients with T2DM versus normal controls (189.3 ± 38.7 vs. 123.0 ± 37 nM) (Beisswenger, et al., 1999). In addition, the levels of MG correlated with rising HbA1c (R = 0.4) (Thornalley, et al., 1989).

4.2.2 Roles of MG in T2DM

4.2.2.1 MG-induced AGEs in T2DM

MG is the most important precursor of AGEs. Numerous studies show that accumulation of intracellular MG and formation of AGEs alter cell function and contribute to the development of T2DM and diabetic complications, such as atherosclerosis, nephropathy, and retinopathy.

In cultured endothelial cells, MG accumulated rapidly under hyperglycemic conditions (Shinohara, et al., 1998). In addition, serum levels of AGEs increased in patients with T2DM and coronary artery disease (Kilhovd, et al., 1999). AGEs induce diabetic atherosclerosis by multiple ways. Argpyrimidine, the fluorescence product of the reaction of MG with arginine residues in protein, has been demonstrated to localize in atherosclerotic lesions, fatty streaks, lipid containing SMCs and macrophages in diabetic patients (Friedman, 1999; Oya, et al., 1999). A correlation of AGEs and severity of atherosclerotic lesions was also shown. AGEs decrease NO availability by quenching NO, impair LDL removal by trapping LDL in the subendothelium and decrease LDL

receptor recognizing AGEs-modified LDL (Bucala, et al., 1994). Furthermore, AGEs enhanced VCAM-1 expression by activating NF-κB. VCAM-1 stimulates the migration of monocytes through endothelium, which is the first step of atherogenesis (Kunt, et al., 1999).

The kidney is a key target of MG and AGEs mediated damage. Mouse renal damage was found after oral administration of MG. A 5-month treatment with MG resulted in elevated levels of collagen in kidney and increased glomerular basement membrane thickness (Golej, et al., 1998). Diabetic mice have significantly elevated renal AGEs, and these abnormalities have been linked to various structural aspects of diabetic nephropathy, including glomerular basement membrane thickening, glomerulosclerosis, and tubulointerstitial fibrosis (Soulis-Liparota, et al., 1995).

MG-induced hydroimidazolone increased selectively in retinas of streptozotocin-induced diabetic rats (Karachalias, et al., 2003). In addition, MG-modified CML was localized in retinal blood vessels of patients with T2DM and was found to correlate with the degree of retinopathy (Stitt, 2001). Furthermore, decreased eNOS expression was observed in retinal vascular endothelial cells exposed to AGEs, which may account for retinal microvascular abnormalities (Chakravarthy, et al., 1998).

4.2.2.2 MG and insulin

Evidence shows that MG destroys pancreatic β-cells, decreases insulin secretion in response to glucose and alters insulin structure and function. MG caused a concentration-dependent increase of apoptotic pancreatic β-cells (Sheader, et al., 2001).

Addition of MG (0.5 or 1 mM) to single isolated rat pancreatic β-cells caused a rapid and marked depolarization, and this effect was reversible upon the removal of MG. MG also led to elevated cytosolic calcium concentration and intracellular acidification in intact rat islets (Cook, et al., 1998). Moreover, acute exposure of isolated mouse islets or β-cells to MG resulted in reduced insulin secretion in response to glucose (Pi, et al., 2007).

The direct effect of MG on human insulin was investigated by Jia *et al.* (Jia, et al., 2006). Human insulin was subjected to electrophoresis in Tricine SDS-PAGE gels after incubation with MG for 3 days. Incubation of insulin (1 μg/μl) with MG (100 μM) resulted in additional bands with lower electrophoretic mobility than native insulin on SDS-PAGE. Mass spectrometry was used in order to achieve more accurate and sensitive determination of MG-induced mass changes. Incubation of 1 μg/μl human insulin with 10 μM MG for 3 days resulted in additional peaks that provided evidence for the formation of MG-insulin. It was further confirmed that MG modified insulin by attaching to the internal arginine residue in the β-chain of insulin. In addition, the formation of this MG-insulin adduct decreased insulin-mediated glucose uptake in different insulin-sensitive cells, such as 3T3-L1 cells (cell line from mouse adipose tissue) and L8 cells (rat skeletal muscle cell line), although MG alone had no effect on glucose uptake. Unlike native insulin, MG-insulin did not inhibit insulin release from pancreatic β-cells. The metabolism of MG-insulin through hepatic cells was also decreased (Jia, et al., 2006). Thus, MG modifies internal arginine residues in the β-chain of insulin, and the formation of an insulin-MG adducts decreases insulin-mediated glucose uptake, impairs autocrine control of insulin secretion, and decreases insulin

clearance. The effect of MG on insulin may contribute to the pathogenesis of T2DM and the development of diabetic complications.

4.2.2.3 MG-induced oxidative stress in T2DM

Growing evidence suggests that MG-induced oxidative damage is responsible for the development of diabetic complications. Stirban and co-workers (Stirban, et al., 2006) tested effects of MG / AGEs rich, heat-processed meal on diabetic patients. T2DM patients without a history of acute cardiovascular events, such as myocardial infarction and unstable angina, during the previous 6 months were recruited. Compared to baseline, MG / AGEs rich, heat-processed food reduced macrovascular flow-mediated dilatation and decreased microvascular reactive hyperemia, indicating macro- and microvascular endothelial dysfunction. The impairment of postprandial flow-mediated dilatation may be the result of a combined effect of reduced NO production and increased NO scavenging, both decreasing NO bioavailability (Stirban, et al., 2006).

Another study of three diabetic populations, the Overt Nephropathy Progressor / Nonprogressor (ONPN) cohort (n = 14), the Natural History of Diabetic Nephropathy study (NHS) cohort (n = 110), and the Pima Indian cohort (n = 45), demonstrated that progression of diabetic nephropathy was significantly correlated with MG levels and oxidative stress (Beisswenger, et al., 2005). The oxidative stress in this study was verified by the reduced GSH levels in red blood cells of diabetic patients. In addition, MG modified renal mitochondrial protein in streptozotocin-treated rats, leading to significantly increased production of mitochondrial $O_2^{\cdot -}$, and oxidative damage (Rosca, et

al., 2005). Furthermore, exposure of human neuroblastoma SH-SY5Y cells to MG was associated with increased ROS production, leading to MG-induced cellular damage (de Arriba, et al., 2006). MG also induced diabetic neuropathy through oxidative stress-mediated activation of p38 MAPK (Fukunaga, et al., 2005).

4.3 $ONOO^-$ and T2DM

4.3.1 Elevated $ONOO^-$ production in T2DM

Increased $ONOO^-$ production and reduced antioxidant defense systems are well established in T2DM. High glucose and MG stimulated a dose-dependent increase in the formation of $ONOO^-$ in retinal endothelial cells by increasing formation of $O_2^{\cdot -}$ and NO (El-Remessy, et al., 2003a; El-Remessy, et al., 2003b). These increases were blocked by the addition of the NOS inhibitor, L-NAME, or $ONOO^-$ scavenger, uric acid. Accelerated renal cortical generation of $ONOO^-$, as well as the reduced NO bioavailability were observed in the early stage of diabetes (Ishii, et al., 2001). In addition, nitrotyrosine levels were significantly higher in diabetic patients in the fasting state and were further elevated in the postprandial state (Ceriello, et al., 2002). Lipid peroxidation, i.e. increased levels of isoprostane, were observed in plasma and urine of type 2 diabetic patients. Isoprostane is produced from arachidonic acid through a nonenzymatic process of lipid peroxidation, which is catalyzed by ROS (Gopaul, et al., 1995; Davi, et al., 1999). In contrast, levels of antioxidant enzymes GPX, SOD and catalase in erythrocytes were significantly decreased in patients with T2DM compared with those from normal subjects (Ramakrishna and Jailkhani, 2008; Flekac, et al., 2008).

4.3.2 $ONOO^-$ and diabetic complications

$ONOO^-$ directly interacts with DNA, proteins and lipids, leading to cellular damage. Overproduction of $ONOO^-$ is considered as a major factor to cause diabetic complications (Rosen, et al., 2001). The role of $ONOO^-$ to induce diabetic complications is highlighted by many studies. Nitrotyrosine, which is a specific marker of $ONOO^-$ formation, directly caused vascular endothelial dysfunction and DNA damage (Mihm, et al., 2000). Furthermore, nitrotyrosine increased apoptosis of endothelial cells, myocytes and fibroblasts in heart biopsies from diabetic patients (Frustaci, et al., 2000). Ultimately, the dysfunction or the degree of cell apoptosis correlated with the levels of nitrotyrosine expressed in those cells. Endothelial dysfunction precedes and predicts more severe microvascular complications in diabetes. In addition, elevated levels of $ONOO^-$ resulted in the enhanced peroxidation of low density lipoprotein (LDL), which promotes atherogenesis in diabetes (Hamilton, et al., 2008). Oxidized LDL is able to transform macrophages and SMCs into foam cells, leading to the formation of atherosclerotic plaques and increased incidence of cardiovascular disease (Bowie, et al., 1993).

In addition to its ability of directly oxidizing DNA, proteins and lipids, $ONOO^-$ impairs cell signal transduction, leading to cellular dysfunction. $ONOO^-$ induced a 9-fold increase in retinal neuron death in retinas of diabetic patients and streptozotocin-induced diabetic rats through nitrating tyrosine residues of nerve growth factor (NGF) TrkA receptor and diminishing phosphorylation of TrkA receptor (Ali, et al., 2008). In

addition, Zou *et al*. have observed that $ONOO^-$ strongly inhibited the phosphorylation and activity of Akt and increased 5'-AMP-activated kinase (AMPK)-dependent Ser^{1179} phosphorylation of eNOS (Zou, et al., 2002).

4.3.3 $ONOO^-$, inflammation and T2DM

Many factors contribute to the development of diabetes and its complications, such as inflammation. A prospective study found that C-reactive protein and interleukin-6 (IL-6), two circulating markers of systemic inflammation, were risk factors for the development of T2DM in apparently healthy middle-aged women (Pradhan, et al., 2001). Low-degree inflammation was observed in the early stage of T2DM. The inflammation increased during the progression of the disease and continued to be enhanced during the development of additional complications (Hwang, et al., 2008).

The relationship between levels of circulating cytokines and blood glucose was investigated. Esposito *et al*. (Esposito, et al., 2002) found that acute hyperglycemia increased circulating concentrations of IL-6, tumor necrosis factor alpha (TNF-α), and interleukin-18 (IL-18) in healthy controls and subjects with impaired glucose tolerance (IGT). However, the elevation of cytokines was greater and lasted longer in patients with IGT compared to non-diabetic subjects. Moreover, antioxidant GSH completely prevented the rise in cytokines induced by hyperglycemia. Since GSH scavenges $ONOO^-$, the result indicates that ROS, particularly $ONOO^-$, mediates hyperglycemia-induced inflammatory cytokine production in humans. In addition, the production of IL-6 induced by high glucose *in vitro,* may be mediated by oxidative stress (Guha, et al.,

2000), and $O_2^{\cdot -}$ might be implicated in promoting inflammation in patients with T2DM (Arnalich, et al., 2000).

NF-κB plays a central role in immune and inflammatory responses. It regulates the expression of genes encoding cytokines and mediators, such as TNF-α, IL-1β, IL-6 and IL-8 (Lee and Burckart, 1998). NF-κB is usually located in the cytosol in an inactivated condition bound to the inhibitory unit IκBα. Degradation of IκBα provokes the activation of NF-κB. ROS signal the degradation of the inhibitory unit IκBα and separate it from the complex. Thus, ROS promote the rapid translocation of active NF-κB into the nucleus, leading to the formation of proflammatory cytokines (Ho and Bray, 1999) (Figure 1-7).

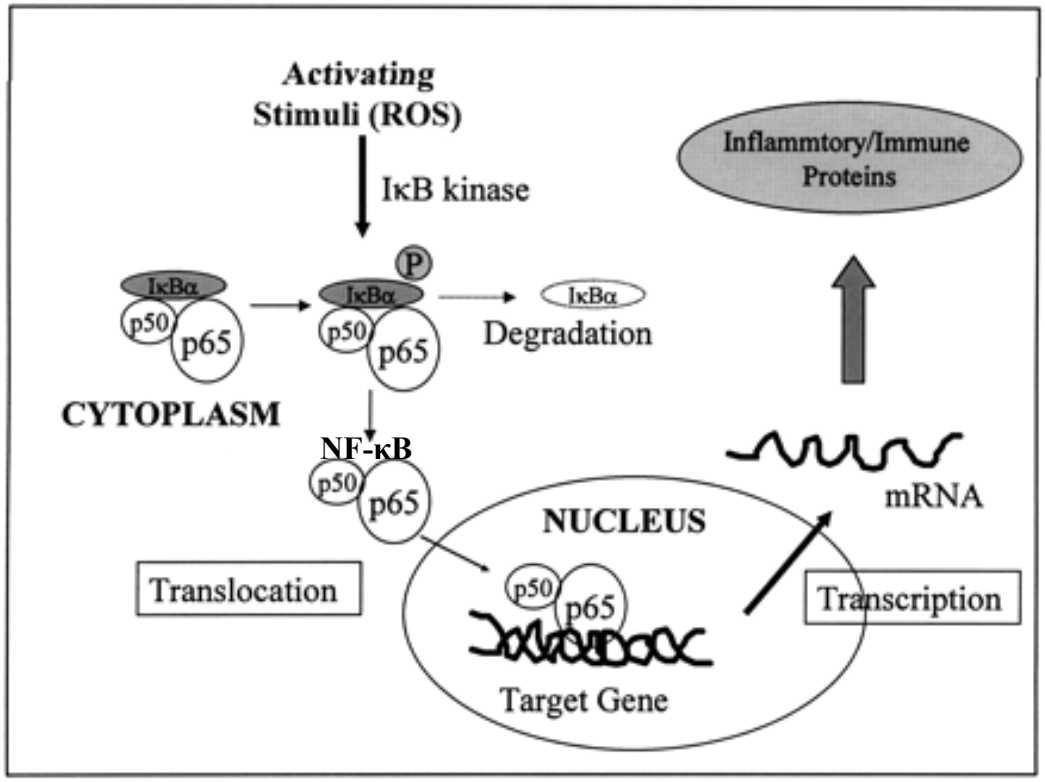

Figure 1-7. Mechanism of ROS-mediated NFκB activation. Activated by ROS, IκB kinase phosphorylates the IκBα subunit (inhibitory protein for NFκB). The phosphorylated IκBα then degrades the IκBα subunit by proteosomes. Degradation of IκBα releases the NFκB p50/p65 complex, allowing the complex to translocate into the nucleus. In the nucleus, the p50/p65 complex binds to the κB-binding sites in promoters of genes encoding immune and inflammatory factors and induces their transcription. (Adapted from *Proceedings of the Society for Experimental Biology and Medicine. 1999; 222(3):205-213*)

Neutrophils are the most abundant type of white blood cells and play a crucial role in innate immunity. Once an inflammatory response is initiated, the neutrophil is the first cell to be recruited to the site of infection or injury, where it phagocytoses bacteria and damaged host tissue. Diabetic patients are at high risk of infection, and the infection is more serious and prolonged once it is occured. It has been suggested that the impaired function of neutrophils accounts for the increased susceptibility to infection observed in those patients (Pickup, et al., 2000). Furthermore, neutrophil dysfunction favors the onset of diabetic angiopathy (Delamaire, et al., 1997), and the development and progression of diabetic nephropathy (Galkina and Ley, 2006).

The mechanism mediating this altered neutrophil function is unclear, although it has been suggested to relate to hyperglycemia (Lawson, et al., 2002). Hyperglycemia leads to persistent activation of neutrophils, as evidenced by the increased activity of neutrophil alkaline phosphatase (Geerlings and Hoepelman, 1999). Alternatively, neutrophils contribute significantly to the initiation and amplification of immune responses through their release of immunoregulatory cytokines (Lloyd and Oppenheim, 1992), including IL-6, TNF-α and interleukin 8 (IL-8).

5. Rational and hypothesis

Studies from our laboratory have demonstrated that exogenous MG induces $ONOO^-$ generation in VSMCs (Chang, et al., 2005; Wu, 2005; Wu and Juurlink, 2002). MG-induced oxidative damage is linked to the pathogenesis of T2DM and its vascular complications. Glucose, a precursor of MG, significantly induces $ONOO^-$ generation mediated by the formation of MG (Hsieh, et al., 2004). Like glucose, fructose is a monosaccharide and also a precursor of MG. It has been shown that a diet rich in fructose can increase blood pressure and induce insulin resistance although the mechanism is unclear (Hallfrisch, et al., 1983).

Mitochondria are major sources of $O_2^{\cdot -}$ generation. MG-modified mitochondria protein in streptozotocin-induced diabetic rat kidney cortex caused a significant increase in $O_2^{\cdot -}$ production (Rosca, et al., 2005). Administration of a MG scavenger improved mitochondrial respiration and decreased oxidative damage to mitochondrial proteins.

MG induced activation of NF-κB p65 as well as $ONOO^-$ production in mesenteric artery SMCs. MG-induced $ONOO^-$ production might be implicated in the activation of NF-κB p65 since the effect of MG was significantly inhibited by NAC, and H_2O_2 induced a similar activation of NF-κB in these cells (Wu, 2005). The activation of NF-κB by other oxidants, such as $O_2^{\cdot -}$, has also been observed in human endothelial cells and rat VSMCs (Ogata, et al., 2000; Canty, et al., 1999). In turn, activated NF-κB induces expression of genes encoding proinflammatory cytokines and mediators. Enhanced levels of proinflammatory cytokines and neutrophil dysfunction have been observed in type 2 diabetic patients.

These observations lead us to hypothesize that MG increases the production of $ONOO^-$ and enhances the levels of proinflammatory cytokines, which may contribute to the development of T2DM and its vascular complications.

6. Objectives and experimental approaches

I. *To investigate the relationships of fructose, MG and $ONOO^-$ production in rat aortic smooth muscle cells (A-10 cells).* We investigated whether fructose, a precursor of MG, induced $ONOO^-$ generation and whether this process was mediated via endogenously increased MG formation. MG levels were measured in A-10 cells cultured in the presence and absence of fructose (2.5-30 μM) for 3-24 h. The generation of $ONOO^-$ was evaluated in the cells treated with fructose or MG in the presence and absence of N-acetyl-L-cysteine (NAC, anti-oxidant) or GSH (scavenger of MG). Since $ONOO^-$ is formed by the reaction of $O_2^{\cdot -}$ with NO at a near equimolar ratio, the fructose- or MG-induced $ONOO^-$ formation was measured in the presence and absence of N(G)-nitro-Larginine methyl ester (L-NAME, an NOS inhibitor), superoxide dismutase (SOD, a $O_2^{\cdot -}$ scavenger), or diphenylene iodonium (DPI, an NAD(P)H oxidase inhibitor). Additionally, the MG-induced expression of iNOS in A-10 cells was also explored.

II. *To test the effect of MG on mitochondria with specific focus on $ONOO^-$ production and mitochondrial enzyme functions in A-10 cells.* Changes in mitochondrial ROS, particularly $ONOO^-$ production, expression of MG-induced CEL, activity of mitochondrial complexes I-IV, MnSOD activity and mitochondrial ATP production in A-10 cells in the presence of exogenous MG (5-100 μM) were investigated. AGEs cross-link breaker alagebrium and non-specific antioxidant NAC were also used in this study.

III. *To determine the effect of MG on cytokine production by, and apoptosis of, neutrophils from type 2 diabetic patients.* Plasma MG levels were measured in type 2 diabetic patients with varying glycated hemoglobin (HbA1c), fasting plasma glucose, and urine albumin / creatinine ratios (UACRs). Proinflammatory cytokines, such as IL-6, IL-8 and TNFα as well as the apoptotic status of neutrophils were determined following different *in vitro* MG treatments.

CHAPTER 2

GENERAL METHODOLOGY

VASCULAR SMOOTH MUSCLE CELL CULTURE

Rat thoracic aortic smooth muscle line (A-10 cells) was obtained from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% bovine serum (BS) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. A-10 cells were seeded either in 100 mm dishes for MG measurement and mitochondria isolation or in 96-well plates for other assays, with an equal amount of cells (10^6/ml) in each well, and cultured to confluence. For cell staining, cells were seeded on cover glass slides or 35 mm glass-bottomed dishes (10^6/ml). Cells were starved in BS-free DMEM for 24 h before exposure to different treatments.

MG MEASUREMENT

There are some reasons leading to the difficulties of MG determination: (1) amount of MG is very little in biological samples, particularly in tissues; (2) multiple and complicated steps are required to purify biological samples in order to quantify MG levels; (3) commercially available MG contains impurities which interfere the accuracy of the result; (4) MG is highly active and readily reacts with other components in samples, leading to the instability of MG levels. Several methods are developed to measure MG levels in biological samples. Most of those methods use High Performance Liquid Chromatography (HPLC). The methods to detect MG amount can be divided into several steps: (1) sample deproteinization; (2) incubation with derivatization agent; (3) chromotographic analysis.

In the presence of proteins, most MG is reversibly bound with proteins and only about 1% of it is free (Lo, et al., 1994). Therefore, it is necessary to use deproteinization

agent, such as perchloric acid (PCA) to liberate bound MG (Lo, et al., 1994). Low pH prevents degradation of DHAP and G-3-P to MG through phosphate elimination, thus, only an acidic agent is suitable to deproteinize MG. PCA is widely used also because many precursors of MG, such as glucose, pyruvate and D-lactate do not increase MG levels in the presence of PCA (McLellan, et al., 1992). However, oxidization of nucleic acid by PCA significantly increases MG amount. Therefore, PCA-precipitated pellet has to be removed by centrifugation before derivatization in order to reduce the interference of nuleic acid. 5-methylquinoxaline (5-MQ) is used as an internal standard because it cannot be formed by nuleic acid degradation and it is available commercially (Chaplen 1996). After deproteinization, MG has to be derivatized because it is impossible to measure MG directly. In most HPLC methods, MG is derivatized to quinoxalines, which can be monitored by a UV detector or fluorescence detector (Chaplen, et al., 1996; Akira, et al., 2004). *o*-phenylenediamine (*o*-PD) is widely used as a derivatization agent (Chaplen 1996, Cameron, D. C). Incubation has to be conducted in the dark to prevent the degradation of other compounds which interferes with the derivatization. In the last step, MG analysis is performed on the HPLC column which is eluted by water with phosphate buffer and acid. Levels of MG vary widely depending on different samples.

In our lab, MG content was determined using an *o*-PD method with modification. In brief, samples were incubated on ice for 10 min with 1/4 volume of PCA and centrifuged (12,000 rpm, 15 min) to remove the PCA-precipitated pellet. The supernatant was supplemented with 100 mM *o*-PD and incubated for 3 h at room temperature. The quinoxaline derivative of MG (2-methylquinoxaline, 2-MQ) and the quinoxaline internal standard (5-methylquinoxaline, 5-MQ) were measured using a Nova-

Pak ® C18 column (3.9 × 150 mm, and 4 μm particle diameter, MA, USA) equipped with a Hitachi high-performance liquid chromatography (HPLC) system (Hitachi Ltd., Mississauga, ON, Canada). The mobile phase was composed of 80% of 10 mM NaH_2PO_4 (pH 4.5) and 20% of HPLC grade acetonitrile in volume. The analysis conditions were as follows: detector wavelength, 315 nm; the flow rate of mobile phase, 1.0 ml/min; typical sample size, 130 μl. Duplicate injections of each sample were made. Samples were calibrated by comparison with 5-MQ standards.

MITOCHONDRIA ISOLATION

Following the instructions of mitochondrion isolation kit from Sigma (Oakville, ON, Canada), cells were lysed using cell lysis solution (1:150, 5 min) and suspended in extraction buffer A. Unbroken cells and nuclei were pelleted by centrifugation at 600 g for 10 min. The supernatant was centrifuged at 15,000 g for 15 min, and the mitochondrial pellet was resuspended in celLytic M cell lysis reagent for MG measurement. The mitochondrial pellet was resuspended in extraction buffer A and freeze-thawed twice for mitochondrial complexes activity determination. Cytochrome C Oxidase Assay Kit from Sigma-Aldrich (Oakville, ON, Canada) was used to determine the integrity of isolated mitochondria. Cytochrome C oxidase is located on the inner mitochondrial membrane and has traditionally been used as a marker for this membrane (Duan, et al., 2003). The activity of cytochrome C oxidase in isolated mitochondria was high, indicating the high integrity and purity of the preparation.

ROS DETERMINATION

$ONOO^-$

The formation of $ONOO^-$ was determined by a DCFH assay. Briefly, cells were loaded with a membrane-permeable, nonfluorescent probe CM-H_2DCFDA (5 μM) for 2 h at 37 °C in BS-free DMEM in the dark. After washing with PBS 3 times, cells were treated with different agents for 6 h, and finally subjected to detection. Once inside the cells, CM-H_2DCFDA becomes membrane-impermeable $DCFH_2$ in the presence of cytosolic esterases, and is further oxidized by $ONOO^-$ to form oxidized DCF which has detectable fluorescence. Oxidized DCF was quantified by monitoring the DCF fluorescence intensity with excitation at 485 nm and emission at 527 nm utilizing a Fluoroskan Ascent plate reader (Thermo Labsystem) and Ascent software, and expressed in arbitrary units.

Cellular NO

DAF-FM is a newly developed reagent for quantification of low concentrations of NO. Cells were preloaded with cell permeable, nonfluorescent DAF-FM (5 μM) in Kreb's buffer for 2 h at 37 °C. After removal of the excess probe and treatment of the cells with different agents, NO production was determined by DAF-fluorescence intensity with excitation at 495 nm and emission at 515 nm in a Spectra MAX Gemini XS plate reader (Molecular Devices) and expressed in arbitrary units. Like CM-H_2DCFDA, DAF-FM is deacetylated by intracellular esterases and then reacts with NO to form a fluorescent benzotriazole (DAF fluorescence).

Plasma NO

Plasma NO was determined as the total concentration of nitrate and nitrite in the plasma using a nitrate/nitrite fluorometric assay kit (Cayman Chemical, Ann Arbor, MI, USA). The fluorescence was detected at excitation and emission wavelengths of 375 nm and 415 nm, respectively, using a Fluoroskan Ascent plate reader (Thermo Labsystem, Amsterdam, Netherlands).

Cellular $O_2^{\cdot -}$

Cellular $O_2^{\cdot -}$ production was measured by lucigenine enhanced chemiluminescence. A-10 cells (10^6/ml) in counting vials were first treated with different agents at 37 °C and then mixed with 25 μM lucigenin for 15 min before being subjected to detection. $O_2^{\cdot -}$ was measured by chemiluminescence intensity detected with a luminomiter (TD-20/20, Tunner Designs, CA, USA) and expressed in arbitrary units.

Mitochondrial ROS (mtROS) and mitochondrial $O_2^{\cdot -}$

The levels of mtROS were determined using molecular probe MitoTracker Red (Busik, et al., 2008). The specific probe MitoSOX was used to detect mitochondrial $O_2^{\cdot -}$ (Schroeder, et al., 2007). A-10 cells were seeded on 35 mm glass-bottom dishes and treated with different agents for 18 h. Then, cells were labeled with MitoTracker Red (300 μM, 15 min) or MitoSOX (2 μM, 20 min). After washing, cells were bathed in DMEM again and subjected to examination under a Confocal Laser Scanning Biological Microscope (Olympus Fluoview 300, Olympus America Inc., Melville, NY, USA) coupled with 40× objective lens. The exposure time of the camera, the gain of the

amplifier and the aperture were fixed at 4.57s/scan, 4.0× and 3 respectively, to allow quantitative comparisons of the relative fluorescence intensity of the cells between groups. 10-14 cells were randomly collected from 4 different pictures of each group. The average fluorescence intensity of each cell was measured using an Image J program (NIH, USA). Data were expressed as mean ± SEM of the fluorescence intensity of those cells.

MEASUREMENT OF MnSOD ACTIVITY

SOD activity of A-10 cells was detected following the instruction of the SOD assay kit from Cayman Chemical (Ann Arbor, MI, USA). KCN at 3 mM was used to inhibit the activity of Cu / Zn SOD, leaving only MnSOD activity to be measured.

IMMUNOCYTOCHEMISTRY

Cells were fixed in 4% formalin for 1 h at room temperature. After permeation with 0.1% Triton X-100 for 5 min, fixed cells were incubated with 3% goat serum for 1 h, and then incubated with primary antibody (anti-iNOS, 1:500; anti-CEL, 1:100; anti-nitrotyrosine, 1:200) at 4°C overnight. Cells then were washed in PBS (0.01 M) for 15 min. For iNOS staining, cells were incubated with diluted biotinylated secondary antibody for 1 h. After washing with PBS, cells were subjected to detection by a Vectastain ABC kit (Vector Laboratories) according to the provided protocol and read using a normal light microscope. For CEL and nitrotyrosine staining, cells were

incubated with diluted fluorescent secondary antibody (FITC-IgG, 1: 200) for 3 h at room temperature. After washing with PBS, cells were mounted on glass slides and observed under a confocal microscope. Fluorescence intensity was measured using Image J program.

DETECTION OF MITOCHONDRIAL COMPLEX ACTIVITY

Mitochondrial complex I activity was determined by monitoring the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm with the addition of assay buffer (10 × buffer containing 0.5 M Tris-HCl at pH 8.1, 1% BSA, 10 μM antimycin A, 3 mM KCN, 0.5 mM coenzyme Q_1) (Long, et al., 2006). Mitochondrial proteins (25 μg/ml) and DCPIP (64 μM) were added to the assay buffer before using. The reaction was started by adding 200 μM NADH and scanned at 600 nm with the reference wavelength of 620 nm for 2 min. Mitochondrial complex III activity was detected by monitoring the reduction of cytochrome C at 550 nm upon the addition of assay buffer (10× buffer contains 0.5 M Tris-HCl at pH 7.8, 2 mM NaN_3, 0.8% Tween-20, 1% BSA, 2 mM decylubiquinol) with 40 μM cytochrome C (Long, et al., 2006). The reaction was started by adding 20 μg/ml mitochondria proteins to the assay buffer and scanned at 550 nm with the reference wavelength of 540 nm for 2 min. Mitochondrial complex IV activity was measured by monitoring the reduction of reduced cytochrome C at 550 nm with the addition of assay buffer (0.5 M phosphate buffer at pH 8.0, 1% BSA and 2% tween) (Long, et al., 2006). Freshly prepared reduced cytochrome C (80 μM) was added to the assay buffer before using. The reaction was started by adding mitochondria protein (20 μg/ml) and scanned

at 550 nm with the reference wavelength of 540 nm for 2 min. All assays were performed at 37 ºC.

ATP DETERMINATION

ATP synthesis was assayed based on a modified method of Atorino *et al.* (Atorino, et al., 2003). Briefly, cells were incubated at 37°C for 30 min in a respiratory buffer (0.02% digitonin, 0.25 M sucrose, 20 mM MOPS, 1 mM EDTA, 5 mM $NaPO_3$, 0.1 % fatty acid-free BSA, 1 mM ATP-free ADP, 5 mM glutamate, and 5 mM malate, pH 7.4). Thereafter, 3% PCA was used to precipitate proteins, and samples were centrifuged at 13,000 rpm for 2 min. Supernatants were taken out to measure ATP after pH was adjusted to 7.8 using 10 M KOH. Data were expressed as nanomoles of ATP per milligram of protein.

HUMAN SUBJECTS

All participants provided informed consent. Twenty non-diabetic subjects and fifty-five T2DM patients were included in this study. Non-diabetic subjects were normal healthy humans. All participants were free of infection and not under any anti-infective or anti-inflammatory medication. The study protocol was approved by the Research Ethics Boards of the University of Saskatchewan and the Saskatoon Health Region.

NEUTROPHIL ISOLATION

Human blood neutrophils were isolated from whole blood using a modification of the method of Gordon *et al*. (Gordon, et al., 2005). In brief, equal volumes of whole

blood and 6% dextran (Abbort Laboratories, QC, Canada) were mixed, then the red blood cells (RBCs) were allowed to sediment for at least 1 h. The leukocyte-rich supernatant was removed and underlaid with 3 mL of density gradient medium (Lymphoprep, Axis-Shield, Oslo, Norway) then centrifuged at 1300 rpm for 30 min. The cell pellet was suspended in 0.2% NaCl for 20 s to quickly lyse the RBCs, then 1.6% NaCl was added to correct the osmolarity of the medium. The cells were sedimented (1300 rpm, 10 min) and the neutrophilic pellet was resuspended in PBS. The neutrophils were washed once and counted.

QUANTIFICATION OF FASTING PLASMA GLUCOSE, BLOOD HbA1c, URINE ALBUMIN AND CREATININE

The measurements of levels of fasting plasma glucose, blood HbA1c, urinary albumin, and creatinine were performed by Chemistry Laboratory at the Royal University Hospital, University of Saskatchewan. Fasting plasma glucose was measured using glucose oxidase method on a Beckman Synchron LX20 (Beckman, Palo Alto, CA, USA). Blood HbA1c was quantitated by ion-exchange high performance liquid chromatography (HPLC) (Bio-Rad Variant II). Urinary albumin (mg/l) and creatinine (mmol/l) were determined by nephelometry (Beckman Array™ Protein System) and the Jaffe rate reaction method (Beckman, Palo Alto, CA, USA), respectively. The unit mg/mmol was used to express UACRs.

CYTOKINE DETERMINATION

Neutrophils (2×10^6/ml) were incubated at 37 °C for 12 h in the presence or absence of MG or other reagents in PBS. After incubation, the supernatant was collected and the levels of tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), and interleukin-6 (IL-6) were determined using a capture ELISA as described previously (Gordon, et al., 2000). Human cytokine-specific ELISA capture detection antibody pairs (R&D, Minneapolis, MN, USA) and 96-well immulon-4 ELISA plates (Dynatech laboratories Inc., Chantilly, VA, USA) were used as recommended by the suppliers. The final steps comprised of incubation with a 1:1000 dilution of streptavidin-conjugated horseradish peroxidase (Gibco, Burlington, Ontario, Canada) followed by the reactions with ABTS–peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). The absorbance was read at 405 nm. Each sample and the standards were run in duplicate.

APOPTOSIS DETECTION

Apoptosis of neutrophils was detected using an active Caspase 3 antibody apoptosis kit (BD, Pharmingen, NJ, USA). Neutrophils (2×10^6/mL) were analyzed by flow cytometry (Beckman Coulter Epics XL, Mississauga, ON, Canada).

CHAPTER 3

FRUCTOSE-INDUCED PEROXYNITRITE PRODUCTION IS MEDIATED BY METHYLGLYOXAL IN VASCULAR SMOOTH MUSCLE CELLS

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ABSTRACT

Methylglyoxal (MG), a highly reactive molecule, has been implicated in the development of insulin resistance. We investigated whether fructose, a precursor of MG, induced $ONOO^-$ generation and whether this process was mediated via endogenously increased MG formation. Fructose significantly increased MG generation in vascular smooth muscle cells (VSMCs) in a concentration and time dependent manner. The intracellular production of MG was increased by 153 ± 23% or 259 ± 28% after cells were treated 6 h with fructose (15 mM or 30 mM), compared with production from untreated cells ($p < 0.01$, $n = 4$ for each group). A significant increase in the production of $ONOO^-$, NO, and $O_2^{\cdot -}$, was found in the cells treated with fructose (15 mM) or MG (10 μM). Fructose- or MG-induced $ONOO^-$ generation was significantly inhibited by MG scavengers, including reduced glutathione or *N*-acetyl-l-cysteine, and by $O_2^{\cdot -}$ or NO inhibitors, such as diphenylene iodonium, superoxide dismutase or *N*-nitro-l-arginine methyl ester. Moreover, an enhanced iNOS expression was also observed in the cells treated directly with MG which was significantly inhibited when co-application with *N*-acetyl-l-cysteine. Our results demonstrated that fructose is capable of inducing a significant increase in $ONOO^-$ production, which is mediated by an enhanced formation of endogenous MG in VSMCs.

Key Words: Methylglyoxal; Fructose; Smooth muscle cell; Peroxynitrite

INTRODUCTION

Increased levels of methylglyoxal (MG) in circulation and tissue have been documented in the insulin resistance syndrome which includes diabetes and hypertension. MG formation is increased in diabetic patients and in cultured human red blood cells under hyperglycemic conditions (Kalapos, 1999). Biochemical and clinical evidence suggests that increased formation of MG in diabetes mellitus is linked to the development of diabetic complications such as micro-vascular damage in the eyes or kidneys (retinopathy or nephropathy), but the exact pathogenic role of this dicarbonyl remains largely unknown. An age-dependent increase in blood pressure coinciding with an elevated MG level in genetic hypertensive rats has also been observed (Wang, et al., 2005; Wang, et al., 2004), even when blood glucose levels were within physiological ranges (Wang, et al., 2004; Chen, et al., 1994; Schmidt, et al., 2004). Like glucose, fructose is a monosaccharide and a precursor of MG. The consumption of fructose or sucrose (1 glucose + 1 fructose) sweeteners used in beverages and processed foods has increased significantly in the last two decades. It has been shown that a diet high in fructose or sucrose can increase blood pressure and induce insulin resistance although the mechanism is unclear (Hallfrisch, et al., 1983; Israel, et al., 1983; Reiser, et al., 1989; Reaven, 1991). Significant increase of serum fructose to 12 μM in diabetic patients compared with a level of 8.1 μM in healthy subjects has been reported (Kawasaki, et al., 2002). An increased activity of aldose reductase, the enzyme which reduces glucose into fructose, has also been linked to the enhanced $ONOO^-$ production in diabetic rats (Obrosova, et al., 2005). As is well known, $ONOO^-$ is an active oxidant and an increase in $ONOO^-$ generation might cause cellular dysfunction through inactivation of enzymes

or ion channels, and/or disturbance of mitochondrial respiration (Virag, et al., 2003). $ONOO^-$ can also mediate the addition of nitrate onto tyrosine residues of proteins to form nitrotyrosine, which has been noted to be elevated in the early phase of type 1 diabetes (Hoeldtke, et al., 2003).

The effect of fructose on $ONOO^-$ production and MG involvement on fructose-mediated $ONOO^-$ formation were postulated, but the actual effects were unknown. To investigate these important regulatory mechanisms of cellular functions, MG levels were measured in VSMCs treated with or without fructose (2.5–30 mM) for 3–24 h. The generation of $ONOO^-$ was evaluated in the cells treated with fructose or MG in the presence or absence of *N*-acetyl cysteine or glutathine (scavenger of MG). Since $ONOO^-$ is formed by the reaction of $O_2^{\cdot -}$ with NO at a near equimolar ratio, the fructose- or MG-induced $ONOO^-$ formation was measured with and without the presence of *N*-nitro-l-arginine methyl ester (an NOS inhibitor), superoxide dismutase (a $O_2^{\cdot -}$ scavenger), or diphenylene iodonium (a NAD(P)H oxidase inhibitor). Additionally, the MG-induced expression of iNOS in A-10 cells was also explored.

MATERIALS AND METHODS

VSMC culture

Rat thoracic aortic SMC line (A-10 cells) was obtained from American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% bovine serum (BS) at 37 °C in a humidified atmosphere of 95% air and

5% CO_2, as described in our previous studies (Chang, et al., 2005; Wu, 2005). A-10 cells were seeded either in 100 mm dishes for MG measurement or in 96-well plates for other assays, with an equal amount of cells (10^6/ml) in each well, and cultured to confluence. For immunocytochemistry staining, cells were seeded on cover glass slides (2×10^6/ml). Cells were starved in BS-free DMEM for 24 h before exposure to different treatments.

MG assay

MG was measured by an *o*-PD method as described previously (Wang, et al., 2004; Chaplen, et al., 1998). In brief, cell pellets were resuspended into ice-cold phosphate buffered saline (PBS), and lysed over ice by sonication (5 s, three times). The sample was then incubated on ice for 10 min with 1/4 volume of perchloric acid (PCA) and centrifuged (12,000 rpm, 15 min) to remove the PCA-precipitated material. The supernatant was supplemented with 100 mM *o*-PD and incubated for 3 h at room temperature. The quinoxaline derivative of MG (2-methylquinoxaline) and the quinoxaline internal standard (5-methylquinoxaline) were measured via Nova-Pak® C18 column (3.9 × 150 mm, and 4 μm particle diameter, MA, USA), with Hitachi high-performance liquid chromatography (HPLC) system (Hitachi, Ltd., Mississauga, ON, Canada).

Measurement of $ONOO^-$

The formation of $ONOO^-$ was determined by a DCFH assay as described previously (Chang, et al., 2005). Briefly, cells were loaded with a membrane-permeable,

nonfluorescent probe CM-H_2DCFDA (5 μM) for 2 h at 37 °C in BS-free DMEM in the dark. After washing with PBS 3 times, cells were treated with or without fructose plus other agents for 6 h, and finally subjected to detection. Once inside the cells, CM-H_2DCFDA becomes membrane-impermeable $DCFH_2$ in the presence of cytosolic esterases, and is further oxidized by $ONOO^-$ to form oxidized DCF which has detectable fluorescence. Oxidized DCF was quantified by monitoring the DCF fluorescence intensity with excitation at 485 nm and emission at 527 nm utilizing a Fluoroskan Ascent plate reader (Thermo Labsystem) and Ascent software, and expressed in arbitrary units.

Determination of NO

DAF-FM is a newly developed reagent for quantification of low concentration of NO. As described in our earlier study (Chang, et al., 2005), cells were preloaded with cell permeable, nonfluorescent DAF-FM (5 μM) in Kreb's buffer for 2 h at 37 °C. After removal of the excess probe and treatment of the cells with different agents, NO production was determined by DAF-fluorescence intensity with excitation at 495 nm and emission at 515 nm in a Spectra MAX Gemini XS plate reader (Molecular Devices) and expressed in arbitrary units. Like CM-H_2DCFDA, DAF-FM is deacetylated by intracellular esterases and then reacts with NO to form a fluorescent benzotriazole (DAF fluorescence).

Detection of $O_2^{\cdot -}$

$O_2^{\cdot -}$ production was measured by lucigenine enhanced chemiluminescence (Wu, et al., 2001). A-10 cells (10^6/ml) in counting vials were first treated with different agents

at 37 °C and then mixed with 25 μM lucigenin for 15 min before being subjected to detection. $O_2^{\cdot -}$ was measured by chemiluminescence intensity detected with a luminometer (TD-20/20, Tunner Designs, CA, USA) and expressed in arbitrary units.

Immunocytochemistry staining

A-10 cells were seeded on glass cover slips followed by treatment with MG or MG plus NAC for 18 h, and subjected to iNOS staining. As described previously (Wu, 2005), treated cells were fixed in 4% formalin for 30 min at room temperature. After permeation with 0.1% Triton X-100 for 30 min, fixed cells were incubated with blocking solution for 1 h, and then incubated with iNOS antibody (1:500; BD transduction laboratories) at room temperature for 2 h. Cells were washed in PBS (0.01 M) for 5 min and incubated with diluted biotinylated secondary antibody for 1 h After washing with PBS, cells were subjected to detection by a Vectastain ABC kit (Vector Laboratories) according to the provided protocol.

Chemicals

Methylglyoxal (MG), *o*-phenylenediamine (*o*-PD), 5-methylquinoxaline, lucigenin, diphenylene iodonium (DPI), superoxide dismutase (SOD), *N*-acetyl-L-cysteine (NAC), reduced glutathione (GSH), *N*-nitro-l-arginine methyl ester (L-NAME), 4, 5-dihydroxy-1,3-benzene-disulfonic acid (Tiron) and mannitol were purchased from Sigma (Oakville, ON, Canada). 2′,7′-dichlorodihydrofluorescein diacetate (DCFH) and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) were obtained from Invitrogen (Burlington, ON, Canada).

Statistical analysis

Data were obtained after the subtraction of the blank (PBS containing different probes in the absence of cells) and expressed as the mean ± SEM from at least three independent experiments. Statistical analysis was performed by the one way analysis of variance (ANOVA). Differences between groups was examined by the Student's unpaired *t*-Test. Values are considered to be statistically significant when $p < 0.05$.

RESULTS

Fructose induced MG generation

Fructose-induced MG production occurred in a concentration and time dependent manner in A-10 cells. When the cells were treated with fructose at the concentration of 15 mM or 30 mM for 6 h, a significant increase in MG formation by 153 ± 23% (4.63 ± 0.42 vs. 1.83 ± 0.26 nmol/mg protein, $p < 0.01$) or 259 ± 28% (6.57 ± 0.37 vs. 1.83 ± 0.26 nmol/mg protein, $p < 0.01$) was observed, in comparison to levels produced by untreated cells. Fructose treatment at 2.5 mM or 5 mM had no significant effect on intracellular MG generation ($p > 0.05$, $n = 4$ for each group, Fig. 3-1A). Fig. 3-1B demonstrates the effect of fructose treatment (15 mM or 30 mM) for 3, 6, 9, and 24 h, on levels of intracellular MG as compared with that of control group ($n = 4$ for each group). MG levels reached a peak at 6 h after fructose treatment and then gradually declined. At 24 h, the level of MG with 15 mM fructose treatment was still 108 ± 19% higher than that of the controls (3.82 ± 0.37 vs. 1.83 ± 0.26 nmol/mg protein, $p < 0.05$, $n = 4$ for each

group); and 30 mM fructose treatment was 197 ± 33% higher than that of the control (5.44 ± 0.46 vs. 1.83 ± 0.26 nmol/mg protein, $p < 0.05$, $n = 4$ for each group). As shown in Fig 1C, the cells were also treated with mannitol (15 or 30 mM) for 6 or 24 h. This mannitol treatment did not cause any change in intracellular MG level, indicating that the fructose-induced MG generation is not due to osmotic changes in the medium.

Fructose induced $ONOO^-$ formation

The DCFH assay was used to measure the intracellular production of oxidized DCF which indicates the formation of $ONOO^-$. As shown in Fig. 3-2A, when the cells were treated with fructose (15 mM) for 6 h, there was a significant increase in the intensity of oxidized DCF by 49.5 ± 14.7% over that of the control group ($p < 0.05$, $n = 8$). This increase was effectively inhibited by 82.3 ± 11.2% when co-treated with GSH (1 mM) ($p < 0.05$, $n = 8$) or by 69.8 ± 17.4% when co-treated with NAC (600 μM) ($p < 0.05$, $n = 8$), in comparison with fructose (15 mM) treatment alone. There was no significant change in oxidized DCF production when the cells were treated with GSH (1 mM) or NAC (600 μM) alone (data not shown).

Since oxidized DCF could also come from the oxidation of $DCFH_2$ by H_2O_2 (Curtin, et al., 2002; Kooy, et al., 1997) and since the generation of $ONOO^-$ requires both NO and $O_2^{\cdot -}$, we treated A-10 cells with fructose (15 mM) in the presence or the absence of $O_2^{\cdot -}$ scavenger SOD, NAD(P)H oxidase inhibitor DPI, or NOS inhibitor L-NAME. In comparison with that from fructose-treated group (Fig. 3-2A), fructose-induced oxidized-

DCF formation was significantly decreased by 86.7 ± 15.6% ($p < 0.05$, $n = 8$) when co-treated with SOD (400 U/ml) or completely abolished when co-treated with DPI (100 μM) ($p < 0.05$, $n = 8$). L-NAME (100 μM) as shown in Fig. 3-2B also significantly reduced the production of fructose-induced oxidized DCF by 79 ± 8.7% ($p < 0.05$, $n = 16$), compared with that from fructose-treated group ($p < 0.05$, $n = 16$ in each group). There was no significant change in oxidized DCF production when cells were treated with SOD (400 U/ml), DPI (100 μM), and L-NAME (100 μM), respectively (data not shown).

Fructose induced generation of $O_2^{\cdot -}$

As shown in Fig. 3-3, the generation of $O_2^{\cdot -}$ increased by 67.8% ($p < 0.05$) in A-10 cells after incubation with fructose (15 mM) for 6 h, compared with that from untreated group. However, $O_2^{\cdot -}$ production in fructose treated cells was completely inhibited when the cells were co-treated with SOD (400 U/ml) ($p < 0.05$, $n = 6$) or DPI (100 μM) ($p < 0.01$, $n = 6$). When the cells were directly treated with MG (10 μM) for 6 h, a significant increase in $O_2^{\cdot -}$ formation was observed ($p < 0.01$, $n = 6$), which was decreased significantly in the presence of SOD (400 U/ml) or DPI (100 μM) ($p < 0.01$, $n = 6$). Neither SOD nor DPI alone had significant effects on the production of $O_2^{\cdot -}$ in A-10 cells (data not shown).

Fructose induced generation of NO

As shown in Fig. 3-4A, NO generation was significantly increased by 63 ± 10.2% after the cells were treated with fructose (15 mM) for 6 h (0.207 ± 0.03 vs. 0.127 ± 0.02,

$p < 0.01$, $n = 16$), in comparison with that from untreated group. Co-application of L-NAME (100 μM) completely prevented fructose-induced NO production ($p < 0.01$, $n = 16$ in each group, Fig. 3-4A). Similarly, increased NO formation was also observed in the presence of MG (10 μM, 6 h) and this MG-induced increase was significantly inhibited by co-application with L-NAME (100 μM) in A-10 cells ($p < 0.01$, $n = 8$, Fig. 3-4A). L-NAME alone had no significant effect on the production of NO in A-10 cells (data not shown).

In addition, application of MG tremendously increased the staining intensity of iNOS in A-10 cells compared with untreated control cells, in which only background levels of staining were observed (Fig. 3-4B). Co-application of NAC (600 μM) effectively prevented the increased iNOS staining induced by MG treatment, while application of NAC alone had no evident effect on iNOS staining in A-10 cells. Similar results were also observed from three other independent experiments.

DISCUSSION

Multiple studies have indicated an increased MG level in different insulin states including diabetes and hypertension (Wang, et al., 2005; Beisswenger, et al., 2005), supporting the importance of MG in the development of insulin resistance. The main source of MG in mammals is anaerobic glycolysis, i.e. non-enzymatic and enzymatic elimination of phosphate from glyceraldehyde-3-phosphate (G-3-P) and dihydroxyacetone phosphate (DHAP) (Koop and Casazza, 1985; Lyles and Chalmers,

1992). MG is also formed during various physiological processes including metabolism of acetone from lipolysis and the break down of threonine from protein catabolism (Lyles and Chalmers, 1992; Yu, et al., 2003). Fructose, a precursor of MG, is metabolized by hexokinase or ketohexokinase to G-3-P and DHAP, which directly form MG. Physiological concentration of fructose in serum is about 8.1 ± 1.0 μM (Kawasaki, et al., 2002). Under specific pathological conditions such as diabetes, its serum concentration can be as high as 12.0 ± 3.8 μM (Kawasaki, et al., 2002). The concentration of fructose used in our present study appears to be much higher than those reported *in vivo* concentrations. However, it is generally acknowledged that to replicate a biological reaction of certain endogenous substances in an *in vitro* system, significantly higher concentrations of these substances have to be used for two reasons. One is that the microenvironment in which the isolated cells are cultured is not optimized as the corresponding *in vivo* environment. Another consideration is that experiments on cultured cells usually take a much shorter time than *in vivo* observation so that a higher concentration is needed to compensate for this short reaction time. In this line, knowing that plasma concentration of methylglyoxal is about 5 μM, 100–500 μM of methylglyoxal was used to study its effect on Jurkat cells (Du, et al., 2001; Du, et al., 2000). MG has also been used at 1 mM on isolated rat pancreatic ß-cells to investigate its effect on Ca^{2+} concentration (Cook, et al., 1998), and at 0.1–10 mM to study its effect on insulin-secreting cells (Sheader, et al., 2001). Increased MG formation may occur when the availability of MG's precursors are increased or scavenging pathways are impaired. In this study, we showed that intracellular MG levels were significantly increased when VSMCs were incubated with fructose (Fig. 3-1). Generation of MG induced by fructose

occurred in a concentration and time dependent manner. Noticeably, type 2 diabetic patients are common with obesity and, 150–225 million people worldwide are affected with type 2 diabetes, according to a recent epidemiologic report (Zimmet, 2003). A diet with high sucrose or fructose has been observed to induce insulin resistance, although without clear mechanisms (Hallfrisch, et al., 1983; Israel, et al., 1983; Reiser, et al., 1989; Reaven, 1991). For instance, an increased blood pressure associated with hyperinsulinemia and hypertriglyceridemia in normal Sprague Dawley rats was observed after only 2 weeks on a high fructose (66%) diet (Hwang, et al., 1987).

MG is very electrophilic and tends to interact readily with certain arginine or lysine residues in proteins, leading to increased glycation of proteins, and therefore the yield of irreversible AGEs is accelerated. It has been shown that MG induces glycation of arginine residues of glutathione reductase, thus inactivating this enzyme and decreasing its scavenging ability for free radicals (Vander Jagt, et al., 1997). Our conclusion that fructose caused a significant increase in $ONOO^-$ (Fig. 2) in A-10 cells is supported by several lines of evidence: 1) The enhanced formation of oxidized DCF was significantly or completely inhibited by $O_2^{\cdot -}$ scavenger SOD or the NAD(P)H oxidase inhibitor DPI (Fig. 3-2A). 2) Fructose-induced oxidization of DCF was markedly inhibited by L-NAME (Fig. 3-2B). 3) Fructose directly increased the generation of NO and $O_2^{\cdot -}$ in A-10 cells, which was inhibited by either L-NAME, SOD or DPI (Fig. 3-3 and Fig. 3-4A). As is well known, $ONOO^-$ is formed by a reaction of NO and $O_2^{\cdot -}$. ONOO− can cross cell membranes-freely (Curtin, et al., 2002) and is an extremely strong and reactive oxidant (Beckman and Koppenol, 1996; Cai and Harrison, 2000). It has been reported that $ONOO^-$ impairs the sarcoplasmic reticulum Ca^{++} pump in pig

coronary artery smooth muscle (Sechi, et al., 1996) and triggers apoptosis in cultured rat aortic VSMCs (Li, et al., 2003). An increased production of $O_2^{\cdot -}$ with reduced NO function leads to an altered blood vessel tone and hypertension (Cuzzocrea, et al., 2004). Increased $O_2^{\cdot -}$ production in the heart, aorta, and polymorphonuclear cells in rats treated with high fructose (60% or 66%) diet was reported (Al-Awwadi, et al., 2005; Delbosc, et al., 2005) and this fructose-induced overproduction of $O_2^{\cdot -}$ was theorized to be linked to an overexpression of cardiac p22 phox (Delbosc, et al., 2005). Inhibition of NAD(P)H oxidase by apocynin restores the impaired endothelial-dependent and -independent responses in type 2 diabetes complicated by NO dysfunction (Hayashi, et al., 2005). The expression of NAD(P)H oxidase in VSMCs has been previously documented (Irani, 2000). The inhibition of fructose-induced $O_2^{\cdot -}$ production in A-10 cells by DPI, revealed in our present study, provided further evidence for the potential importance of NAD(P)H oxidase in redox balance in VSMCs. However, the role of NAD(P)H oxidase in this context cannot be concluded yet as DPI may also have molecular targets other than NAD(P)H oxidase.

In addition, the defects of the antioxidant system have been observed in the high fructose fed model. Activities of red cell Cu / Zn-SOD, Se-glutathione peroxidase and blood GSH are significantly lower in rats fed with high fructose (Faure, et al., 1999). In our study, oxidized DCF was significantly decreased after the co-application of fructose with MG scavenger GSH or NAC (Fig. 3-2). An increase in formation of NO or $O_2^{\cdot -}$ was observed when the cells were directly treated with 10 μM of MG (Fig. 3-3 and Fig. 3-4A). An enhanced iNOS positive staining was also observed when the cells were treated with MG, in comparison with the untreated group (Fig. 3-4B). Therefore, our data

clearly indicate that fructose-induced $ONOO^-$ is mediated by an increased formation of MG in VSMCs. It is tempting to portray MG as a linker between fructose metabolism and cellular signal transductions, or a regulator in redox tone and redox cell signaling.

In summary, our results indicate that fructose treatment increased the formation of $ONOO^-$ via increased NO and $O_2^{\cdot -}$ production in A-10 cells, and this effect was directly mediated by an elevated intracellular concentration of MG. An endogenous overproduction of MG and MG-induced ROS will eventually impair vascular function and contribute to the development of insulin resistance.

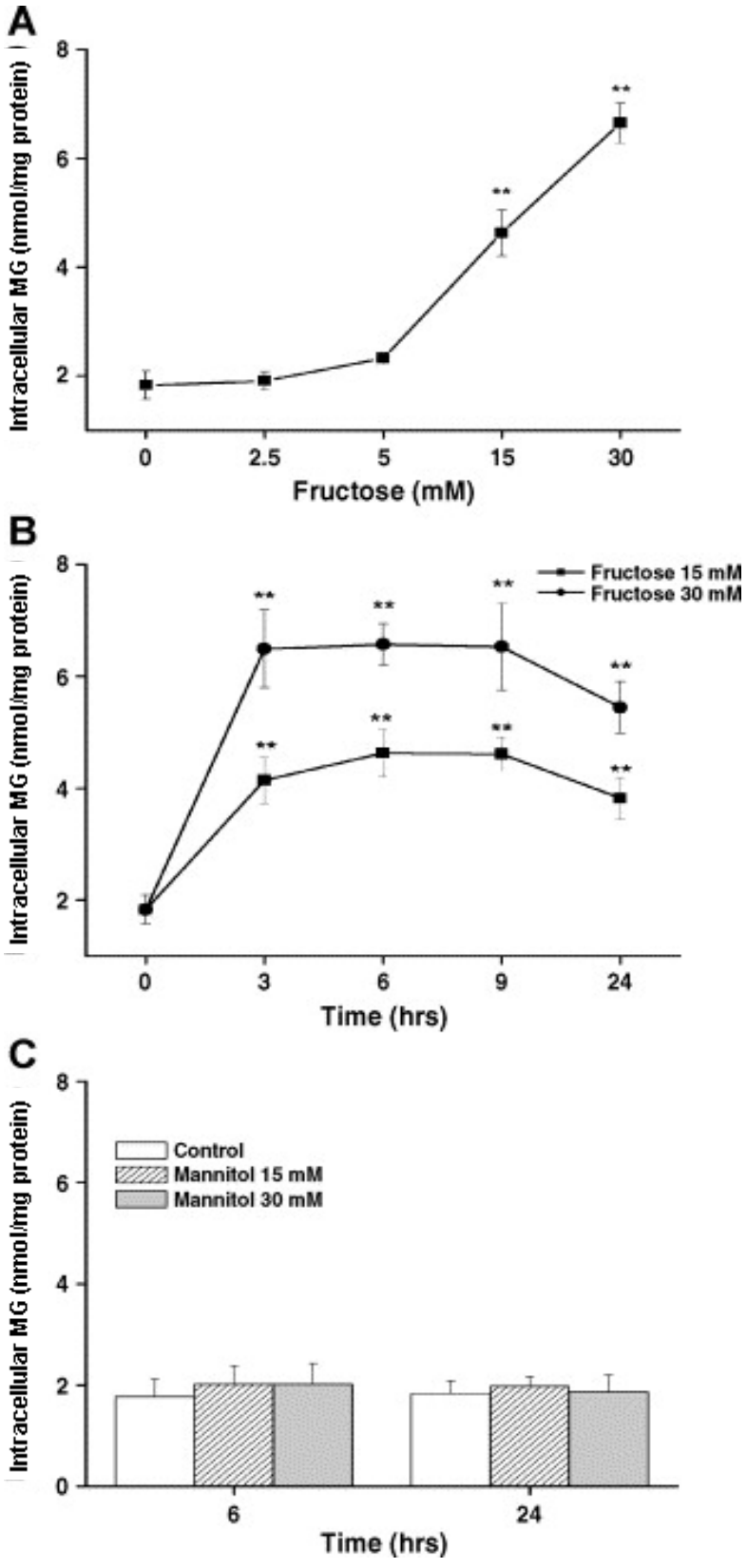

A
Intracellular MG (nmol/mg protein)
8
6
4
2
0
2.5
5
15
30
Fructose (mM)
**
B
Intracellular MG (nmol/mg protein)
Fructose 15 mM
Fructose 30 mM
0
3
6
9
24
Time (hrs)
C
Intracellular MG (nmol/mg protein)
Control
Mannitol 15 mM
Mannitol 30 mM
0
6
24
Time (hrs)

Figure 3-1. Concentration- and time-dependent MG generation in A-10 cells treated with fructose. A. MG levels in A-10 cells were assessed after 6 h incubation with fructose at the concentration of 0, 2.5, 5, 15 or 30 mM. B. MG levels in A-10 cells were measured after treatment with fructose (15 or 30 mM) for 0, 3, 6, 9, and 24 h, respectively. C. MG levels were determined after the cells were treated with mannitol (15 or 30 mM) for 6 or 24 h. $n = 4$ for each group in A and B. * $p < 0.05$ or ** $p < 0.01$ vs. control group.

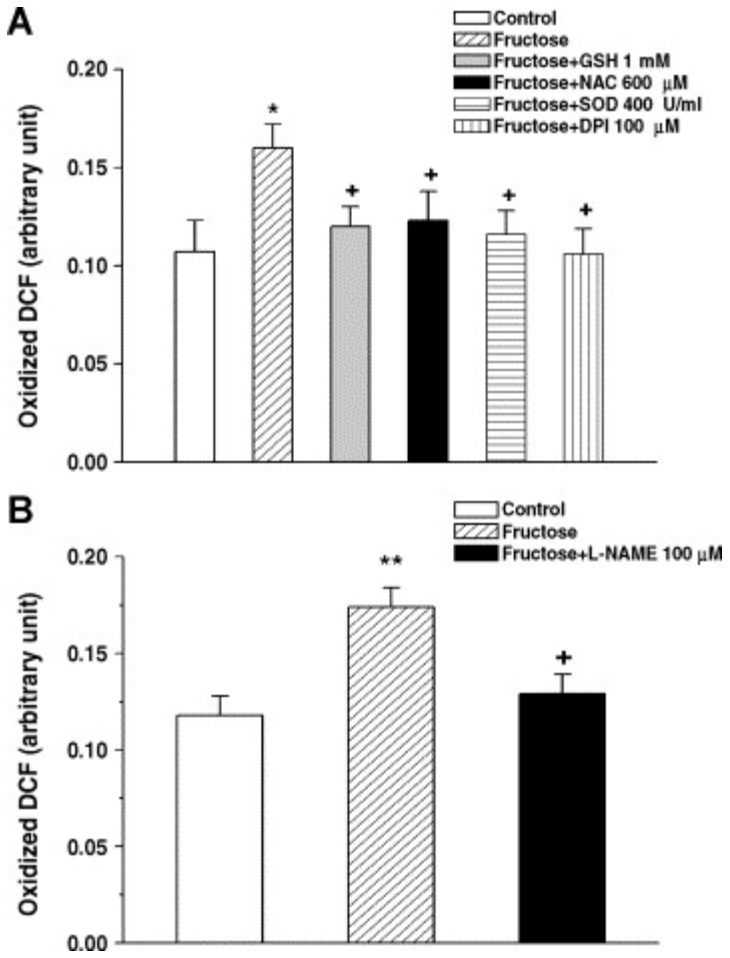


Figure 3-2. Effects of fructose and other agents on production of $ONOO^-$. A. The production of $ONOO^-$ was determined after A-10 cells were incubated for 6 h with fructose (15 mM) in the presence or absence of GSH (1 mM), NAC (600 μM), SOD (400 U/ml) or DPI (100 μM). B. Fructose (15 mM)-induced generation of $ONOO^-$ was quantified with or without L-NAME (100 μM). $n = 8$ for each group in A and $n = 16$ for each group in B. $*p < 0.05$ or $**p < 0.01$ vs. control group; $+p < 0.05$ vs. fructose group.

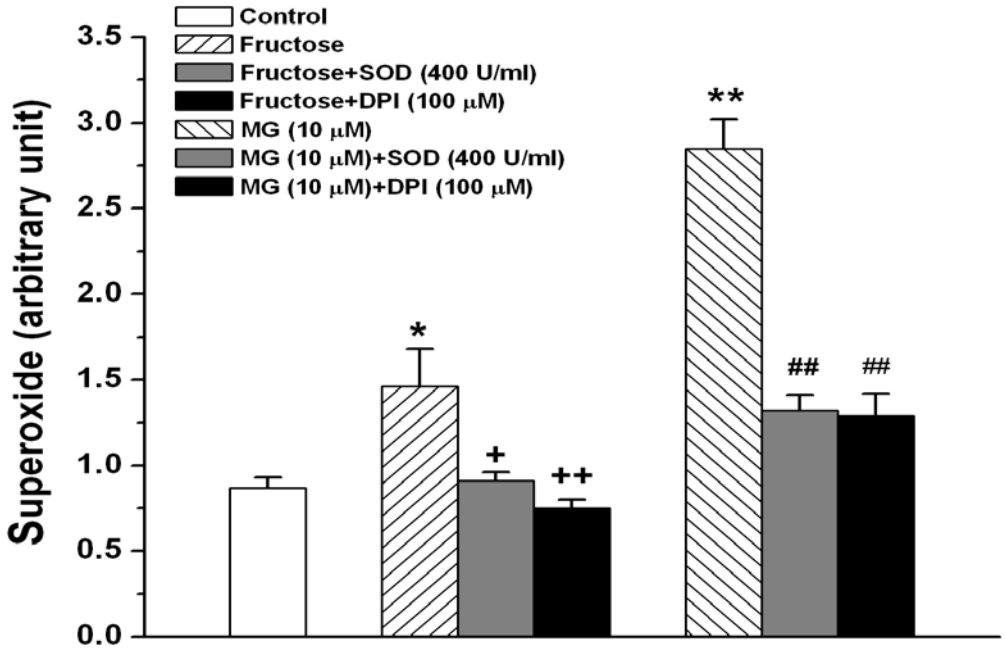
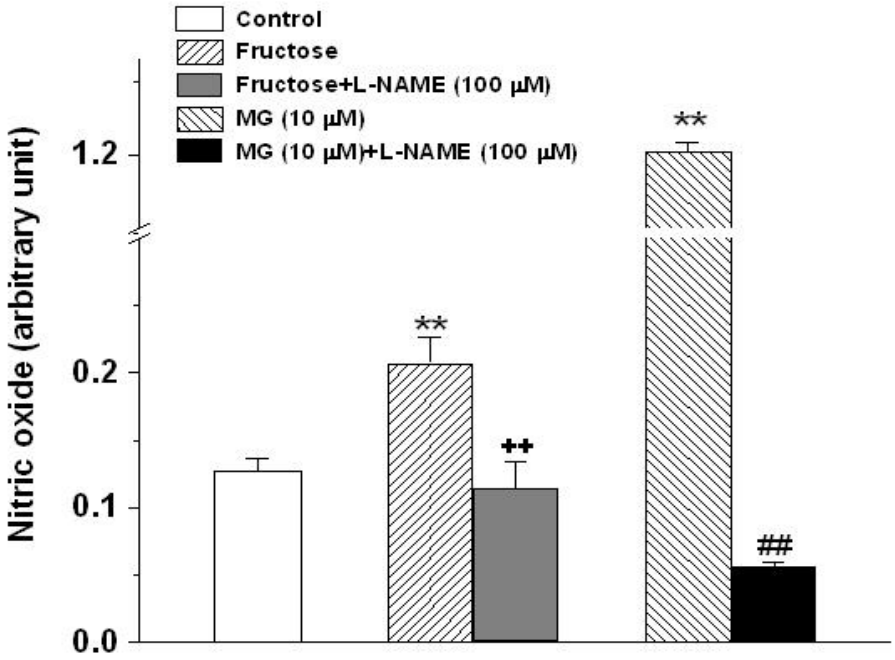


Figure 3-3. Effects of fructose, MG and other agents on $O_2^{\cdot -}$ production. $O_2^{\cdot -}$ was determined after A-10 cells were incubated for 6 h with fructose (15 mM) or MG (10 μM) in the presence or absence of SOD (400 U/ml) or DPI (100 μM). $n = 6$ for each group. $^{*}p < 0.05$ or $^{**}p < 0.01$ vs. control group; $+p < 0.05$ and $++p < 0.01$ vs. fructose group; $\#\#p < 0.01$ vs. MG group.

A


Control
Fructose
Fructose+L-NAME (100 μM)
MG (10 μM)
MG (10 μM)+L-NAME (100 μM)
Nitric oxide (arbitrary unit)
1.2
0.2
0.1
0.0
**
**
++
##

B

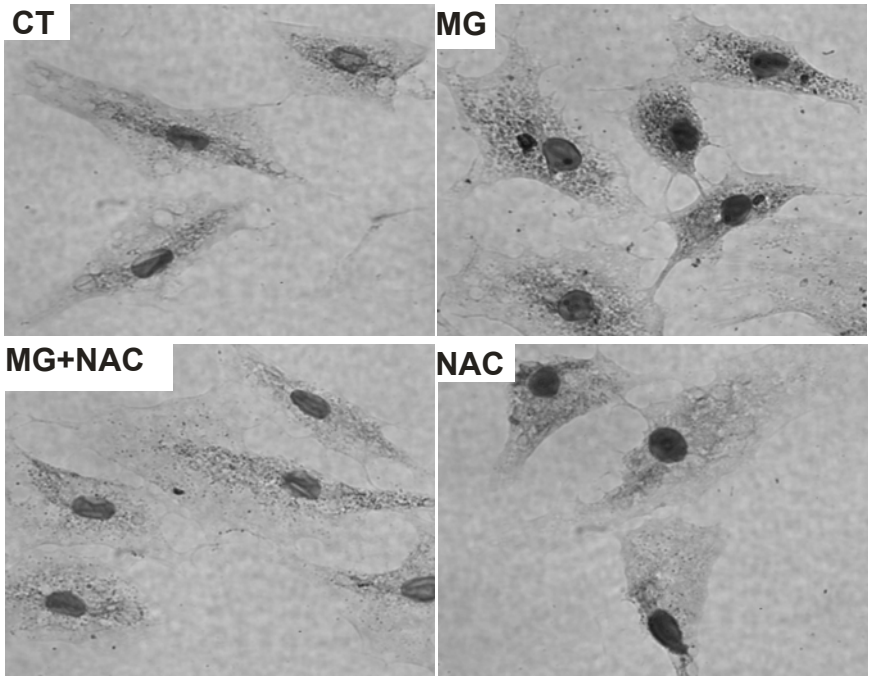

CT
MG
MG+NAC
NAC

Figure 3-4. Effects of fructose, MG and other agents on NO production. A. The concentration of NO was assayed after A-10 cells were incubated for 6 h with fructose (15 mM) or MG (10 μM) in the presence or absence of L-NAME (100 μM). $n = 16$ for fructose and fructose + L-NAME groups, $n = 8$ for MG and MG + L-NAME groups. ** $p < 0.01$ vs. control group, ++$p < 0.01$ vs. fructose group, and ##$p < 0.01$ vs. MG group. B. MG induced iNOS expression in A10 cells. A10 cells seeded on cover glass slips were treated with MG (100 μM) for 18 h, then stained with mouse anti-iNOS monoclonal antibody. More positive staining appeared in MG-treated cells and MG-induced iNOS expression was inhibited by co-applying NAC (600 μM).

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CHAPTER 4

METHYLGLYOXAL-INDUCED MITOCHONDRIAL DYSFUNCTION IN VASCULAR SMOOTH MUSCLE CELLS

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ABSTRACT

The mitochondrial effect of methylglyoxal (MG) with specific foci on peroxynitrite ($ONOO^-$) production, manganese superoxide dismutase (MnSOD) activity, and mitochondrial functions in vascular smooth muscle A-10 cells were investigated. Mitochondrial MG content was significantly increased after A-10 cells were treated with exogenous MG, and so did advanced glycated endproducts (AGEs) formation, indicated by the appearance of N^{ε}-(carboxyethyl) lysine, in A-10 cells. The levels of mitochondrial reactive oxygen species (mtROS) and $ONOO^-$ were significantly increased by MG treatment. Application of $ONOO^-$ specific scavenger uric acid lowered the level of mtROS. MG significantly enhanced the production of mitochondrial superoxide ($O_2^{\cdot-}$) and nitric oxide (NO), which were inhibited by SOD mimic 4-hydroxy-tempo and mitochondrial nitric oxide synthase (mtNOS) specific inhibitor 7-nitroindazole, respectively. The activity of MnSOD was decreased by MG treatment. Furthermore, MG decreased respiratory complex III activity and ATP synthesis in mitochondria, indicating an impaired mitochondrial respiratory chain. AGEs cross-link breaker alagebrium reversed all aforementioned mitochondrial effects of MG. Our data demonstrated that mitochondrial function is under the control of MG. By inhibiting complex III activity, MG induces mitochondrial oxidative stress and reduces ATP production. These discoveries will help unmask molecular mechanisms for various MG-induced mitochondrial dysfunction-related cellular disorders.

Key Words: methylglyoxal; mitochondria; peroxynitrite; complex III; alagebrium

INTRODUCTION

Mitochondria are the powerhouse of mammalian cells. When electrons pass through complexes I – IV of the electron transport chain (ETC), 2-5% of electrons leak out of the ETC and interact with oxygen to form superoxide ($O_2^{\cdot -}$) in mitochondria, which accounts for about 85% of total intracellular $O_2^{\cdot -}$ (Chance, et al., 1973; Droge, 2002). Electron leakage most often occurs at complex I and complex III of the ETC, and the amount of $O_2^{\cdot -}$ increases dramatically if these complexes are inhibited (Turrens, 2003). Under physiological condition, $O_2^{\cdot -}$ is converted to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (MnSOD), which is the primary antioxidant defensive enzyme in mitochondria (Li, et al., 2006). This anti-oxidant system ensures the clearance of free radicals and protects cells against oxidative damage. Mitochondria also contain specific nitric oxide synthase (mtNOS), which catalyzes the production of nitric oxide (NO) (Epperly, et al., 2007). A considerable amount of NO generated from mtNOS reacts with $O_2^{\cdot -}$ to form peroxynitrite ($ONOO^-$) (Dedkova, et al., 2004). $ONOO^-$ is a highly reactive oxidant, damaging proteins, DNA, and lipids (Valko, et al., 2007). Mitochondrial oxidative stress is tightly related to the pathophysiology of type 2 diabetes and associated complications (Kim, et al., 2008).

Methylglyoxal (MG) is a dicarbonyl compound which readily reacts with certain proteins to form advanced glycated endproducts (AGEs), like *N*-carboxyethyl-lysine (CEL). This rapid interaction contributes to the pathogenesis of insulin resistance syndrome, such as diabetes and hypertension (Wang, et al., 2008; Goh and Cooper, 2008;

Jia, et al., 2006). We have previously shown that MG induced the generation of reactive oxygen species (ROS) in hypertensive rat vascular smooth muscle cells (VSMCs) and animal tissues (Wu and Juurlink, 2002; Desai and Wu, 2008). We also found that MG (Chang, et al., 2005) or fructose (a precursor of MG) (Wang, et al., 2006) induced the production of $ONOO^-$ in cultured rat thoracic aortic smooth muscle cells (A-10 cells).

To date, the role of MG in the regulation of mitochondrial function is unclear. We hypothesized that MG affects mitochondrial function by interfering with respiratory complexes and altering mitochondrial $ONOO^-$ production. In the present study, changes in mitochondrial ROS production, activity of mitochondrial complex, and MnSOD activity in A-10 cells in the presence of exogenous MG were investigated. AGEs crosslink breaker alagebrium and non-specific antioxidant *N*-acetyl-L-cysteine (NAC) were also used in this study.

MATERIALS AND METHODS

Chemicals and antibodies

Anti-nitrotyrosine antibody and bovine serum were purchased from Invitrogen Corporation (Burlington, ON, Canada). Anti-CEL antibody was obtained from Novo Nordisk (A/S, Denmark). Alagebrium was from Alteon Inc. (Parsippany, NJ, USA). Cell culture medium, FITC IgG fluorescent antibody, MG, NAC, *o*-phenylenediamine (*o*-PD), 2-methylquinoxaline, 5-methylquinoxaline, KCN, 2,6-dichlorophenolindophenol

(DCPIP), rotenone, thenoyltrifluoroacetone (TTFA), antimycin A, coenzyme Q1, cytochrome C, NaN_3, tween, NADH, decylubiquinol, digitonin, sucrose, MOPS, EDTA, $NaPO_3$, fatty acid-free BSA, ATP-free ADP, glutamate and malate were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Cell culture

A-10 cells, which is a aortic smooth muscle cell line from rats, was obtained from American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2, as described in our previous study (Wang, et al., 2006). Cells of passages 3 to 8 were used in this study.

Isolation of mitochondria

Following the instruction of Mitochondrion Isolation Kit from Sigma-Aldrich (Oakville, ON, Canada), cells were lysed using cell lysis solution (1:150, 5 min) and suspended in extraction buffer A. Unbroken cells and nuclei were pelleted by centrifugation at 600 g for 10 min. The supernatant was centrifuged at 15,000 g for 15 min, and the mitochondrial pellet was resuspended in celLytic M cell lysis reagent for MG measurement. The mitochondrial pellet was resuspended in extraction buffer A and freeze-thawed twice for mitochondrial complexes activity determination. Cytochrome C Oxidase Assay Kit from Sigma-Aldrich (Oakville, ON, Canada) was used to determine

the integrity of isolated mitochondria. Cytochrome C oxidase is located on the inner mitochondrial membrane and has traditionally been used as a marker for this membrane (Duan, et al., 2003). The activity of cytochrome C oxidase in isolated mitochondria was high, indicating the high integrity and purity of the preparation.

MG content determination

MG content was determined using an *o*-PD method as described previously (Wang, et al., 2006). In brief, mitochondria isolated from A-10 cells were incubated on ice for 10 min with 1/4 volume of perchloric acid (PCA) and centrifuged (12,000 rpm, 15 min) to remove the PCA-precipitated mitochondrial debris. The supernatant was supplemented with 100 mM *o*-PD and incubated for 3 h at room temperature. The quinoxaline derivative of MG (2-methylquinoxaline) and the quinoxaline internal standard (5-methylquinoxaline) were measured using a Nova-Pak ® C18 column (3.9 × 150 mm, and 4 μm particle diameter, MA, USA) equipped with a Hitachi high-performance liquid chromatography (HPLC) system (Hitachi Ltd., Mississauga, ON, Canada).

Detection of mitochondrial ROS (mtROS) and mitochondrial $O_2^{\cdot-}$

Mitochondria produce a variety of ROS, such as $ONOO^-$, NO and $O_2^{\cdot-}$. MitoTracker Red CM-H_2XRos and MitoSOX from Invitrogen Corporation (Burlington,

ON, Canada) were used to detect the levels of mtROS and mitochondrial $O_2^{\cdot -}$ (Busik, et al., 2008; Schroeder, et al., 2007). A-10 cells were seeded on 35 mm glass-bottom dishes and treated with different agents for 18 h. Then, cells were labeled with MitoTracker Red (300 μM, 15 min) or MitoSOX (2 μM, 20 min). After washing, cells were bathed in DMEM again and subjected to examination under a Confocal Laser Scanning Biological Microscope (Olympus Fluoview 300, Olympus America Inc., Melville, NY, USA) coupled with 40× objective lens. The exposure time of the camera, the gain of the amplifier and the aperture were fixed at 4.57s/scan, 4.0× and 3 respectively, to allow quantitative comparisons of the relative fluorescence intensity of the cells between groups. 10-14 cells were randomly collected from 4 different pictures of each groups. The average fluorescence intensity of each cell was measured using Image J program (NIH, USA). Data were expressed as mean ± SEM of the fluorescence intensity of those cells.

Measurement of MnSOD activity and NO level

SOD activity of A-10 cells was detected following the instructions of the SOD Assay Kit from Cayman Chemical (Ann Arbor, MI, USA). KCN at 3 mM was used to inhibit the activity of Cu/Zn SOD, leaving only MnSOD activity to be measured. For NO detection (Wang, et al., 2006), cells were preloaded with 5 μM membrane permeable DAF-FM (Invitrogen Corporation, Burlington, ON, Canada) in Kreb's buffer for 2 h at 37°C. After removal of the excess probe and with different treatments, DAF-

fluorescence intensity, reflecting intracellular NO level, was measured with excitation at 495 nm and emission at 515 nm in a Fluoroskan Ascent plate reader (Thermo Labsystem, Helsinki, Finland).

Immunocytochemistry staining

A-10 cells were seeded on glass cover slips with different treatments for 18 h, and subjected to immuno-staining. As described previously (Wang, et al., 2006), cells were fixed in 4% formalin for 1 h at room temperature. After permeation with 0.1% Triton X-100 for 5 min, fixed cells were incubated with 3% goat serum for 1 h, and then incubated with primary antibody (anti-CEL, 1:100; anti-nitrotyrosine, 1:200) at 4°C overnight. Cells were washed in PBS (0.01 M) for 15 min and incubated with diluted fluorescent secondary antibody (FITC-IgG, 1: 200) for 3 h at room temperature. After washing with PBS, cells were mounted on glass slides and observed under a confocal microscope. Fluorescence intensity was measured using Image J program.

Detection of the activities of complex I , complex III, and complex IV

Mitochondrial complex I activity was determined by monitoring the reduction of DCPIP at 600 nm with the addition of assay buffer (10 × buffer containing 0.5 M Tris-HCl at pH 8.1, 1% BSA, 10 μM antimycin A, 3 mM KCN, 0.5 mM coenzyme Q_1) (Long, et al., 2006). Mitochondrial proteins (25 μg/ml) and DCPIP (64 μM) were added

to the assay buffer before using. The reaction was started by adding 200 μM NADH and scanned at 600 nm with the reference wavelength of 620 nm for 2 min. Mitochondrial complex III activity was detected by monitoring the reduction of cytochrome C at 550 nm upon the addition of assay buffer (10× buffer contains 0.5 M Tris-HCl at pH 7.8, 2 mM NaN_3, 0.8% Tween-20, 1% BSA, 2 mM decylubiquinol) with 40 μM cytochrome C (Long, et al., 2006). The reaction was started by adding 20 μg/ml mitochondria proteins to the assay buffer and scanned at 550 nm with the reference wavelength of 540 nm for 2 min. Mitochondrial complex IV activity was measured by monitoring the reduction of reduced cytochrome C at 550 nm with the addition of assay buffer (0.5 M phosphate buffer at pH 8.0, 1% BSA and 2% tween) (Long, et al., 2006). Freshly prepared reduced cytochrome C (80 μM) was added to the assay buffer before using. The reaction was started by adding mitochondria protein (20 μg/ml) and scanned at 550 nm with the reference wavelength of 540 nm for 2 min. All assays were performed at 37 ºC.

Determination of ATP synthesis

ATP synthesis was assayed based on a modified method of Atorino *et al* (Atorino, et al., 2003). Briefly, cells were incubated at 37°C for 30 min in a respiratory buffer (0.02% digitonin, 0.25 M sucrose, 20 mM MOPS, 1 mM EDTA, 5 mM $NaPO_3$, 0.1 % fatty acid-free BSA, 1 mM ATP-free ADP, 5 mM glutamate, and 5 mM malate, pH 7.4). Thereafter, 3% PCA was used to precipitate proteins, and samples were centrifuged at 13,000 rpm for 2 min. Supernatants were taken out to measure ATP after pH adjusted to

7.8 using 10 M KOH. ATP was detected using ATP Bioluminescent Assay Kit from Sigma-Aldrich (Oakville, ON, Canada). Data were expressed as nanomoles of ATP per milligram of protein.

Statistical analysis

Data were expressed as mean ± SEM from at least three independent experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA). Differences between groups were examined by Student's unpaired *t*-Test. Values are considered to be statistically significant when $p < 0.05$.

RESULTS

Effect of MG on mtROS generation

After A-10 cells were treated with exogenous MG (30 μM) for 18 h, mitochondrial MG content increased by 50.7% (0.205 ± 0.012 vs. 0.136 ± 0.014 nmol/mg mitochondrial protein, $p < 0.01$, n = 4 for each group). Alagebrium (50 μM) had no effect on basal content of mitochondrial MG but its presence decreased the effect of exogenous MG on mitochondrial MG content (0.14 ± 0.009 vs. 0.205 ± 0.01 nmol/mg mitochondrial protein, $p < 0.01$, n = 4 for each group). NAC (600 μM) had no effect on mitochondrial MG content (data not shown).

MG increased the fluorescence intensity of CEL in a concentration-dependent manner. At 30 μM, MG increased the fluorescence intensity of CEL by 321% (Fig. 4-1, A and B). Co-treatment with alagebrium (50 and 100 μM) decreased the effect of 30 μM MG (Fig. 4-1, A and C). NAC (600 μM) did not show any effect on the staining of CEL (data not shown).

Exposure of cells to MG (5 to 100 μM) caused a significant concentration-dependent increase in mtROS generation. The production of mtROS increased dramatically with 30 μM MG and reached a plateau with 100 μM MG (Fig. 4-2, A and B). Co-incubation of NAC (600 μM) significantly decreased mtROS generation induced by MG (Fig. 4-2, A and B). Alagebrium (50 and 100 μM) and $ONOO^-$ specific scavenger uric acid (50 μM) inhibited mtROS generation induced by 30 μM MG (Fig. 4-2, A, C and D).

Effects of MG on NO and nitrotyrosine generation

The effect of MG on NO generation was evaluated by DAF-FM, a specific probe used for quantitating a low concentration of NO. As shown in Fig. 4-3, MG (30 μM) increased the production of NO by 48% ($p < 0.01$). Alagebrium (50 μM), NAC (600 μM), and mtNOS inhibitor 7-nitroindazole (50 μM) significantly reduced MG-increased NO generation.

Nitrotyrosine is formed by $ONOO^-$-mediated nitration of tyrosine residues of proteins. As shown in Fig. 4A and 4C, MG (20 and 30 μM) significantly increased the fluorescence intensity of nitrotyrosine in A-10 cells by 176-191%. The addition of NAC (600 μM) significantly inhibited the formation of nitrotyrosine induced by MG. Co-incubation of alagebrium (50 μM) also significantly reduced the fluorescence intensity of nitrotyrosine induced by MG (30 μM) (Fig. 4-4, A and D). Nitrotyrosine and mitotracker were co-localized in the tested cells as indicated by the overlap of yellow and red-green images (Fig. 4-4B).

Effect of MG on mitochondrial $O_2^{\cdot-}$ generation

MitoSOX, a specific probe to detect mitochondrial $O_2^{\cdot-}$ level, was used in this assay. MG (30 μM) increased mitochondrial $O_2^{\cdot-}$ production by 69.9% ($p < 0.01$), compared with untreated cells. Co-incubation of alagebrium (50 μM) and SOD mimic 4-hydroxy-tempo (Tempol, 500 μM) decreased mitochondrial $O_2^{\cdot-}$ production induced by MG treatment by 57% ($p < 0.01$) and 85.8% ($p < 0.01$), respectively (Fig. 4-5, A and B).

Effect of MG on MnSOD activity

MG (5-30 μM) decreased the activity of MnSOD, the first line enzyme to scavenge $O_2^{\cdot-}$ in mitochondria. MG at 30 μM decreased MnSOD activity by 24.5% ($p <$

0.05) (Fig. 4-6A). Alagebrium (10-100 μM) normalized MG-decreased MnSOD activity (Fig. 4-6B). NAC (600 μM) had no effect on MnSOD activity (data not shown).

Effect of MG on mitochondrial functions

MG (30 μM) treatment for 18 h had no obvious effect on the activity of complex I or complex IV, but significantly decreased complex III activity by 11.7% ($p < 0.05$), as shown in Fig. 4-7A. Alagebrium (50 μM) inhibited the effect of MG on complex III by 64.61% ($p < 0.05$). NAC (600 μM) did not have an effect on complex III activity (data not shown).

In order to confirm the effect of MG on mitochondrial ETC complexes, complex inhibitors were used to treat cells for 2 h in the absence or presence of MG. Mitochondrial $O_2^{\cdot -}$ generation was thereafter determined using the specific probe MitoSOX. Rotenone (0.5 μM and 1 μM), TTFA (5 μM and 10 μM), antimycin A (3 μM and 5 μM) and KCN (0.5 mM and 1 mM), which are respective blockers of complex I, complex II, complex III and complex IV, significantly increased production of mitochondrial $O_2^{\cdot -}$ in A-10 cells (Fig. 4-7B). No difference was observed between effects of two concentrations of each blocker. Therefore, these inhibitors appear to maximally inhibit the respective complexes. Interestingly, MG (30 μM) further increased rotenone (1 μM), TTFA (10 μM) and KCN (1 mM)-induced mitochondrial $O_2^{\cdot -}$ generation by 48.11%, 52.6% and 40.2%, respectively, in comparison with the cells treated with the inhibitor alone. However, the addition of MG (30 μM) did not change complex III

inhibitor (antimycin A)-induced mitochondrial $O_2^{\cdot-}$ generation. These results suggested that MG targeted on complex III to induce mitochondrial $O_2^{\cdot-}$ generation (Fig. 4-7B). MG (30 μM) significantly lowered ATP production by 44.8% (4.76 ± 0.74 vs. 8.62 ± 0.24 nmol/mg protein, $p < 0.01$). Alagebrium (50 μM) restored ATP synthesis inhibited by MG by 78.0% (Fig. 4-8).

DISCUSSION

MG causes crosslink among lysine, cysteine, and arginine residues of selective proteins to form AGEs, like CEL, altering the structure of proteins and their functions (Wang, et al., 2008). Higher levels of MG have been found in diabetic patients than in healthy controls (Wang, et al., 2007). In the present study, we observed that mitochondrial MG content was significantly increased after the cells were treated with exogenous MG. It appears that MG can move across plasmalemma and mitochondrial membrane to attack different molecular targets. Once inside the cells, MG induces glycation of many proteins in the cytosol, mitochondria and other vesicles. The formation of CEL in mitochondria may result in the dysfunction of mitochondrial proteins, and furthermore, increase mtROS generation. Alagebrium, an AGEs crosslink breaker (Desai and Wu, 2007), not only decreased CEL formation, but also diminished MG levels in mitochondria. The result indicates that alagebrium scavenges MG and inhibits glycation directly, although the mechanism is unknown. This discovery also echoes the observation obtained by Nobecourt *et al.* (Nobecourt, et al., 2008).

The physiological concentration of plasma MG in rats is approximately 5 μM (Nagaraj, et al., 2002). Our previous study detected the plasma MG levels of 33.6 μM in 20-week-old SHR and 14.2 μM in age-matched WKY rats (Wang, et al., 2004). Plasma levels of MG increased from 3.3 μM in healthy humans to 5.9 μM in type 2 diabetic patients (Wang, et al., 2007). In addition, cultured cells may produce more MG since MG concentration up to 310 μM was detected in cultured Chinese hamster ovary cells (Chaplen, et al., 1998). Furthermore, up to10 mM MG had been used to investigate its effect on insulin secreting cells and insulin signaling pathways in rat L6 myoblasts (Sheader, et al., 2001; Riboulet-Chavey, et al., 2006). Thus, MG (30 μM) used in the present study is not only the physiological relevant concentration, but also suitable to mimic the insulin resistance environment in rat aortic smooth muscle cells.

Our previous work has shown that MG induced overproduction of $O_2^{\cdot -}$, NO, and $ONOO^-$ in rat VSMCs (Chang, et al., 2005). The present study demonstrated that mitochondria are targets of MG for this pro-oxidative action. More specifically, we demonstrated that MG increased mitochondrial $ONOO^-$ production in VSMCs. Several lines of evidence support this conclusion. (1) Uric acid, a specific scavenger of $ONOO^-$, significantly decreased MG-induced mtROS generation. (2) Increased staining of nitrotyrosine was observed in MG treated A-10 cells, and the expression of nitrotyrosine was mostly co-localized with mitochondrial marker staining. (3) MnSOD is the major enzyme which catalyzes $O_2^{\cdot -}$ degradation in mitochondria and protects mitochondria against oxidative stress. Our results show that MG reduced the activity of MnSOD in mitochondria of VSMCs. (4) MG-induced mitochondrial $O_2^{\cdot -}$ production was inhibited by Tempol. As a SOD mimic, Tempol is more stable and membrane-permeable than

MnSOD itself (Chatterjee, et al., 2000). (5) Located on the inner mitochondrial membrane, mtNOS is considered as the alpha-isoform of neuronal nitric oxide synthase (nNOS) and is responsible for NO production in mitochondria (Kanai, et al., 2001; Elfering, et al., 2002). MG-induced intracellular NO was decreased by 7-nitroindazole. The latter is the specific inhibitor of mtNOS (Carreras, et al., 2002) and can prevent mitochondrial structural damage mediated by increased mitochondria NO generation in the developing brain (Giusti, et al., 2008). Together with MG-induced mitochondrial $O_2^{\cdot -}$, the stimulation of mtNOS by MG also contributes to $ONOO^-$ formation.

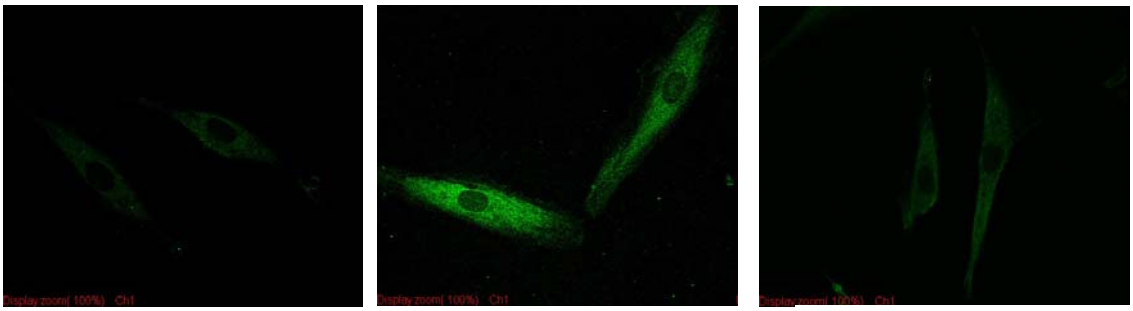
Of particular importance is our observation that MG selectively damaged complex III activity, not complex I or complex IV. This effect may underlie MG-inhibited ATP synthesis and MG-enhanced ROS production. Further evidence for the inhibition of complex III by MG was derived from the failure of MG to increase mitochondria $O_2^{\cdot -}$ generation in the presence of antimycin A, a specific blocker of complex III. That alagebrium restored MG-inhibited complex III activity suggests that the complex III is glycated by MG. Complex III, which is also called cytochrome C reductase, transfers electrons from ubiquinone to cytochrome C. The inhibition of complex III by MG may disrupt the ETC, rendering more electrons leaking out to form $O_2^{\cdot -}$. Consequently, hydrogen electrochemical gradient across the inner mitochondrial membrane is weakened, and the driving force for ATPase to synthesize ATP provided by hydrogen influx across the inner mitochondrial membrane is reduced. Cellular integrity and function are therefore compromised. Although we did not directly measure the activity of complex II in the presence of MG, our experiments with complex II inhibitor, TTFA, indicate that complex II is not a major site of mitochondrial $O_2^{\cdot -}$ generation in A-10 cells.

Furthermore, after complex II is maximally inhibited by TTFA, MG still induced mitochondrial $O_2^{\cdot-}$ production. This shows that the effect of MG on superoxide production does not depend on complex II. Evidence shows that mitochondrial dysfunction, especially elevated production of mtROS resulted from complex III inhibition, is closely linked with the pathogenesis of insulin resistance (Kim, et al., 2008). Moreover, normalization of mitochondrial superoxide production blocked the diabetic hyperglycemia damage in bovine aortic endothelial cells (Nishikawa, et al., 2000). Therefore, complex III dysfunction-induced mitochondrial oxidative stress plays an important role in the pathophysiology of insulin resistance syndrome.

In addition, our study demonstrated that alagebrium reversed all harmful effects of MG on mitochondria of cultured cells. Compared with alagebrium, the beneficial effect of NAC is limited. It reduced MG-induced ROS, NO, and nitrotyrosine production, but did not affect mitochondrial functions.

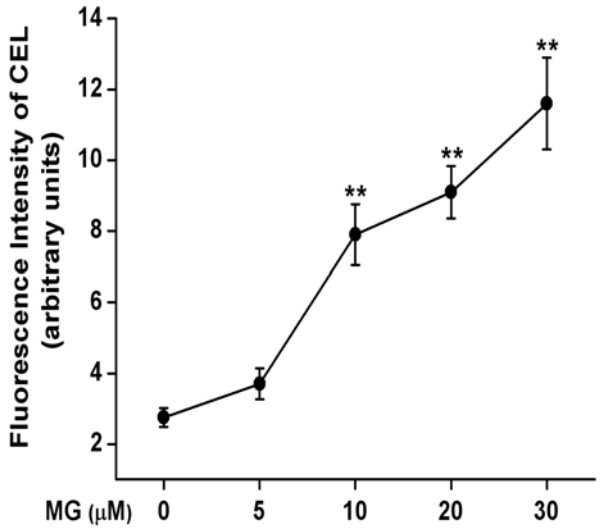
In summary, our study demonstrates that MG plays a critical role in regulating mitochondrial functions of VSMC. Respiratory complex III is the major and selective target of MG in mitochondria. Together with reduced MnSOD activity and disruption of the ETC in the presence of MG largely explain the increased oxidative stress and decreased ATP production in many MG-related cellular disorders. These novel observations provide new inside in the physiological importance and pathophysiological implications of the interaction of MG with mitochondria functions. It also sheds light on pathogenesis of and treatment for many mitochondrial-originated cellular disorders encountered in insulin resistance syndrome.

A



Control **MG (30 µM)** **MG (30 µM) + Alagebrium (50 µM)**

B



C

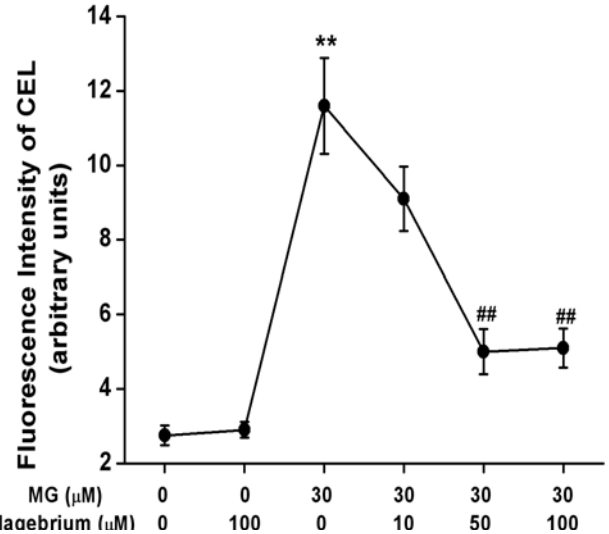
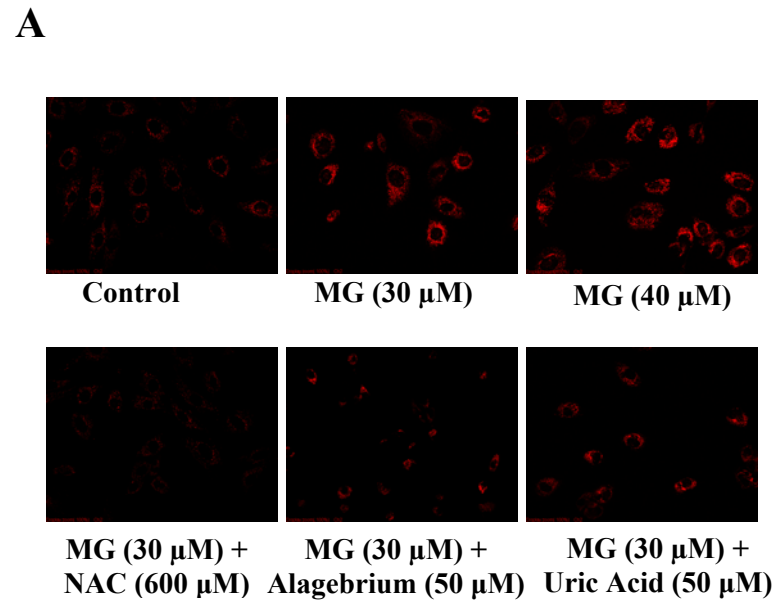
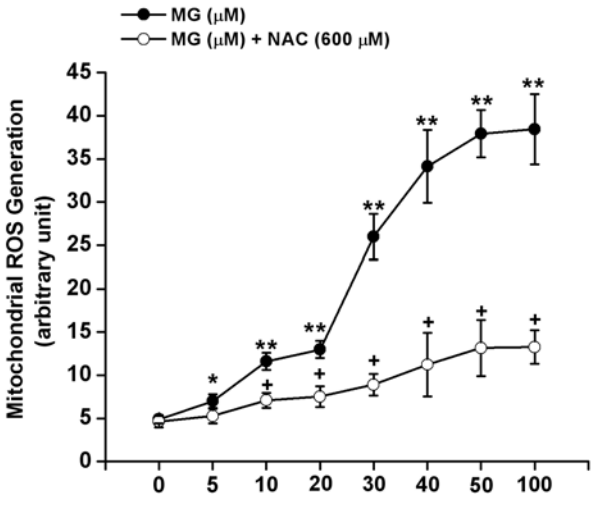
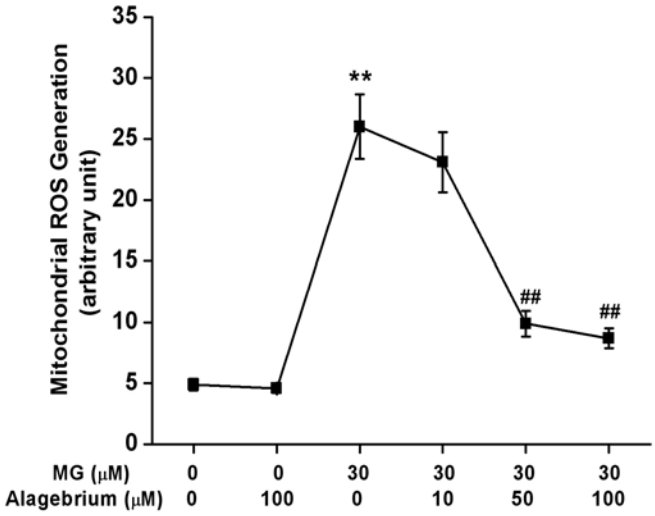


Figure 4-1. Effect of MG on the fluorescence intensity of CEL in A-10 cells. (A) MG increased the staining of CEL in A-10 cells, which was decreased by alagebrium. (B) Cells were treated with MG (5-30 μM). (C) Cells were co-treated with alagebrium (10-100 μM) and MG (30 μM). After treated with different agents for 18 h, cells were stained using anti-CEL (1:100 at 4ºC overnight) and secondary fluorescent antibody (FITC-IgG, 1:200 at room temperature for 3 h) and read under Confocal microscope. Fluorescence intensity was analyzed using Image J program. ** $p < 0.01$ vs. cells without any treatment; ## $p < 0.01$ vs. cells treated with MG (30 μM) alone. n = 12.


A
Control
MG (30 μM)
MG (40 μM)
MG (30 μM) + NAC (600 μM)
MG (30 μM) + Alagebrium (50 μM)
MG (30 μM) + Uric Acid (50 μM)


B
MG (μM)
MG (μM) + NAC (600 μM)
Mitochondrial ROS Generation (arbitrary unit)
0 5 10 20 30 40 50 100


C
Mitochondrial ROS Generation (arbitrary unit)
MG (μM) 0 0 30 30 30 30
Alagebrium (μM) 0 100 0 10 50 100

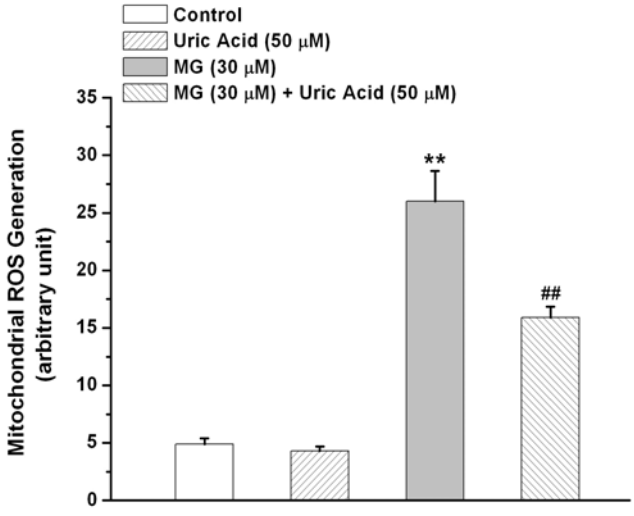

D
Control
Uric Acid (50 μM)
MG (30 μM)
MG (30 μM) + Uric Acid (50 μM)
Mitochondrial ROS Generation (arbitrary unit)

Figure 4-2. Effect of MG on mitochondrial ROS generation in A-10 cells. (A) MG enhanced mitochondrial ROS generation, which was decreased by *N*-acetyl-L-cysteine (NAC), alagebrium, and uric acid. (B) Cells were treated with MG (5-100 μM) in the presence or absence of NAC (600 μM). (C) Cells were co-treated with alagebrium (10-100 μM) and MG (30 μM). (D) Cells were co-treated with uric acid (50 μM) and MG (30 μM). After 18 h treatment with different agents, cells were loaded with molecular probe MitoTracker Red (300 μM, 15 min) and read under Confocal microscope. Fluorescence intensity was analyzed using Image J program. * $p < 0.05$ and ** $p < 0.01$ vs. cells without any treatment; + $p < 0.01$ vs. MG treatment alone at the same concentration; ## $p < 0.01$ vs. cells treated with MG (30 μM) alone. n = 10-14.

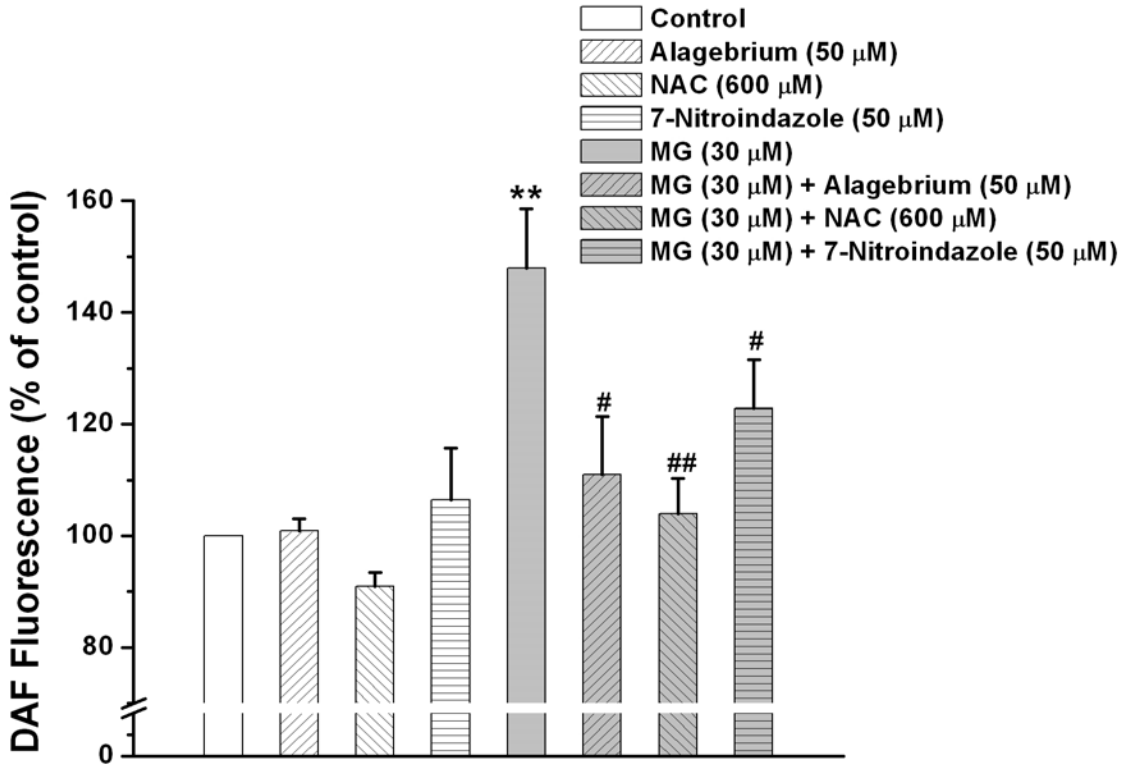
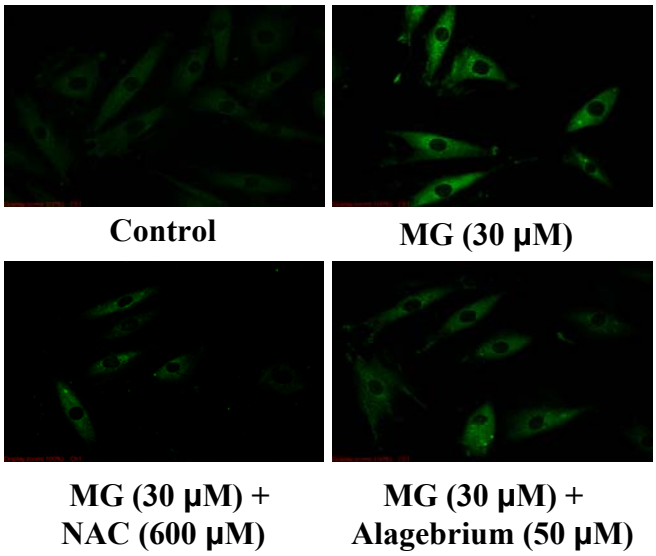
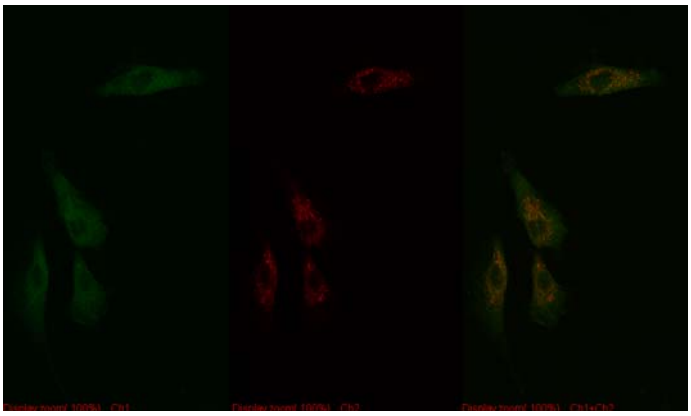


Figure 4-3. Effect of MG on NO production in A-10 cells. Cells were treated with different agents for 18 h. Molecular probe DAF-FM (5 μM, 2h) was used to detect cellular levels of NO. ** $p < 0.01$ vs. control; # $p < 0.05$ and ## $p < 0.01$ vs. cells treated with MG (30 μM) alone. n = 8. NAC, *N*-acetyl-L-cysteine.

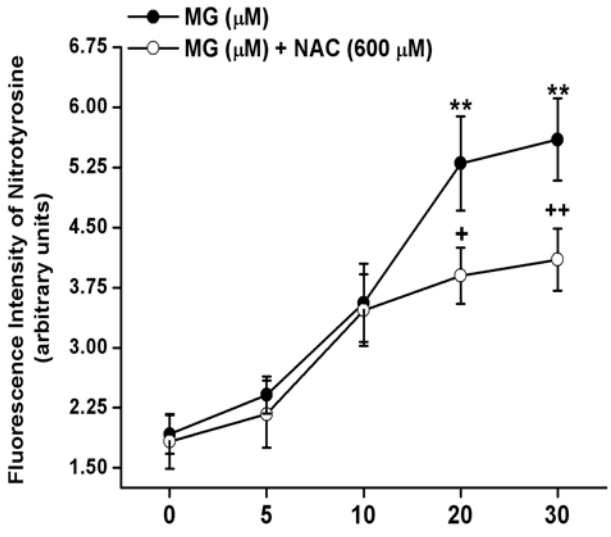
A


Control
MG (30 μM)
MG (30 μM) + NAC (600 μM)
MG (30 μM) + Alagebrium (50 μM)

B


Nitrotyrosine
Mitotracker
Merge

C


MG (μM)
MG (μM) + NAC (600 μM)
Fluorescence Intensity of Nitrotyrosine (arbitrary units)
6.75
6.00
5.25
4.50
3.75
3.00
2.25
1.50
0
5
10
20
30
**
**
+
++

D

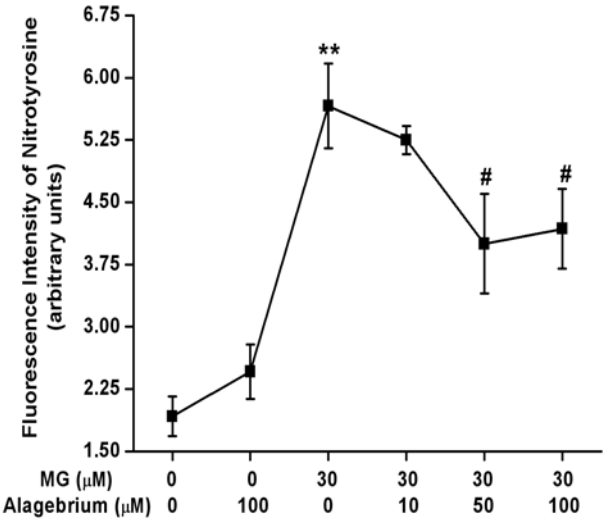
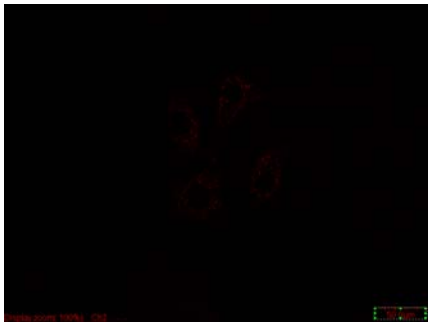
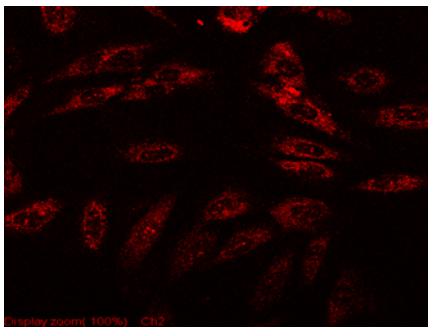

Fluorescence Intensity of Nitrotyrosine (arbitrary units)
6.75
6.00
5.25
4.50
3.75
3.00
2.25
1.50
**
#
#
MG (μM) 0 0 30 30 30 30
Alagebrium (μM) 0 100 0 10 50 100

Figure 4-4. Effect of MG on the fluorescence intensity of nitrotyrosine in A-10 cells. (A) MG increased nitrotyrosine staining, which was inhibited by alagebrium and *N*-acetyl-L-cysteine (NAC). (B) Cells were co-stained with anti-nitrotyrosine and MitoTracker Red to determine whether increased nitrotyrosine was located in mitochondria. (C) Cells were treated with MG (5-30 μM) in the presence or absence of NAC (600 μM). (D) Cells were co-treated with alagebrium (10-100 μM) and MG (30 μM). Cells were treated with different agents for 18 h. Double cell staining of MitoTracker Red (300 μM, 15 min) and nitrotyrosine (anti-nitrotyrosine 1: 200 at 4ºC overnight; FITC-IgG 1:200 at room temperature for 3 h) were conducted. Cells were read under Confocal microscope. Fluorescence intensity was measured using Image J program. ** $p < 0.01$ vs. cells without any treatment; + $p < 0.05$ and ++ $p < 0.01$ vs. MG treatment alone at the same concentration; # $p < 0.05$ vs. cells treated with MG (30 μM) alone. n = 10-14.

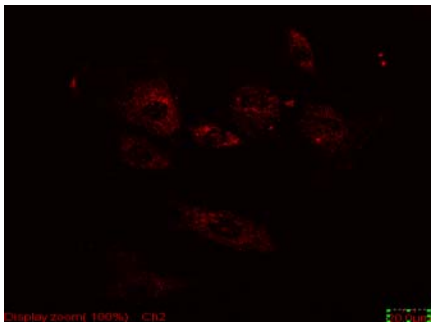
A



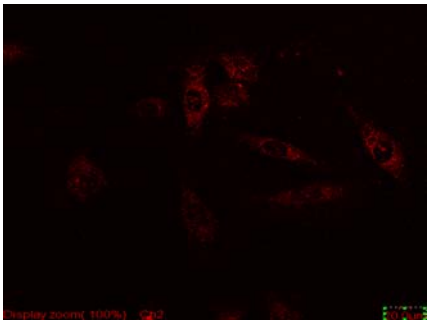
Control



MG (30 µM)



MG (30 µM) +
Alagebrium (50 µM)



MG (30 µM) +
Tempol (500 µM)

B

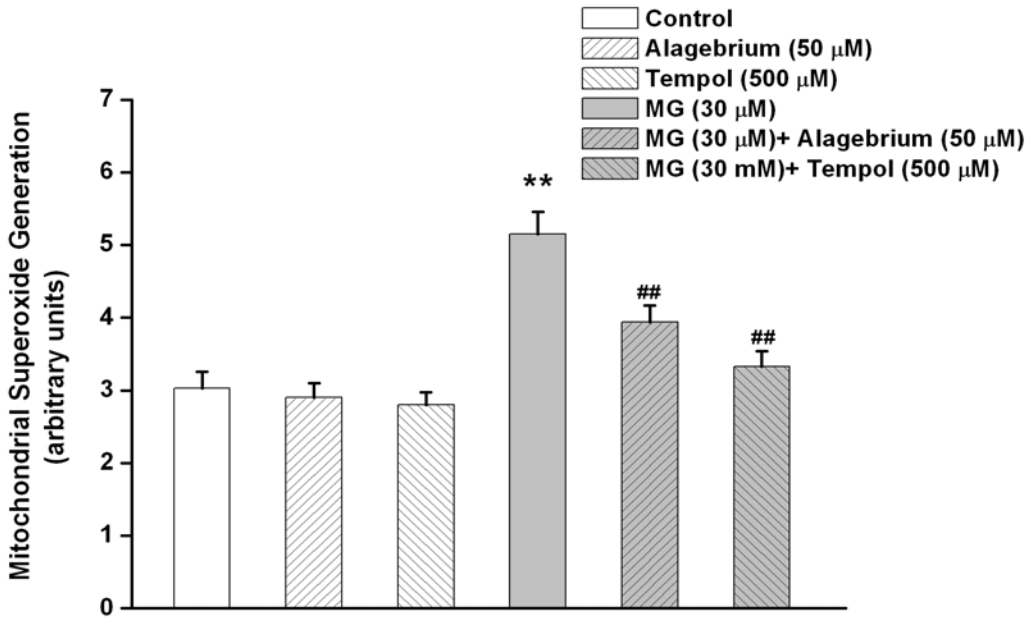
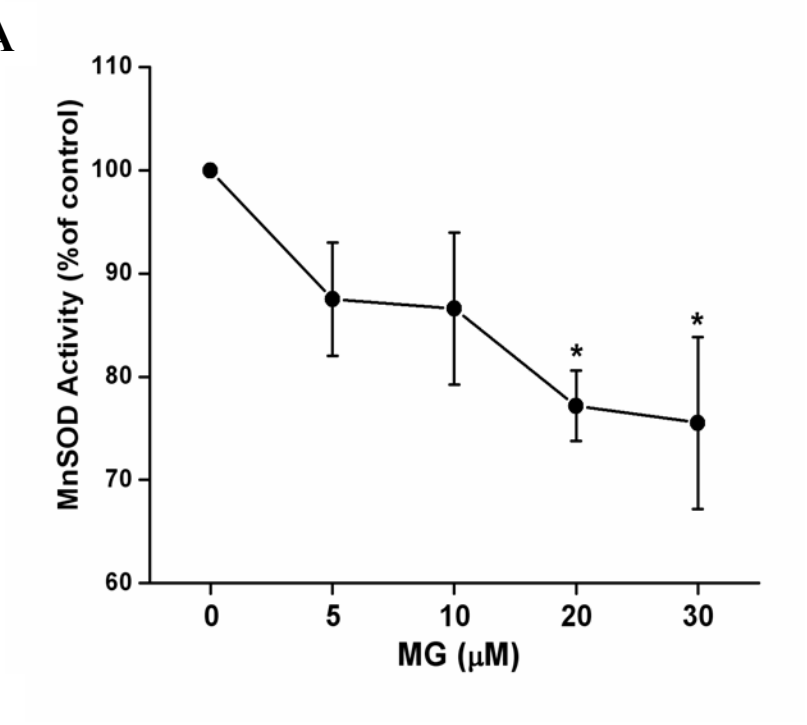

Control
Alagebrium (50 μM)
Tempol (500 μM)
MG (30 μM)
MG (30 μM)+ Alagebrium (50 μM)
MG (30 mM)+ Tempol (500 μM)
Mitochondrial Superoxide Generation
(arbitrary units)
0
1
2
3
4
5
6
7
**
##
##

Figure 4-5. Effect of MG on mitochondrial $O_2^{\cdot -}$ generation in A-10 cells. (A) MG increased MitoSOX signal in mitochondria, which was decreased by alagebrium and 4-hydroxy-tempo (Tempol). (B) Mitochondrial $O_2^{\cdot -}$ generation in A-10 cells. After treated with different agents for 18 h, cells were loaded with molecular probe MitoSOX (2 μM, 20 min) and read under Confocal microscope. Fluorescence intensity was measured using Image J program. ** $p < 0.01$ vs. control; ## $p < 0.01$ vs. cells treated with MG (30 μM) alone. n = 12.

A


MnSOD Activity (%of control)
110
100
90
80
70
60
0
5
10
20
30
MG (μM)
*
*

B

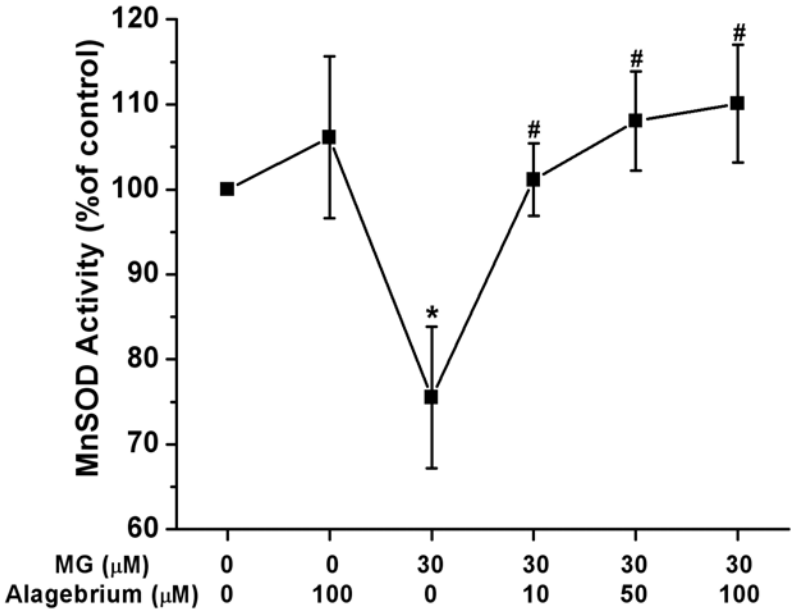
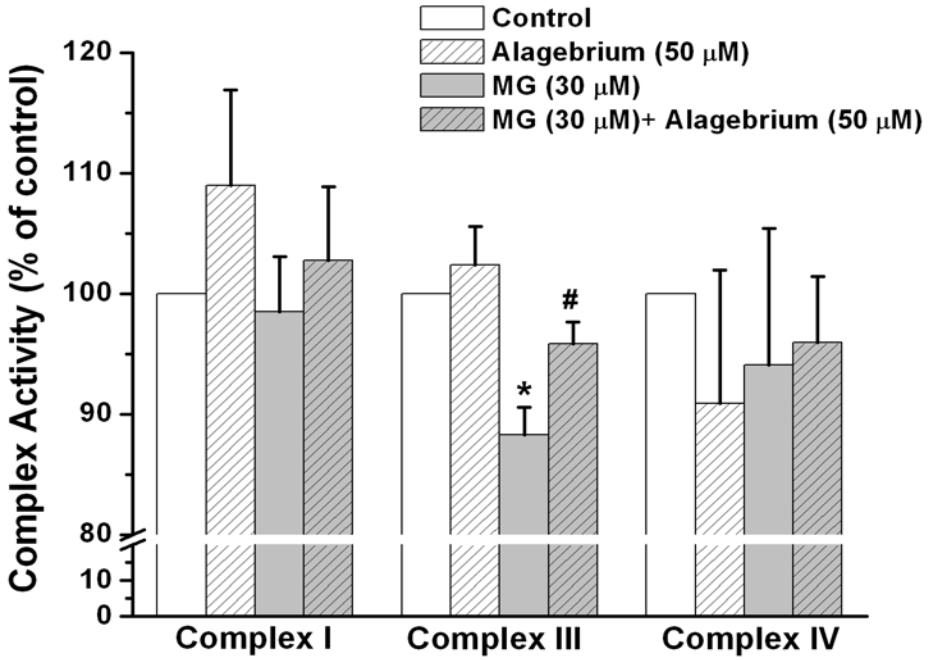

MnSOD Activity (%of control)
120
110
100
90
80
70
60
*
#
#
#
MG (μM) 0 0 30 30 30 30
Alagebrium (μM) 0 100 0 10 50 100

Figure 4-6. Effect of MG on MnSOD activity in A-10 cells. (A) MG (5-30 μM) decreased MnSOD activity in A-10 cells. (B) MnSOD activity in A-10 cells co-treated with alagebrium (10-100 μM) and MG (30 μM). Cells were treated with different agents for 18 h. SOD Assay Kit was used to detect SOD activity. KCN at 3 mM was used to inhibit the activity of Cu/Zn SOD, leaving only MnSOD activity to be measured. * $p <$ 0.05 vs. cells without any treatment; # $p < 0.05$ vs. cells treated with MG (30 μM) alone. n = 4.


Control
Alagebrium (50 μM)
MG (30 μM)
MG (30 μM)+ Alagebrium (50 μM)
Complex Activity (% of control)
120
110
100
90
80
10
0
*
#
Complex I
Complex III
Complex IV

B

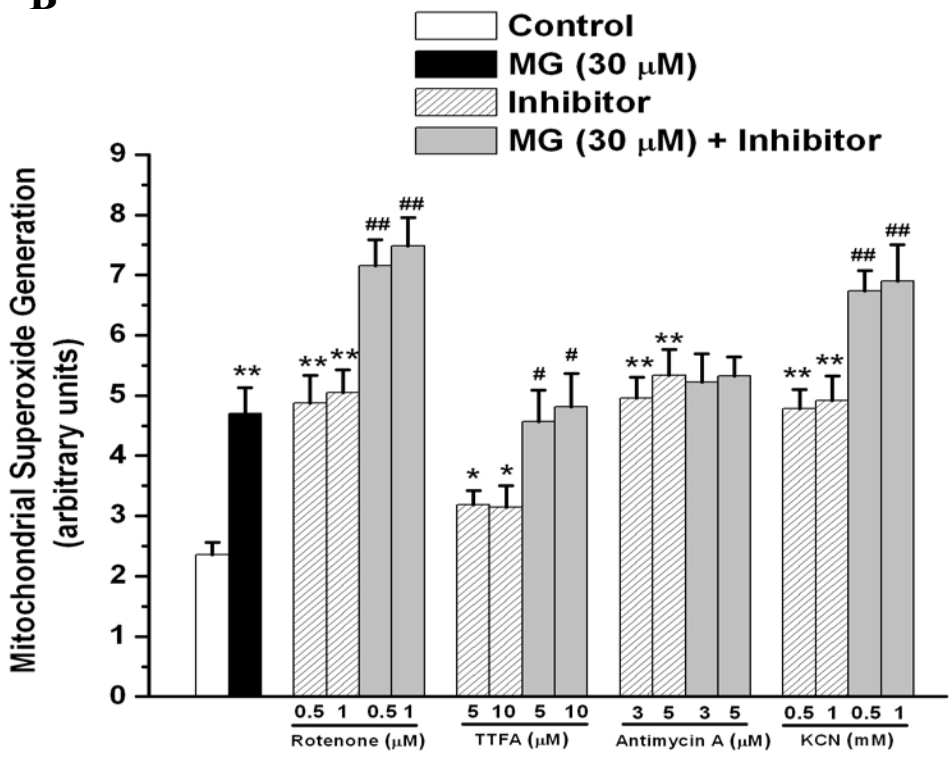

Control
MG (30 μM)
Inhibitor
MG (30 μM) + Inhibitor
Mitochondrial Superoxide Generation (arbitrary units)
0
1
2
3
4
5
6
7
8
9
0.5 1 0.5 1
Rotenone (μM)
5 10 5 10
TTFA (μM)
3 5 3 5
Antimycin A (μM)
0.5 1 0.5 1
KCN (mM)

Figure 4-7. Effect of MG on mitochondrial complexes in A-10 cells. (A) Effect of MG on activities of complex I, complex III and complex IV in A-10 cells. Cells were treated with different agents for 18 h. * $p < 0.05$ vs. control; # $p < 0.05$ vs. cells treated with MG (30 μM) alone. n = 4. (B) Effect of MG on mitochondrial $O_2^{\cdot-}$ generation in the presence of different inhibitors of respiratory complexes. A-10 cells were treated with different agents for 2 h. Rotenone, thenoyltrifluoroacetone (TTFA), antimycin A and KCN are inhibitors of complexes I, II, III and IV, respectively. * $p < 0.05$ and ** $p < 0.01$ vs. control; # $p < 0.05$ and ## $p < 0.01$ vs. inhibitor alone treated cells. n = 12.

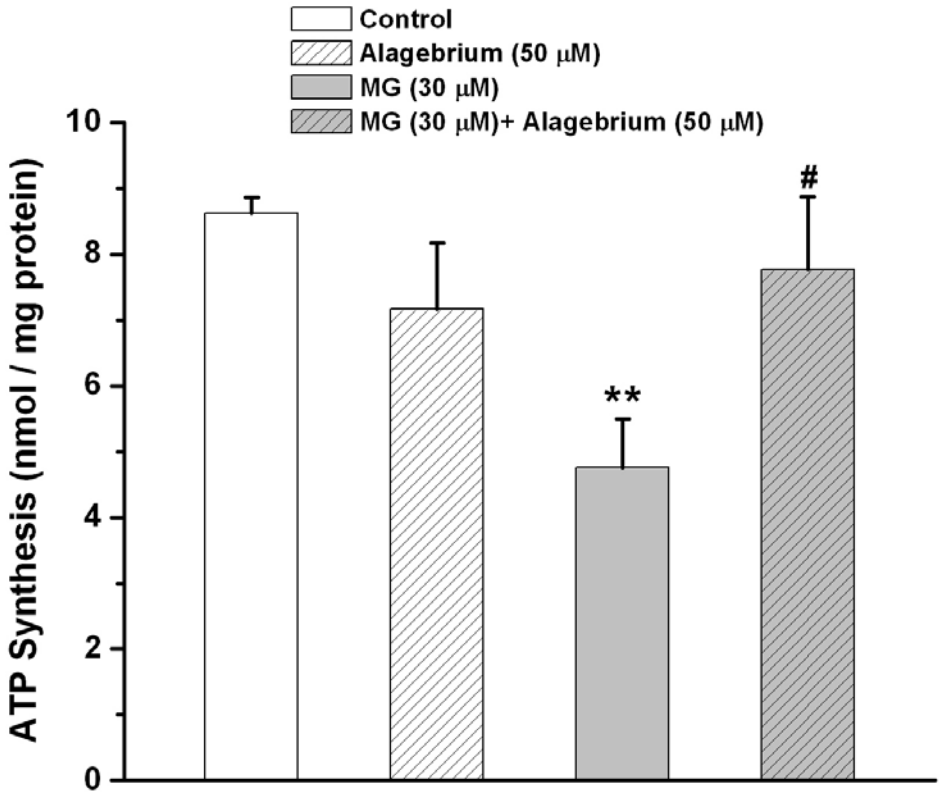


Figure 4-8. Effect of MG on ATP synthesis (30 min) in mitochondria of A-10 cells. Cells were treated with different agents for 18 h, and ATP levels were determined using ATP Bioluminescent Assay Kit. ** $p < 0.01$ vs. control; # $p < 0.05$ vs. cells treated with MG (30 μM) alone. n = 4.

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CHAPTER 5

PROINFLAMMATORY AND PROAPOPTOTIC EFFECTS OF METHYLGLYOXAL ON NEUTROPHILS FROM PATIENTS WITH TYPE 2 DIABETES MELLITUS

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ABSTRACT

To determine the effect of methylglyoxal (MG) on cytokine production by, and apoptosis of, neutrophils from type 2 diabetes mellitus (T2DM) patients. The levels of plasma MG, cytokines released by isolated neutrophils and the apoptotic status of neutrophils were determined. The higher level of plasma MG in T2DM patients was correlated positively with glycated hemoglobin levels, fasting plasma glucose levels and urine albumin/creatinine ratios. The basal levels of cytokines released from neutrophils were markedly higher in patients. MG treatment of the neutrophils isolated from diabetic patients either did not alter, or decreased, the production of cytokines. In contrast, MG induced the release of cytokines from neutrophils of non-diabetics. Moreover, the neutrophils from T2DM patients showed a greater proclivity for apoptosis, which was further increased by *in vitro* MG treatment. MG stimulated neutrophils to release more cytokines, which might play a role in the development of infection in T2DM.

Key Words: Methylglyoxal; Type 2 diabetes mellitus; Neutrophil; Cytokine

INTRODUCTION

Methylglyoxal (MG) is a metabolite of glucose. It is a highly reactive compound that can modify cellular proteins and nucleic acids, leading to changes in cellular function. MG and MG-induced formation of advanced glycated endproducts (AGEs) have been implicated in the development of insulin resistance, such as diabetes and hypertension (Desai and Wu, 2007; Vlassara, et al., 2002).

Neutrophils are the most abundant type of white blood cells and play a crucial role in innate immunity. Once an inflammatory response is initiated, the neutrophil is the first cell to be recruited to the site of infection or injury, where it phagocytoses bacteria and damaged host tissue. The neutrophils of diabetic patients display increased necrosis and enhanced production of reactive oxygen species (T2DM) (Shurtz-Swirski, et al., 2001), increased apoptosis (Type 1 Diabetes Mellitus, T1DM) (Tennenberg, et al., 1999), and significantly lower neutrophil chemotactic responses (both T1DM and T2DM) (Delamaire, et al., 1997). In general, patients with diabetes are at high risk of infections, which are more serious and prolonged. It is notable that the circulating levels of proinflammatory cytokines are elevated in diabetic patients (Pickup, et al., 2000), and it has been suggested that the impaired functions of neutrophils contribute to the increased susceptibility to infections observed in these patients.

The mechanism mediating this altered neutrophil function is not clear, although it has been suggested that it is related to hyperglycemia (Lawson, et al., 2002). Hyperglycemia, or the presence of AGEs, leads to persistent activation of neutrophils, as

evidenced by the increased activity of neutrophil alkaline phosphatase (Geerlings and Hoepelman, 1999). Numerous investigations have documented an increase in the formation of MG and AGEs in diabetes, and these could be causally related to the development of diabetic complications such as nephropathy, retinopathy, and vascular disease (Wu, 2006). However, whether MG has a direct effect on neutrophils remains unknown.

The aim of the present study was to explore the effect of MG on cytokine release from, and apoptosis of, neutrophils from T2DM patients. Plasma MG levels were measured in T2DM patients with varying glycated hemoglobin (HbA1c), fasting plasma glucose, and urine albumin / creatinine ratios (UACRs). Levels of nitric oxide (NO) and cytokines were determined, as was the apoptotic status of neutrophils following different *in vitro* MG treatments.

MATERIALS AND METHODS

Chemicals

MG, *o*-phenylenediamine, 2-methylquinoxaline, 5-methylquinoxaline, metformin, and reduced glutathione (GSH) were purchased from Sigma Chemical Co. (Oakville, ON, Canada).

Subjects

All participants provided informed consent. Twenty non-diabetic subjects and fifty-five T2DM patients were included in this study. Non-diabetic subjects were normal healthy human. All participants were free of infection and not under any anti-infective or anti-inflammatory medication. The study protocol was approved by the Research Ethics Boards of the University of Saskatchewan and the Saskatoon Health Region.

Quantification of fasting plasma glucose, blood HbA1c, urine albumin, and creatinine

The measurements of levels of fasting plasma glucose, blood HbA1c, urinary albumin, and creatinine were performed by the Chemistry Laboratory at the Royal University Hospital, University of Saskatchewan. Fasting plasma glucose was measured using glucose oxidase method on a Beckman Synchron LX20 (Beckman, Palo Alto, CA, USA). Blood HbA1c was quantitated by ion-exchange high performance liquid chromatography (HPLC) (Bio-Rad Variant II). Urinary albumin (mg/l) and creatinine (mmol/l) were determined by nephelometry (Beckman Array™ Protein System) and the Jaffe rate reaction method (Beckman, Palo Alto, CA, USA), respectively. The unit mg/mmol was used to express UACRs.

Measurement of MG

MG was measured using an *o*-phenylenediamine method as described previously (Wang, et al., 2006). The quinoxaline derivative of MG (2-methylquinoxaline) and a quinoxaline internal standard (5-methylquinoxaline) were measured with a Hitachi HPLC

system (Hitachi Ltd., Mississauga, ON, Canada) using a Nova-Pak® C18 column (3.9 × 150 mm and 4 μm particle diameter, MA, USA).

Quantitation of NO

NO was determined as the total concentration of nitrate and nitrite in the plasma using a nitrate/nitrite fluorometric assay kit (Cayman Chemical, Ann Arbor, MI, USA). The fluorescence was detected at excitation and emission wavelengths of 375 nm and 415 nm, respectively, using a Fluoroskan Ascent plate reader (Thermo Labsystem, Amsterdam, Netherlands).

Neutrophil isolation

Neutrophils were isolated from whole blood using a modification of the method of Gordon *et al*.(Gordon, et al., 2005). In brief, equal volumes of whole blood and 6% dextran (Abbort Laboratories, QC, Canada) were mixed, then the red blood cells (RBCs) were allowed to sediment for at least 1 h. The leukocyte-rich supernatant was removed and underlaid with 3 ml of density gradient medium (Lymphoprep, Axis-Shield, Oslo, Norway) then centrifuged at 1300 rpm for 30 min. The cell pellet was suspended in 0.2% NaCl for 20 s to quickly lyse the RBCs, then 1.6% NaCl was added to correct the osmolarity of the medium. The cells were sedimented (1300 rpm, 10 min) and the neutrophilic pellet was resuspended in PBS. The neutrophils were washed once and counted.

Determination of cytokines

Neutrophils (2×10^6/ml) were incubated at 37 °C for 12 h in the presence or absence of MG or other reagents in PBS. After the incubation, the supernatant was collected and the levels of tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), and interleukin-6 (IL-6) were determined using a capture ELISA as described previously(Gordon, et al., 2000). Human cytokine-specific ELISA capture detection antibody pairs (R&D, Minneapolis, MN, USA) and 96-well immulon-4 ELISA plates (Dynatech laboratories Inc., Chantilly, VA, USA) were used as recommended by the suppliers. The final steps comprised of incubation with a 1:1000 dilution of streptavidin-conjugated horseradish peroxidase (Gibco, Burlington, Ontario, Canada) followed by the reactions with ABTS–peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). The absorbance was read at 405 nm. Each sample and the standards were run in duplicate.

Determination of apoptosis

Apoptosis of neutrophils was detected using an active Caspase 3 antibody apoptosis kit (BD, Pharmingen, NJ, USA). Neutrophils (2×10^6/ml) were analyzed by flow cytometry (Beckman Coulter Epics XL, Mississauga, ON, Canada).

Statistical analysis

The cytokines and apoptosis data were analyzed using a one-way ANOVA. Differences between groups were examined by the Student's unpaired *t*-test. The remainder of the data were analyzed using nonparametric methods (Spearman rank

correlation coefficient and Mann–Whitney *U* test) as the data were not normally distributed. Analyses were performed using SPSS version 15 (SPSS, Chicago, IL, USA). Data were expressed as mean ± SE. Values were considered to be statistically significant when $p \leq 0.05$.

RESULTS

Characteristics of study subjects

The mean duration of diagnosed diabetes in our T2DM patients was 10.1 ± 1.0 years. The mean blood levels of HbA1c (7.21 ± 0.19%) and fasting plasma glucose (9.68 ± 0.67 mM) in T2DM patients ($n = 55$) were significantly higher than those in non-diabetic subjects (5.02 ± 0.11% and 4.78 ± 0.25 mM, respectively; $p < 0.01$; $n = 20$). Elevated levels of NO were observed in the plasma of the T2DM patients compared with the non-diabetic subjects (36.7 ± 3.7 vs. 28.2 ± 0.4 μM, respectively; $p < 0.01$) (Table 5-1A).

Elevated plasma MG levels in type 2 diabetes

The plasma levels of MG in the T2DM patients were 77% higher than those in the non-diabetic subjects (5.9 ± 0.7 vs. 3.3 ± 0.4 μM, respectively; $p < 0.01$) (Fig. 5-1A). Furthermore, the levels of plasma MG were increased significantly in T2DM patients with more significant hyperglycemia (HbA1c > 7%) compared to the patients with HbA1c < 7% (Table 5-1B). The plasma levels of MG correlated positively with the

blood levels of HbA1c ($n = 55$, Fig. 5-1B) and the fasting plasma glucose levels in the T2DM patients ($n = 22$, Fig. 5-1C). A correlation was also observed between plasma levels of MG and the UACRs ($n = 22$, Fig. 5-1D) in the T2DM patients with nephropathy. There was no correlation between plasma levels of MG and total cholesterol, triglyceride, glomerular filtration rate or serum creatinine in the T2DM patients examined (data not shown).

Production of cytokines

Ten non-diabetic normal healthy subjects (M/F: 5/5; age: 61.1 ± 3.43 years; age range: 46–73) and 10 T2DM patients (M/F: 5/5; age: 58.3 ± 2.99 years; age range: 46–70) free of metformin or any anti-infective or anti-inflammatory treatment were selected to donate the blood for neutrophil isolation. The basal level of TNF-α release from neutrophils was markedly higher in T2DM patients in comparison with non-diabetic subjects (68.6 ± 2.6 vs. 57.8 ± 3.6 pg/ml, respectively; $p < 0.05$, $n = 10$) (Fig. 5-2A), as was release of IL-8 (92.9 ± 2.4 vs. 85.9 ± 2.7 pg/ml, respectively; $p < 0.05$, $n = 10$) and IL-6 (129.3 ± 6.0 vs. 103.5 ± 3.1 pg/ml, respectively; $p < 0.01$, $n = 10$). The *in vitro* release of TNF-α, IL-8, and IL-6 by neutrophils of non-diabetic subjects was significantly upregulated, in a dose-dependent fashion, by incubation with MG ($p < 0.05$, $n = 10$), although MG reduced the release of these mediators by cells from diabetic subjects (Fig. 5-2). Maximal release of TNF-α and IL-8 by MG-stimulated non-diabetic neutrophils (20 μM MG) achieved the equivalent of basal levels of cytokine released by cells from the T2DM patients (Figs. 5-2A and B).

The impact on neutrophil cytokine release of stimulating the cell with GSH (400 U/ml) and/or metformin (100 μM) was assessed, either alone or together with MG (5 or 20 μM). GSH (400 U/ml) or metformin (100 μM) alone had no effect on cytokine generation by cells from non-diabetic subjects, but decreased basal production of IL-8 and IL-6 by neutrophils from T2DM patients (Fig. 5-3). Addition of GSH (400 U/ml) or metformin (100 μM) did not alter the deleterious effect of MG on the production of TNF-α, IL-8, and IL-6 by neutrophils from T2DM patients. However, GSH (400 U/ml) or metformin (100 μM) significantly inhibited the impact of 20 μM MG on TNF-α, IL-8, and IL-6 expression by neutrophils from non-diabetic subjects (Fig. 5-3).

Apoptosis detection

Flow cytometry showed that neutrophils from T2DM patients were more susceptible to apoptosis than cells from non-diabetic subjects. At 12 h after purification, the levels of Caspase 3 cleavage were significantly greater among neutrophils isolated from T2DM patients than from non-diabetic subjects ($6.7 \pm 0.5\%$ vs. $3.5 \pm 0.3\%$, respectively; $p < 0.01$, $n = 7$, Fig. 5-4). *In vitro* MG treatments similarly increased apoptosis among neutrophils from both T2DM patients and non-diabetic subjects (Fig. 5-4B).

DISCUSSION

Patients with diabetes often experience more serious and prolonged infections (Delamaire, et al., 1997). It has been suggested that a neutrophil dysfunction in these

patients favors such infections, as well as the onset of diabetic angiopathy (Delamaire, et al., 1997), and the development and progression of diabetic nephropathy (Galkina and Ley, 2006).

Among other ways, neutrophils contribute significantly to the initiation and amplification of immune responses through their release of immunoregulatory cytokines (Lloyd and Oppenheim, 1992). TNF-α and IL-6 belong to a group of cytokines that are involved in the upregulation of inflammatory reactions. IL-8 functions to chemotactically attract and stimulate neutrophils, while TNF-α is well known to induce cell apoptosis (Cowburn, et al., 2005). Increased release of cytokines by neutrophils would provide evidence that these cells are activated in diabetic patients, and we did observe an increased basal release of TNF-α, IL-8, and IL-6 by cells from T2DM patients. This increased production of cytokines was not further enhanced by treatment with 5 and 10 μM MG (Fig. 5-2), although the generation of cytokines by neutrophils from non-diabetic subjects was significantly enhanced by MG treatments (5–20 μM). The generation of TNF-α and IL-8 by optimally MG-stimulated normal neutrophils achieved the basal levels of cytokine release by neutrophils of T2DM patients (Fig. 5-2). Conversely, MG (20–30 μM) inhibited the production of cytokines by neutrophils from T2DM patients. More important than the absolute values at each individual assay point shown in Fig. 5-2 are the trends of these responses and clearly the response trends of the neutrophils to MG are totally opposite between non-diabetic and diabetic subjects. The mean duration of diabetes of those patients is about 10 years and their mean level of plasma MG is about 6 μM (Table 5-1A). The persistent and chronic elevation of MG in chronic diabetic patients changes the reaction of neutrophils to MG from hyperactivity at

the beginning to tolerance eventually. With the progress of diabetes, especially in the chronic diabetic patients, neutrophils are tolerant or burn out with the impaired functions (Delamaire, et al., 1997). It is reasonable to postulate that the persistent *in vivo* MG stimulation of the circulating neutrophils of T2DM patients induces their expression of these cytokines as we observed, but that further *in vitro* MG stimulation was actually supermaximal and thereby lead to impairment within these cells of the inflammatory cytokine response. On the other hand, *in vitro* addition of MG to neutrophils from normal subjects, which would not have been constitutively exposed to MG *in vivo*, could thereby augment this cytokine response.

We have shown that the mean plasma MG levels were 6.07 μM in T2DM patients (HbA1c > 7%), a 26.7% increase over those in T2DM patients with HbA1c < 7% (4.79 μM). Results from the United Kingdom Prospective Diabetes Study show that when HbA1c levels increase by 1%, there is a 30% increase in the risk of new microvascular complications or the progression of existing complications in T2DM patients (UKPDS, 1998). Thus, our data suggest that the elevated levels of plasma MG might be associated with the development of T2DM complications. The plasma levels of MG were higher than 10 μM in some patients with high blood HbA1c levels, high fasting plasma glucose levels or high UACRs (Fig. 5-1). With chronic exposure to such elevated levels of MG, the circulating neutrophils of type 2 diabetes patients could be stimulated to generate increased levels of proinflammatory cytokines and thereby potentially facilitate pathogenic inflammatory processes.

Perhaps not unexpectedly, metformin and GSH, considered as scavengers of MG (Wang, et al., 2006; Beisswenger, et al., 1999), significantly inhibited the release of cytokines by neutrophils following MG treatment (5–20 μM) in non-diabetic subjects. Interestingly, when the neutrophils from T2DM patients, which might be thought of as chronically stimulated with MG *in vivo*, were treated with metformin or GSH alone, IL-8 and IL-6 levels were lowered (Fig. 5-3). However, metformin and GSH did not have any discernible effect on neutrophils from T2DM patients that were treated with additional MG *in vitro*. It is possible that the persistent MG stimulation of neutrophils in T2DM patients, potentially lead to saturated release of cytokines. In this scheme, MG-induced neutrophil dysfunction may become irreversible and the newly added metformin and GSH failed to prevent the cellular damage made by the pre-existing MG.

We observed that neutrophils from T2DM patients showed a higher percentage of apoptosis. This could be related to the chronically elevated MG levels in these patients, which we have shown induce constitutive TNF release by their neutrophils, and conceptually this could augment neutrophil apoptotic responses (Cowburn, et al., 2005). Indeed, TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL receptor expression have been reported in human neutrophils (Kamohara, et al., 2004), although the low levels of neutrophil apoptosis observed in diabetics may well not be a causative factor in diabetic inflammation.

The association between diabetes and inflammation could be related in part to the effects of MG on neutrophil proinflammatory cytokine production, as could the susceptibility of diabetic patients to infections. Neutrophil-driven endothelial injury

involves both activation of these leukocytes and their adhesion to the endothelium and both of these are accompanied by upregulation of the integrins CD11a and CD11b on the neutrophils (van Oostrom, et al., 2004). It is well recognized that diabetic nephropathy is integrally related to pathologic changes in the microvasculature of the glomeruli, which are of course critical in the filtration of blood. It is also known that CD11b expression on the neutrophils of diabetic patients precedes early diabetic nephropathy (microangiopathy) and the development of vascular complications, suggesting that the neutrophils are poised in a proinflammatory mode in these individuals (Mastej and Adamiec, 2006). It is possible that MG-induced TNF-α and other inflammatory cytokine production induces the production of an array of chemokines (e.g., monocyte chemoattractant protein-1 (Segerer, et al., 2000)), which chemoattract inflammatory cells, including neutrophils, monocytes, and other leukocytes, into renal tissues and thereby further foster renal inflammatory processes. Thus, MG-induced cytokine generation and release might contribute to inflammatory processes and diabetic complications.

MG induced generation of TNF-α, IL-8, and IL-6 might be realized through activation of the nuclear factor kappa B (NF-κB) signalling pathway. NF-κB generation is associated with increased expression of numerous inflammatory cytokine genes, including TNF-α, IL-8, and IL-6 (Barnes and Karin, 1997), as well as augmented secretion of IL-8, at least, from macrophages (Bhattacharyya, et al., 2002). It has been reported that MG activates NF-κB p65 induction and increases intercellular adhesion molecule-1 expression in vascular smooth muscle cells from normal and spontaneously hypertensive rats (Wu, 2005; Wu and Juurlink, 2002) and that MG upregulates expression of other inflammatory markers, including nervous growth factor and of IL-1β

in hippocampal neuronal cells (Di Loreto, et al., 2004). The molecular mechanisms by which MG induces the generation of proinflammatory cytokines by neutrophils remains an interesting area, although one that has yet to be thoroughly explored.

In summary, MG formation was increased in T2DM patients, especially those with blood HbA1c levels greater than 7%. This increased MG level was closely linked to the severity of hyperglycemia and the UACRs. MG-stimulated neutrophils upregulated their release of TNF-α, IL-6, and IL-8, which could lead to a proinflammatory state and play a role in the development of complications seen in T2DM. The mechanisms for MG-induced increase in production of proinflammatory cytokines in neutrophils are complex. The role of MG in the development of infection in type 2 diabetic patients, therefore, should be further investigated.

Table 5-1A. Basic characteristics of non-diabetes and patients with T2DM

Subject	Non-diabetes	T2DM
n	20	55
Gender (M/F)	15/5	32/23
Age (years)	61.9 ± 3.2	61.4 ± 1.6
Range	(29–82)	(34–80)
Duration (years)		10.1 ± 1.0
Range		(0.5-38)
HbA1c (%)	5.02 ± 0.11	7.21 ± 0.19*
Fasting plasma glucose (mM)	4.78 ± 0.25	9.68 ± 0.67*
NOx (μM)	28.2 ± 0.4	36.7 ± 3.7*

* $p < 0.01$ vs. non-diabetic subjects.

Table 5-1B. Plasma levels of MG in non-diabetes and T2DM with different blood levels of HbA1c

Subjects	*n*	MG (μM)
Non-diabetes	20	3.34 ± 0.38
T2DM with HbA1c < 7%	17	4.79 ± 0.36$^{\#}$
T2DM with HbA1c > 7%	38	6.07 ± 0.44*,$^{\#\#}$

$^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ vs. non-diabetic subjects. $p < 0.05$ vs. T2DM patients with HbA1c < 7%.

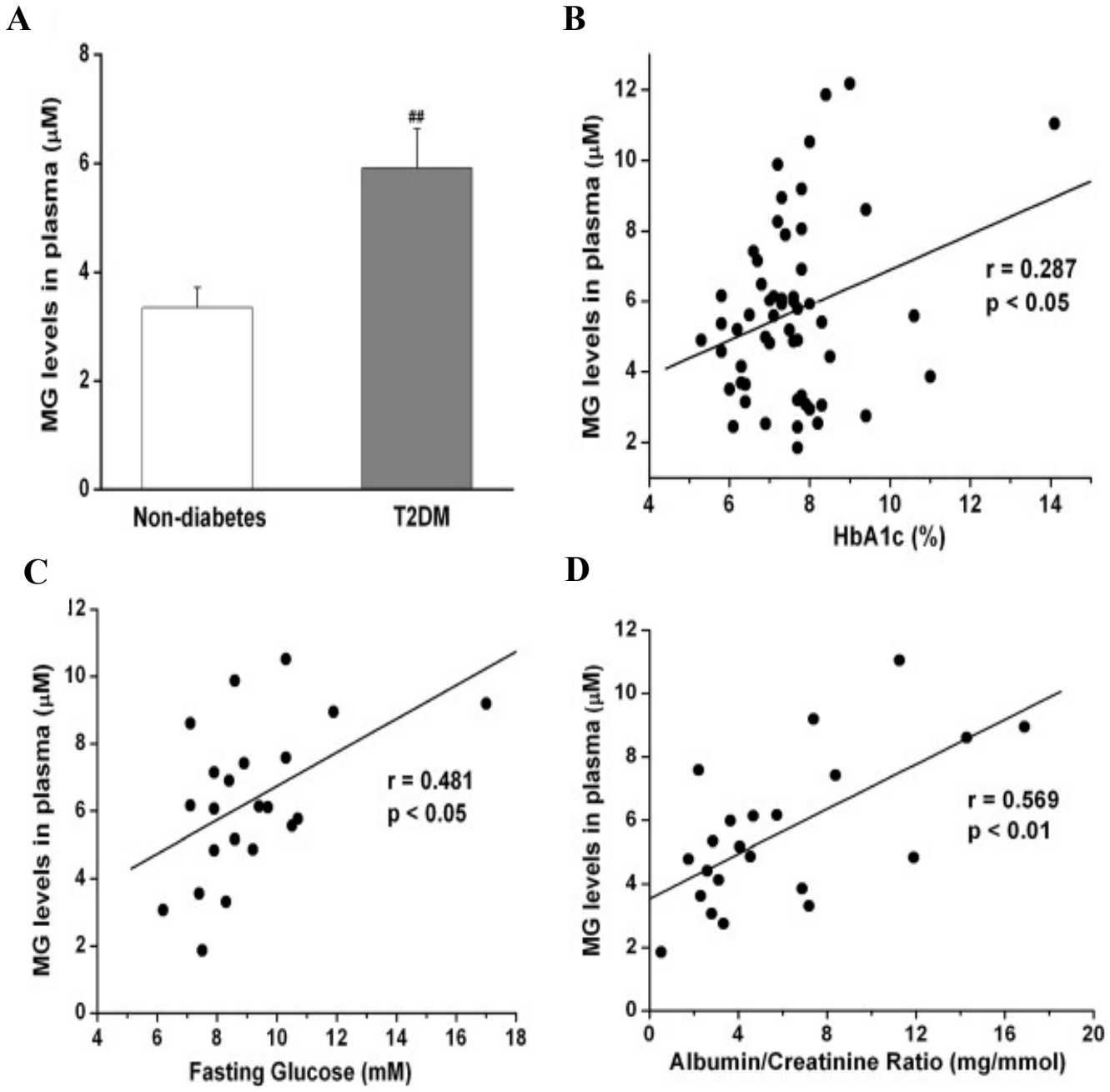

A
MG levels in plasma (μM)
##
Non-diabetes
T2DM
B
MG levels in plasma (μM)
r = 0.287
p < 0.05
HbA1c (%)
C
MG levels in plasma (μM)
r = 0.481
p < 0.05
Fasting Glucose (mM)
D
MG levels in plasma (μM)
r = 0.569
p < 0.01
Albumin/Creatinine Ratio (mg/mmol)

Figure 5-1. Elevated plasma MG levels in type 2 diabetes. (A) Plasma levels of MG in non-diabetic subjects ($n = 20$) and T2DM patients ($n = 55$). $^{\#\#}p < 0.01$ vs. non-diabetic subjects. (B) Correlation of plasma levels of MG and blood HbA1c in T2DM patients. $n = 55$. (C) Correlation of plasma levels of MG and fasting plasma glucose in T2DM patients. $n = 22$. (D) Correlation of plasma levels of MG and UACRs in T2DM patients. $n = 22$. 22 patients sampled for the results shown in panels c and d were not identical. Each value shown in panels b–d represents the result from one patient.

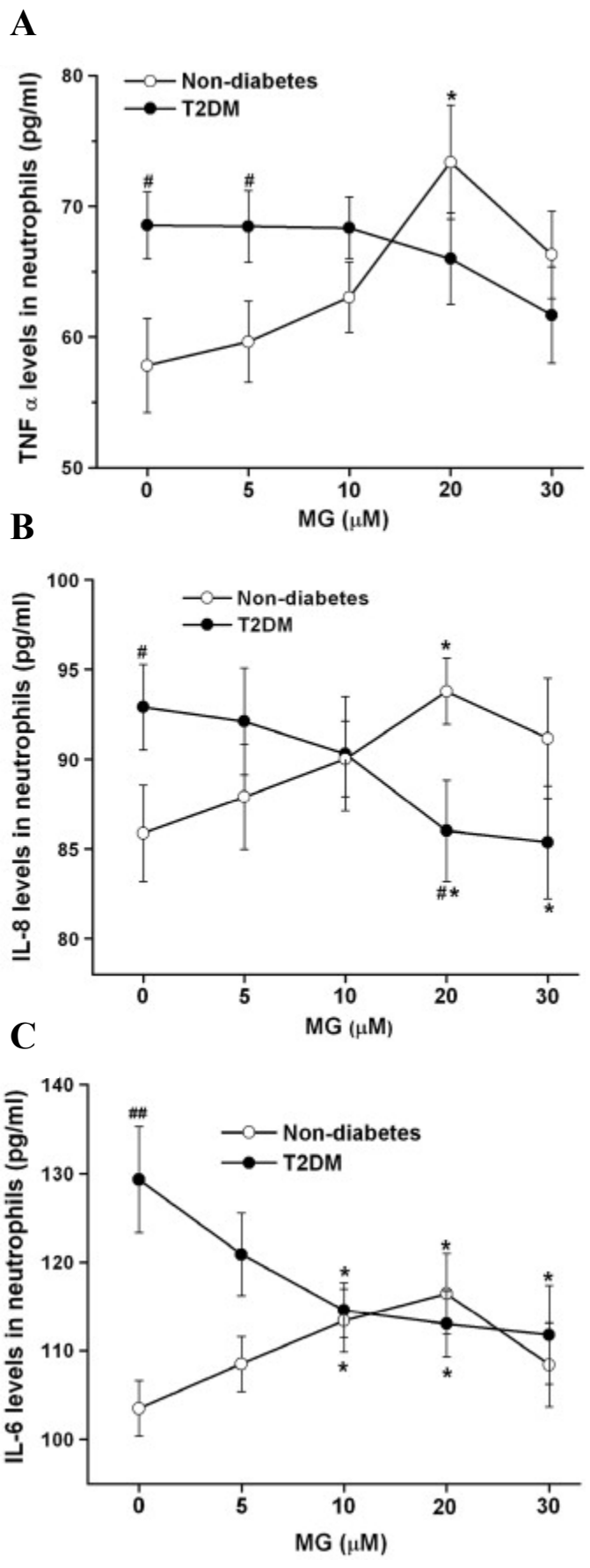

A
Non-diabetes
T2DM
TNF α levels in neutrophils (pg/ml)
MG (μM)
B
Non-diabetes
T2DM
IL-8 levels in neutrophils (pg/ml)
MG (μM)
C
Non-diabetes
T2DM
IL-6 levels in neutrophils (pg/ml)
MG (μM)

Figure 5-2. The effect of MG on the release of TNF-α, IL-8, and IL-6 from neutrophils in non-diabetic subjects and T2DM patients. The levels of cytokine released from neutrophils were determined after exposure to MG (0–30 μM) treatment for 12 h. $n = 5–10$ for each group in A, B, and C. * $p < 0.05$ vs. neutrophils without any treatment from non-diabetic subjects or T2DM patients; $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ vs. neutrophils from non-diabetic subjects with the same treatment.

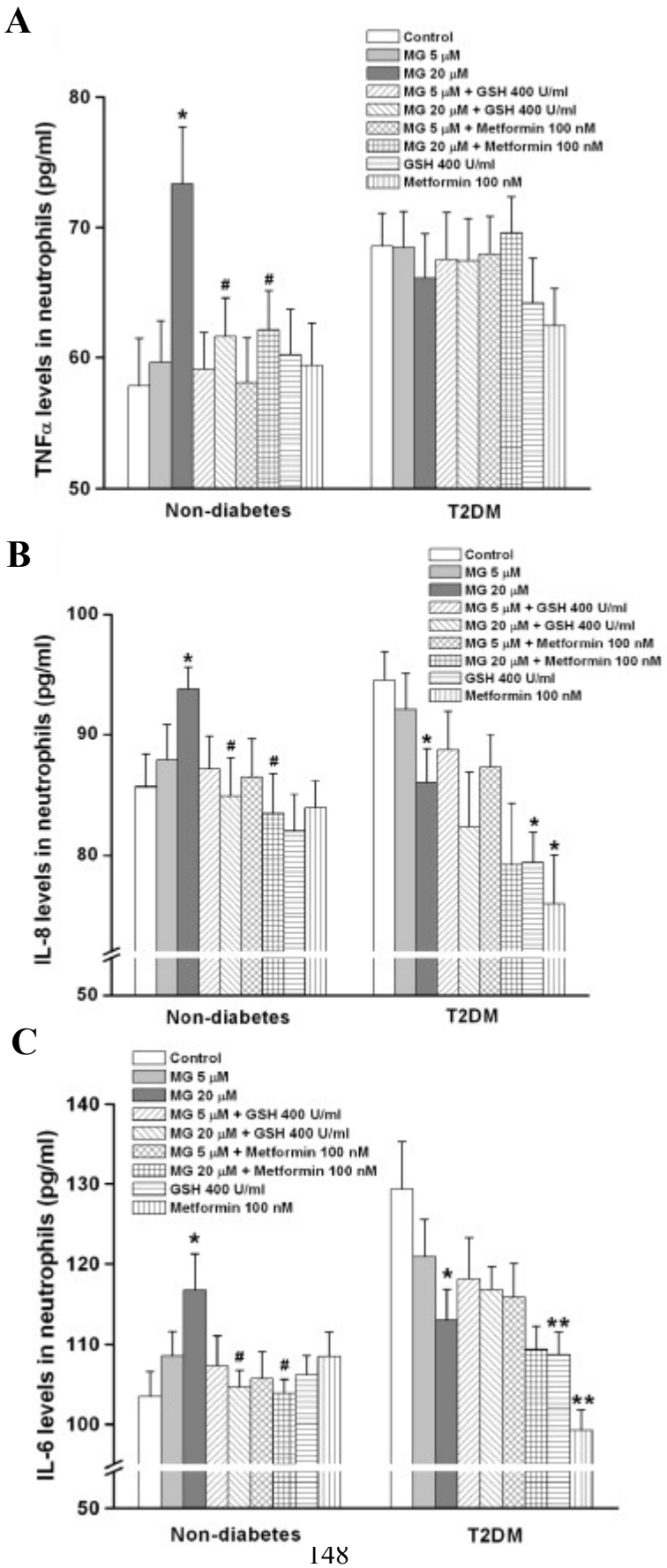
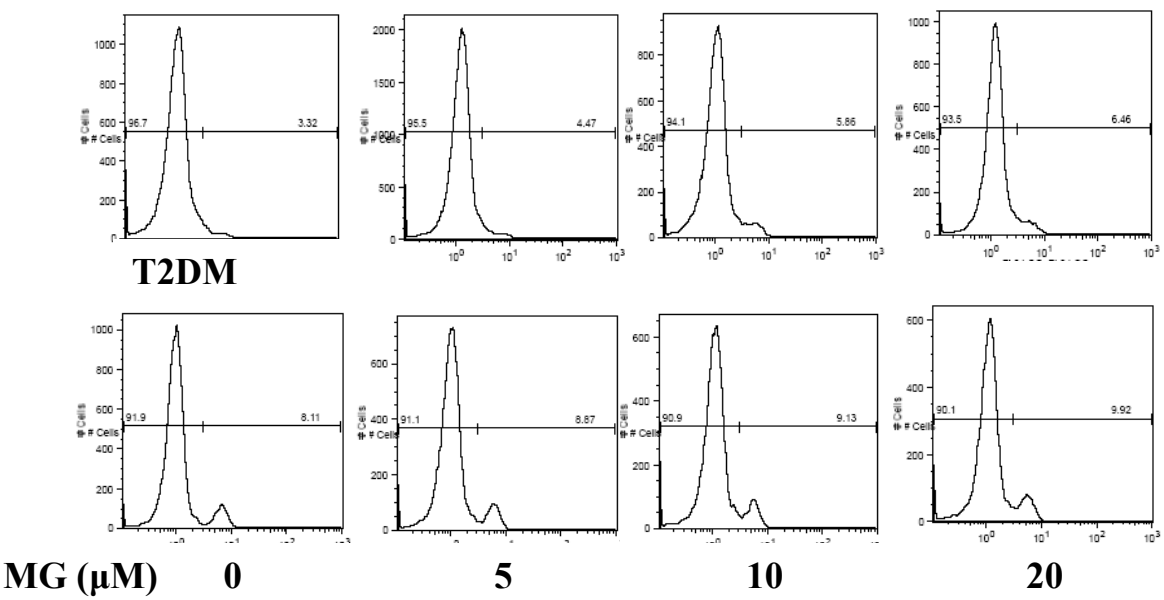

A
Control
MG 5 μM
MG 20 μM
MG 5 μM + GSH 400 U/ml
MG 20 μM + GSH 400 U/ml
MG 5 μM + Metformin 100 nM
MG 20 μM + Metformin 100 nM
GSH 400 U/ml
Metformin 100 nM
TNFα levels in neutrophils (pg/ml)
80
70
60
50
Non-diabetes
T2DM
B
IL-8 levels in neutrophils (pg/ml)
100
90
80
50
C
IL-6 levels in neutrophils (pg/ml)
140
130
120
110
100
50

Figure 5-3. Levels of TNF-α, IL-8, and IL-6 in neutrophils from non-diabetic subjects and T2DM patients with MG (5 or 20 μM) treatment for 12 h in the absence or presence of GSH (400 U/mL) or metformin (100 μM). $n = 5–10$ for each group in A, B, and C. * $p < 0.05$ and ** $p < 0.01$ vs. neutrophils without any treatment in non-diabetic subjects and T2DM patients groups; $^{\#}p < 0.05$ vs. neutrophils treated by MG (20 μM).

A


Non-diabetes
96.7
3.32
95.5
4.47
94.1
5.86
93.5
6.46
T2DM
91.9
8.11
91.1
8.87
90.9
9.13
90.1
9.92
MG (μM) 0 5 10 20

B

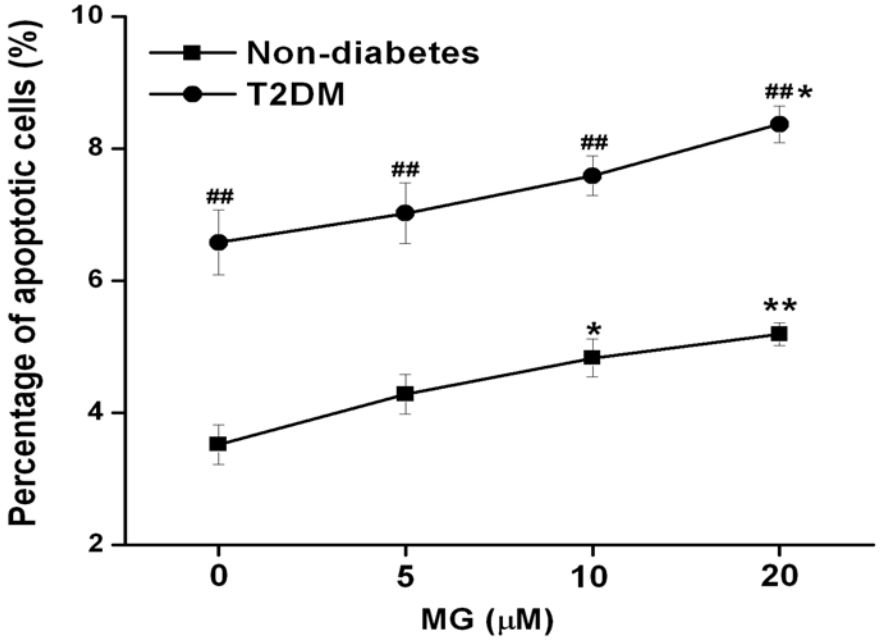

Non-diabetes
T2DM
Percentage of apoptotic cells (%)
MG (μM)
##
##
##
###
*
**

Figure 5-4. MG-induced changes in Caspase 3 cleavage levels in neutrophils. (A) Histograms of Caspase 3 cleavage levels of neutrophils after MG (0–20 μM) treatment *in vitro* for 12 h. (B) The changes in Caspase 3 cleavage levels of neutrophils after MG (0–20 μM) treatment *in vitro* for 12 h. Caspase 3 cleavage was detected with the active Caspase 3 antibody and determined by flow cytometry. $n = 7$ in each group. * $p < 0.05$ and ** $p < 0.01$ vs. neutrophils without any treatment from non-diabetic subjects and T2DM patients; $^{\#\#}$ $p < 0.01$ vs. neutrophils from non-diabetic subjects with the same treatment.

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CHAPTER 6

DISCUSSION AND CONCLUSIONS

General discussion

Since the discussion related to specific results have been given in chapter 3-5, the general discussion of the results, therefore, is present in this chapter.

The role of MG in pathophysiology of T2DM and development of diabetic complications is widely investigated. MG-induced oxidative stress is postulated to contribute to the pathogenesis of insulin resistance. However, the effect of endogenous MG on $ONOO^-$ generation, mechanism of MG-induced oxidative damage, and the role of MG in neutrophil dysfunction and inflammatory status in patients with T2DM have not been thoroughly studied.

Our present studies showed that MG levels were significantly increased when VSMCs were incubated with fructose. Generation of MG by fructose occurred in a concentration- and time- dependent manner. Fructose, via the formation of MG, significantly induced the production of $O_2^{\cdot -}$, NO, and $ONOO^-$. Fructose- or MG-induced $ONOO^-$ generation was significantly inhibited by MG scavenger GSH, and by $O_2^{\cdot -}$ or NO inhibitors, such as diphenylene iodonium, SOD or L-NAME. Moreover, an enhanced iNOS expression was also observed in the cells treated directly with MG. Our data demonstrate that enhanced MG formation causes overproduction of $ONOO^-$, which eventually impairs vascular function and contributes to diabetic complications.

The mechanisms of MG-induced $ONOO^-$ generation in VSMCs were further explored. Since mitochondria are major sources of $O_2^{\cdot -}$ formation, effect of MG on

mitochondrial enzymes and functions was tested. We found that MG (30 μM) significantly enhanced mitochondrial $O_2^{\cdot -}$, NO, and $ONOO^-$ production. MG also significantly inhibited respiratory complex III activity and decreased MnSOD activity. Our data demonstrate that by inhibiting complex III activity, MG induces mitochondrial oxidative stress and reduces mitochondrial ATP generation, which indicates the dysfunction of ETC and mitochondria. The findings further confirm that MG-induced mitochondrial dysfunction and oxidative stress play a critical role in the pathogenesis of T2DM and associated complications.

We also tested the role of MG in patients with chronic T2DM. The higher levels of plasma MG in T2DM was correlated positively with HbA1c, fasting plasma glucose and urine albumin / creatinine ratios. In addition, we found that MG induced the release of cytokines from neutrophils of non-diabetics. In contrast, MG treatment of the neutrophils isolated from type 2 diabetic patients either did not alter, or decreased the production of cytokines. Moreover, the neutrophils from patients with T2DM showed a greater proclivity for apoptosis, which was further increased by *in vitro* MG treatment. These data suggest that MG not only directly causes oxidative damages in diabetic patients, but also initiates or promotes the inflammatory process in T2DM.

Our present studies show that MG increased $ONOO^-$ production, reduced antioxidant ability and enhanced the levels of circulating proinfammatory cytokines. The effects of MG contribute to the development of T2DM.

1. Fructose, endogenous MG and T2DM

The main source of MG in mammals is anaerobic glycolysis, i. e. non-enzymatic and enzymatic elimination of phosphate from G-3-P and DHAP (Koop and Casazza, 1985; Lyles and Chalmers, 1992). Glucose and fructose are major precursors of DHAP and G-3-P. However, most *in vitro* studies focused on the relationship of glucose with MG. For example, Padayatti *et al*. reported that retinal endothelial cells incubated with 30 mM D-glucose produced significantly higher levels of intracellular MG than control cells or cells incubated with 30 mM L-glucose (Padayatti, et al., 2001). L-glucose is unable to induce intracelluar MG generation because it is not permeable to endothelial cells. Our study, for the first time, demonstrated that fructose (15-30 mM) significantly induced endogenous MG formation in VSMCs. Fructose is metabolized by hexokinase or ketohexokinase to G-3-P and DHAP, which directly form MG. The consumption of fructose and sucrose (1 fructose and 1 glucose) in beverages and processed food is increased dramatically. Serum fructose in diabetic patients significantly increased to 12 μM compared with a level of 8.1 μM in healthy subjects (Kawasaki, et al., 2002). Diet with high fructose can induce insulin resistance. Increased blood pressure, hyperinsulinemia and hypertriglyceridemia have been observed in normal SD rats fed with high fructose (66%) diet for 2 weeks (Hwang, et al., 1987). Nagai and coworkers successfully established a rat model which is on the diet of fructose to mimic the metabolic condition of T2DM (Nagai, et al., 2009). Furthermore, 15% fructose diet for 5 weeks resulted in a significantly higher insulin and glucose response in human subjects (Hallfrisch, et al., 1983). The mechanism of fructose-induced insulin resistance is still unclear.

The current study showed that fructose, via the production of MG, induced $ONOO^-$ formation in VSMCs. This effect of fructose was inhibited by MG scavenger GSH. $ONOO^-$ is formed via the reaction of NO and $O_2^{\cdot -}$. $ONOO^-$ crosses cell membranes freely and causes protein, lipid and DNA damage. It also has been reported that $ONOO^-$ impaired the sarcoplasmic reticulum Ca^{2+} pump in pig coronary artery SMCs and triggered apoptosis in cultured rat aortic SMCs (Sechi, et al., 1996). The observation of fructose-induced $ONOO^-$ generation via MG formation in VSMCs strongly suggests that overproduction of endogenous MG contributes to the oxidative stress occurred in diabetic patients. Moreover, the consumption of high fructose diet should be limited in order to prevent the development or slow down the progression of T2DM and its complications.

2. MG, mitochondria $ONOO^-$ and T2DM

As mentioned earlier, endogenous MG induced cellular $ONOO^-$ generation in VSMCs. Interestingly, we further found that MG significantly induced mitochondrial $O_2^{\cdot -}$, NO, and $ONOO^-$ production in VSMCs. Increased mitochondrial NO production reacts with $O_2^{\cdot -}$ to form $ONOO^-$. Mitochondrial $ONOO^-$ formation was reported to be related to the pathophysiology of T2DM and its complications (Kim, et al., 2008). $ONOO^-$ can directly break DNA stands and activate nuclear enzyme poly (ADP-ribose) polymerase (PARP). PARP induces the activation of multiple pathways including activation of NF-κB, PKC, AGEs generation, which are believed to be the main

molecular mechanisms of diabetic complications (Szabo, 2009). mtROS also induced the activation of PKC, formation of AGEs and accumulation of sorbitol in endothelial cells (Nishikawa and Araki, 2007). In addition, mtROS caused glomerular hyperfiltration and diabetic nephropathy through activation of COX-2 gene transcription and PGE2 overproduction (Kiritoshi, et al., 2003). mtROS suppressed the first phase of glucose-induced insulin secretion in pancreatic β cells (Sakai, et al., 2003). Furthermore, normalization of mitochondrial $O_2^{\cdot -}$ production blocked hyperglycemia-induced damage (Nishikawa, et al., 2000).

The activity of mitochondrial complex III was decreased after MG treatment. As shown in figure 1-5, complex III transfers electrons from ubiquinone to cytochrome C and pumps protons from the inside to the outside of the mitochondrial inner membrane to make the electrochemical gradient. This gradient drives ATPase to catalyze ATP production. Complex III is responsible for the generation of $O_2^{\cdot -}$. Inhibition of complex III blocks the electron transportation and increases the leaking out of electrons to form $O_2^{\cdot -}$ in the mitochondria. Consequently, the hydrogen electrochemical gradient across the inner mitochondrial membrane is weakened, and ATP synthesis is reduced. Cellular integrity and function are therefore compromised. Hyperglycemia is closely linked with the impaired complex III activity. Hu *et al*. reported that high glucose impaired mitochondrial proteins, including complex III in cardiac myocytes (Hu, et al., 2009). Significantly decreased complex III activity was also observed in the kidney of early diabetes (Munusamy, et al., 2009). In addition, complex-III was inhibited in mouse retina during the chronic development of diabetes (Kanwar, et al., 2007). Therefore, complex III dysfunction plays an important role in the pathophysiology of insulin

resistance syndrome. Our study, for the first time, shows that MG inhibits mitochondrial complex III activity in VSMCs, which explains the increased mitochondrial ROS production and reduced ATP generation in diabetes and its complications.

In addition, we found that MG decreased MnSOD activity in VSMCs. MnSOD is the key enzyme eliminating $O_2^{\cdot -}$ in mitochondria. Normal activity of MnSOD ensures normal mitochondrial function. Increased mitochondrial damage during aging has been observed in partial MnSOD deficiency ($MnSOD^{+/-}$) mice (Wallace, 2002). Mutational inactivation of genes encoding MnSOD caused neonatal lethal dilated cardiomyopathy, which was ameliorated by treatment with MnSOD mimics (Wallace, 2002). Genotypic studies showed that deficiency of MnSOD Ala16Val polymorphism was associated with the development of T2DM and increased the risk of diabetic nephropathy in Japanese-Americans and in Chinese (Nakanishi, et al., 2008; Liu, et al., 2009). On the other hand, overexpression of MnSOD in endothelium inhibited expression of retinal vascular endothelial growth factor in diabetic mice and prevented hyperglycemia-induced increases in mitochondrial $O_2^{\cdot -}$ levels and membrane permeability and the decrease in complex III activity (Goto, et al., 2008). Those results strongly implicated the role for MnSOD in the pathogenesis of retinopathy in diabetes (Kanwar, et al., 2007). Furthermore, elevation of MnSOD improved respiration and normalized mass in diabetic mitochondria. MnSOD also protected the morphology of diabetic hearts and completely normalized contractility in diabetic cardiomyocytes (Shen, et al., 2006). MG-induced decrease of MnSOD activity in our study contributes to the enhanced mitochondrial ROS production.

MG-inhibited complex III activity and antioxidant defense enzyme MnSOD provide the new view of physiological importance and pathophysiological implications of the interaction of MG with mitochondria functions. It also sheds light on pathogenesis of, and treatment for many mitochondrial-originated cellular disorders encountered in insulin resistance syndrome.

3. MG, inflammation and T2DM

Immunologic abnormalities, such as inflammation, are associated with T2DM and diabetic complications. It is notable that the circulating levels of proinflammatory cytokines, such as IL-6 and TNF-α, are significantly elevated in patients with T2DM (Pickup, et al., 2000). Neutrophils are the first line of cell defense against various infections. They contribute significantly to the initiation and amplification of immune response through their release of proinflammatory cytokines (Lloyd and Oppenheim, 1992). Neutrophils in patients with T2DM display increased necrosis, enhanced production of ROS (Shurtz-Swirski, et al., 2001) and significantly lower chemotactic responses (Delamaire, et al., 1997). In the current study, MG significantly induced the release of TNF-α, IL-8 and IL-6 by neutrophils from healthy control. MG might induce cytokine generation via the activation of NF-κB. Previous study in our lab showed that MG activated NF-κB p65 induction and increased intercellular adhesion molecule-1 expression in VSMCs from normal rats and SHR (Wu, 2005; Wu and Juurlink, 2002). NF-κB generation is associated with the increased expression of numerous inflammatory

cytokine genes, including TNF-α, IL-8 and IL-6 (Barnes and Karin, 1997). On the other hand, our study showed that MG increased plasma NO levels and reduced red blood cell GSH in patients with T2DM, indicating the existence of MG-induced oxidative stress in diabetic patients. As mentioned earlier, ROS degrade the inhibitory IκBα unit, and provoke the rapid translocation of active NF-κB into nucleus, leading to the formation of proflammatory cytokines (Ho and Bray, 1999). In the current study, an increased basal level of cytokines released by neutrophils from patients with T2DM was observed. High levels of plasma MG in diabetic patients might stimulate neutrophils to produce more cytokines. On the contrary, MG treatment inhibited the production of cytokines by neutrophils from T2DM patients. As the average duration of patients in the present study is about 10 years, the persistent and prolonged elevation of MG in diabetic patients hyperactivates neutrophils. It is reasonable to postulate that further *in vitro* MG stimulation was actually supermaximal, converting neurophils from proinflammatory state to dysfunctional state, and thereby lead to reduced inflammatory cytokine response.

MG stimulates neutrophils and upregulates their release of TNF-α, IL-8 and IL-6. TNF-α and IL-6 are cytokines upregulating inflammatory reactions. IL-8 chemotactically attracts and stimulates neutrophils, while TNF-α is well known to induce cell apoptosis (Cowburn, et al., 2005). Activated NF-κB has been observed in patients with T2DM (Aljada, et al., 2001). Thus, MG-induced oxidative stress in patients with T2DM may implicate in MG-mediated NF-κB-cytokine pathway. The proinflammatory effect of MG initiates an inflammatory state in T2DM and plays an important role in the development of diabetic complications. Mechanisms of MG-induced production of proinflammatory cytokines in neutrophils need to be further explored.

4. Scavengers of MG andT2DM

Scavengers of MG show positive effects to attenuate or postpone diabetic complication. Aminoguanidine, metformin and alagebrium all decreased MG-mediated glycation of ApoA-I in discoidal rHDL and preserved normal HDL function in diabetes (Nobécourt, et al., 2008). Several MG scavengers, GSH, metformin and alagebrium have been used in the current studies. First, fructose- or MG-induced cellular $ONOO^-$ generation was significantly inhibited by GSH. Second, alagebrium significantly reversed all effects of MG on mitochondrial ROS generation, CEL expression, MnSOD activity and complex III activity. Third, metformin and GSH successfully inhibited the release of cytokines by neutrophils following MG treatment.

GSH is the cofactor of MG degradation in the glyoxalase enzyme pathway. High availability of GSH results in the rapid detoxification of MG and prevents MG accumulation in cells. Metformin was observed to significantly reduce circulating MG levels in patients with T2DM (Beisswenger, et al., 1999). Metformin also directly traped MG and reduced its levels *in vitro* (Ruggiero-Lopez, et al., 1999).

Aminoguanidine (1 g/L in drinking water) prevented the development of albuminuria, mesangial expansion and glomerular basement membrane thickening in kidneys of diabetic rats (Yamauchi 1997). A five-year study of diabetic dogs showed that aminoguanidine therapy (20–25 mg/kg) prevented the development of retinopathy in these animals. There were decreased retinal microaneurysms, acellular capillaries, and pericyte loss in aminoguanidine treated animals compared with those in diabetic controls

(Kern and Engerman, 2001). Aminoguanidine (25 and 50 mg/kg/day i.p.) prevented decreased nerve blood flow and improved nerve conduction velocity in streptozotocin-induced diabetic rats. Effect of aminoguanidine on nerve conduction was in a dose-dependent fashion (Kihara, et al., 1991).

Alagebrium has been studied in various diabetic animal models and has been shown to attenuate diabetic nephropathy, cardiac dysfunction and atherosclerosis (Coughlan, et al., 2007). In streptozotocin diabetic apo E knockout mouse, which is a diabetic nephropathy model with not only albuminuria and glomerular injury but also significant tubulointerstitial injury with prominent fibrosis and macrophage infiltration, alagebrium reduced albuminuria and renal structural injury (Lassila, et al., 2004). Alagebrium treatment also attenuated damage of ROS to diabetic kidneys. Application of alagebrium reduced mitochondrial superoxide generation and enhanced MnSOD activity in diabetic kidneys (Coughlan, et al., 2007). The effect of alagebrium on cardiac function has been demonstrated in different models. Alagebrium treatment increased ventricular collagen solubility in diabetic aged dogs, reduced left ventricular collagen content and improved diastolic function in aged SHR (Susic, et al., 2004), and finally, improved large artery stiffness and reduced arterial pulse pressure in clinical trials (Liu, et al., 2003). Furthermore, alagebrium reduced atherosclerotic plaque formation in streptozotocin diabetic apo E knockout mice (Lassila, et al., 2004).

Taken all together, our results further confirmed the pro-oxidant and pro-inflammatory roles of MG. MG scavengers, such as GSH, metformin and alagebrium have beneficial effect and prevent or inhibit MG-induced vascular oxidative damage and

the inflammatory state. Application of MG scavengers may prevent the development or slow down the progression of T2DM and its vascular complications.

5. Limitations of study

Glucose can be transported through cell membranes via the facilitation of glucose transporter (GLUT). The genetic investigation of GLUT is still undergoing, and the GLUT family is expanding. Currently, this big family contains 3 subgroups: GLUT 1-4, GLUT 5, 7, 9, 11 and GLUT 6, 8, 10, 12. GLUT 5, 7, 9 and 11 are identified as fructose transporters. Studies show that GLUT 5 is located in the epithelial brush border of upper small intestine, brain, kidney, skeletal muscle and adipose cells. GLUT 7 is on liver and kidney and GLUT 11 is located on heart and skeletal muscle (Joost, et al., 2001). In addition, MG is a small molecule and the mechanism of MG crossing through cell membrane is still unclear. To date, no study is available to clarify if MG crosses plasma membrane freely or via transporters. The mechanisms of MG crossing through smooth muscle cells and neutrophils need further exploration.

Several laboratories are investigating MG besides our group. Different results were obtained due to the diversity of MG concentrations and experimental procedures. For instance, Speer and coworkers in Eriksson's lab incubated isolated mitochondria with MG (2 mM) for 5 min and found MG transiently suppressed mitochondrial permeability transition (Speer, et al., 2003). Authors also tested mitochondrial oxygen consumption which indicates mitochondrial respiratory rate and found that MG did not affect mitochondria respiration rate. Basically, our story is different from Speer's. We focused

on the effect of MG on mitochondrial oxidative stress. Levels of free radicals and activities of mitochondrial enzymes were measured. Our results show that MG induced mtROS generation and reduced activities of complex III and MnSOD. In addition, up to 10 mM MG had been used to investigate its effect on insulin secreting cells and insulin signaling pathways in rat L6 myoblasts (Sheader, et al., 2001; Riboulet-Chavey, et al., 2006). It is not reasonable to compare our results with theirs because we used MG (30 μM) to treat cells for 18 h. The physiological concentration of plasma MG in rats is approximately 5 μM (Nagaraj, et al., 2002). Our previous study detected the plasma MG levels of 33.6 μM in 20-week-old SHR and 14.2 μM in age-matched WKY rats (Wang, et al., 2004). Plasma levels of MG increased from 3.3 μM in healthy humans to 5.9 μM in type 2 diabetic patients (Wang, et al., 2007). Moreover, cultured cells may produce more MG since MG concentration up to 310 μM was detected in cultured Chinese hamster ovary cells (Chaplen, et al., 1998). Thus, MG (30 μM) used in the present study is not only the physiological relevant concentration, but also suitable to mimic the insulin resistance environment in rat aortic smooth muscle cells. High concentrations of MG, such as 2 mM or 10 mM, are not physiological levels and not proper for mimicking physiological conditions.

CONCLUSIONS

Our studies demonstrate the important role of MG-induced oxidative stress in T2DM. MG mediated fructose-induced production of NO, $O_2^{\cdot -}$, and $ONOO^-$ in VSMCs. Fructose- or MG-induced $ONOO^-$ generation was significantly inhibited by the MG

scavenger GSH, and by $O_2^{\cdot -}$ or NO inhibitors. Moreover, an enhanced iNOS expression was also observed in the cells treated directly with MG. We also observed that MG significantly enhanced mitochondrial $O_2^{\cdot -}$, NO, and $ONOO^-$ production, increased the expression of MG-induced AGE (CEL) and $ONOO^-$ modified protein (nitrotyrosine) expression, and decreased MnSOD activity. Furthermore, MG significantly inhibited respiratory complex III activity and reduced mitochondrial ATP generation, indicating the dysfunction of ETC and mitochondria.

In addition, the higher levels of plasma MG in T2DM was correlated positively with HbA1c, fasting plasma glucose and urine albumin / creatinine ratios. MG induced the release of cytokines from neutrophils of non-diabetics. In contrast, MG treatment of the neutrophils isolated from type 2 diabetic patients either did not alter, or decreased the production of cytokines. Moreover, the neutrophils from patients with T2DM showed a greater proclivity for apoptosis, which was further increased by *in vitro* MG treatment. These data suggested that MG initiates or promotes the inflammatory process in T2DM.

In conclusion, our data demonstrate that MG-induced oxidative stress plays a critical role in the pathogenesis of T2DM. Increased $ONOO^-$ production, decreased antioxidant defense ability, impaired mitochondrial function and enhanced circulating proinflammatory cytokines contribute to MG-induced oxidative stress, the development of T2DM and its vascular complications.

SIGNIFICANCE OF THE STUDY

More than 2 million Canadians have diabetes and this number is expected to reach 3 million by 2010. Approximatly 90% of diabetic patients have T2DM. The personal costs of diabetes are mainly due to the increased incidence of complications such as neuropathy, nephropathy, cardiovascular disease, retinopathy, and stroke. Approximately 80% of people with diabetes will die as a result of heart disease or stroke. Overall, diabetes contributes to the deaths of over 40, 000 Canadians each year. Moreover, the financial burden of diabetes and its complications is huge. The direct cost for each diabetic patient for medication and supplies ranges from $1,000 to $15,000 a year. It is estimated that diabetes will cost the Canadian healthcare system $15.6 billion a year by 2010 and rise to $19.2 billion by 2020 (Canadian Diabetes Association, http://www.diabetes.ca/about-diabetes/what/prevalence/).

Clarifying the role of MG in the development of T2DM is significant and may lead to discoveries of new mechanisms and methods for the prevention and treatment of T2DM and associated complications. The derived novel discoveries can be directly transformed to pharmaceutical anti-diabetic drugs and to new diagnostic methods for early diagnosis and follow-up the progression of T2DM.

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 MG-modified mitochondrial proteins. The activities and expression levels (mRNA and protein) of different mitochondrial enzymes involved in ROS generation will be determined and quantified using Real-Time PCR and Western blot analysis.
2. To investigate other mechanisms of MG-induced oxidative stress besides mitochondrial protein modification. Those mechanisms include p38 MARK and NF-κB mediated signaling pathways.
3. To investigate the mechanisms of MG-induced proinflammatory cytokines generation. Different parameters regulating cytokine production in neutrophils will be explored, including mRNA expression of NFκB and the IκBα unit.

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