EXAMINATION OF ADVANCED GLYCATION END-PRODUCTS IN CHILDREN WITH TYPE 1 DIABETES

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Examination of Advanced Glycation End-products in Children with Type 1 Diabetes

by

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ABSTRACT

Type 1 diabetes mellitus (T1DM) results from the autoimmune destruction of the insulin producing β cells in the pancreas. In T1DM, insulin deficiency induces abnormal metabolism of glucose, lipids and protein that may result in elevation of reactive aldehydes methylglyoxal and glyoxal. Hyperglycemia and high levels of methylglyoxal and glyoxal can modify cell protein, promoting the formation of advanced glycation end-products (AGEs), which may contribute to the development of diabetic complications. In this research, novel high-performance liquid chromatograph coupled with tandem mass spectrometric detection (LC-MS/MS) methods to measure plasma methylglyoxal, glyoxal and some of AGEs were developed. Plasma methylglyoxal, glyoxal and one AGE (MG-H1) were measured in young humans with complication-free T1DM. The activity of the ubiquitous membrane enzyme, Na^+/K^+-ATPase, was also assessed. Fifty-six patients with T1DM (DM group), 6 to 22 years, and 18 non-diabetics (ND group), 6 to 21 years, were enrolled in the study. Mean hemoglobin A1C (%) was higher in the DM group (8.5 ± 1.3; mean ± standard deviation) as compared to the control group (5.0 ± 0.3). The mean plasma methylglyoxal (nmol/L) and glyoxal level (nmol/L), respectively, were found higher in the DM group (842 ± 238, 1052 ± 515) versus the control group (439 ± 90, 328 ± 208). Plasma free AGE, MG-H1 (mg/L), was also found to be higher in the DM group (2.7 ± 1.1) versus the ND group (1.7 ± 0.9), and weakly correlated with methylglyoxal levels but not glycemia as determined by A1C. Erythrocyte membrane Na^+/K^+-ATPase

activity (nmol NADH oxidized/ min/mg protein) was elevated in the DM group (4.47 ± 0.98) compared to the ND group (2.16 ± 0.59). A1C correlated with plasma methylglyoxal and glyoxal, and both aldehydes correlated with each other. A high correlation of A1C with Na^+/K^+-ATPase activity, and a regression analysis which showed that A1C was a good predictor of this enzyme activity, suggests that glucose may play a role in promoting membrane alterations. Increased plasma methylglyoxal, glyoxal, plasma free MG-H1 and erythrocyte Na^+/K^+-ATPase activity may predict the occurrence of future diabetic complications which may be prevented by early aggressive insulin treatment.

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LIST OF ABBREVIATIONS

A1C	Hemoglobin A1C
AGEs	Advanced glycation end-products
AP-1	Activator Protein-1
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AUC	Area under the curve
BSA	Bovine serum albumin
CBC	Complete Blood Count
CE	Collision energy
CEC	Carboxyethyl-cysteine
CEL	Nε-carboxyethyl-lysine
cGMP	Cyclic guanosine monophosphate
CID	Collision induced dissociation
CMC	Carboxymethyl-cysteine
CTLA-4	Cytotoxic T lymphocyte antigen 4
CVD	Cardiovascular disease
D4-lysine	L-Lysine-(4,4,5,5-D4)
DAG	Diacylglycerol

DAN	Diaminonaphthalene
DETPA	Diethylenetriaminepenta acetic acid
DHAP	Dihydroxyacetone phosphate
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethyleneglycol bis (2-aminoethyl-ether) tetraacetic acid
ENOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal-regulated kinase ½
ESI	Eelectrospray ionization
G3P	Glyceraldehyde-3-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC-MS	Gas chromatography/mass spectrometry
G-H1	Glyoxal-derived hydroimidazolones
GOLD	Glyoxal-derived lysine dimer
HFBA	Heptafluoro butyric acid
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatograph
IA-2	Islet antigen-2
ICAM-1	Intracellular adhesion molecule-1
IL-1	Interleukin-1
JNK	Jun N-terminal kinase

LC-MS	Liquid chromatography- mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low density lipoprotein
LYP	Lymphoid protein tyrosine phosphatase
MAPK	Mitogen-activated protein kinases
MEK	MARK/ERK kinase
MG	Methylglyoxal
MG-H	Methylglyoxal-derived hydroimidazolone
MOLD	Methylglyoxal-derived lysine dimer
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
mSin3A	Mammalian Sin3A
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
ND	Non-diabetes
NF-κB	Nuclear factor κB
NFPA	Nonafluoropentanoic acid
NO	Nitric oxide
$ONOO^-$	Oxidizing peroxynitrite anion
p38	Protein kinase 38
PDGF	Platelet derived growth factor

PEP	Phosphoenolpyruvate
PKC	Protein kinase C
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RAGEs	Receptor of advanced glycation end-products
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SHR	Spontaneously hypertensive rats
SPE	Solid phase extraction
SSAO	Semicarbazide-sensitive amine oxidase
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
t-BOC arginine	t-butyloxycarbonyl arginine
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TNF-α	Tumor necrosis factor – α
Tris	Tris(hydroxymethyl)amino-methane
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelium growth factor
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell
WKY	Wistar-Kyoto rats

CHAPTER 1

INTRODUCTION AND OVERVIEW

Type 1 Diabetes Mellitus (T1DM) is a common chronic condition in children that is characterized by hyperglycemia. Over time this results in complications like hypertension, nephropathy, retinopathy, and neuropathy. Hemoglobin A1C (A1C) is a marker of glycemia and correlates with the risk of complications (Valeri et al., 2004). Some patients with good glycemic control develop severe complications, while others whose glycemic control may be inadequate remain relatively free of complications for long periods of time (Forbes et al., 2005; Snieder et al., 2001). To date there is no biochemical test that can reliably predict clinical outcome in an individual patient. Determining a sensitive predictor or etiological basis of diabetic complications would allow for appropriate early interventions with reduction in morbidity and mortality in individuals with diabetes mellitus (DM) (Forbes et al., 2005).

Advanced glycation end-products (AGEs) form as a result of a series of chemical reactions following glycation. In DM, hyperglycemia and altered glucose metabolism lead, to an excess generation of reactive aldehydes, like methylglyoxal and glyoxal (Ahmed and Thronalley, 2007). Both of these dicarbonyls are known to cause AGE formation by non-enzymatic reaction with the amino side chains of arginine or lysine, or the sulfhydryl groups of cysteines in proteins. AGEs and their precursors are found in many tissues including plasma. AGE formation may affect the structure and function of cellular proteins and membrane pump enzymes like Na^+/K^+-ATPase that control cellular

ions, and contribute to the pathogenesis of diabetic complications. In this study we developed methods to measure methylglyoxal and the structurally related dicarbonyl, glyoxal, and free AGE residues in plasma. The relationships between A1C, plasma methylglyoxal and glyoxal levels, and free AGE residues was assessed in young individuals with uncomplicated T1DM. We also explored the relationship between these factors and Na^+/K^+-ATPase activity in the red blood cell (RBC) membrane.

1.1 Type 1 Diabetes Mellitus (T1DM)

1.1.1 Diabetes Mellitus (DM)

DM is characterized by persistent and variable hyperglycemia (high blood glucose levels). The cost of DM to healthcare and society in general is tremendous, and estimated at US$5.2 billion in Canada alone (Dawson et al., 2002). This includes an estimated $573 million directly spent on DM care and another $637 million spent on diabetes related cardiovascular disease.

DM is subdivided into 3 types depending on etiology. T1DM is caused by an absolute deficiency in insulin due to autoimmune destruction of islet ß cells in the pancreas. Type 2 DM (T2DM) is associated with obesity and is characterized by tissue insulin resistance and inadequate insulin secretion. This may eventually progress through a number of phases finally resulting in ß cell burnout and absolute insulin deficiency. A third type is gestational DM, a condition related to T2DM but presents as hyperglycemia

only during pregnancy (Canadian Diabetes Association Clinical Practice Guidelines, 2003).

T1DM, also known as insulin-dependent DM, childhood DM, or juvenile-onset DM, most commonly presents in children and adolescents. The typical age of onset is less than 25 years (Pepper, 2006). Also, in contrast to T2DM, T1DM occurrence is typically in individuals who are lean rather than obese (Ganong, 2003; Myers, 2005). The work of this thesis focuses on T1DM. T1DM constitutes approximately 10% of all individuals with DM and occurs mainly in populations of Europe and North America (Champe et al., 2005; Gillespie, 2006; Habermann, 2006). T1DM is increasing in incidence globally at a rate of about 3% per year (EURODIAB ACE Study Group, 2000). There are isolated populations (such as in Newfoundland and Labrador) where T1DM is higher (Newhook, 2004). In Newfoundland the incident rate of T1DM is approximately 36 per 100,000 compared with about 25 per 100,000 or less elsewhere in Canada. Like all types of DM, T1DM is associated with increased risk for and a high incidence of certain complications. Hence, DM in general has been considered a syndrome of metabolic abnormalities (i.e. metabolic disorder of glucose, protein, lipids, water and electrolytes), microvascular disease (i.e. retinopathy, neuropathy, and nephropathy), and macrovascular disease (i.e. atherosclerosis) (Myers, 2005; Champe, et al., 2005). DM is the leading cause of adult blindness and amputation, and is a major cause of renal failure, heart disease, and stroke (Champe et al., 2005). Cardiovascular disease (CVD) is the main cause of premature death among people with DM – about 65% of people with DM die from heart disease or stroke (Geiss, 1995). T1DM patients are often young at the time of diagnosis. Although

the pathogenic factors are active early on, complications usually develop later as the disease progresses and are not as common during early stages.

1.1.2 Potential Causes and Clinical Presentation of T1DM

The precise triggers of the autoimmune destruction of islet ß cells resulting in T1DM remain elusive (Wasserfall and Atkinson, 2006). However, some have suggested a role for $CD4^+$ and $CD8^+$ T lymphocytes and infiltrating macrophages in the destructive process (Foulis et al., 1991; Roep, 2003). B lymphocytes are also involved by producing autoantibodies (Brusko et al., 2005).

The key determinants for the development of T1DM seem to involve a genetic predisposition modified by environmental factors (Knip, 2003). The following susceptibility genes have been suggested to be involved in T1DM (Gillespie, 2006): the human leukocyte antigen (HLA) on chromosome 6 especially DR4-DQ8 and DR3-DQ2 (Cudworth and Woodrow, 1975; Risch, 1987; Todd, 1995); the shorter number of tandem repeats, in a variable number of tandem repeats region, in the insulin gene promoter on chromosome 11 (Bennett et al., 1995); an allele of the gene for a negative regulator of T-lymphocyte activation, cytotoxic T lymphocyte antigen 4 (CTLA-4) found on chromosmone 2q33 (Gillespie, 2006); and a variant of PTPN22, the gene encoding lymphoid tyrosine phosphatase (Bottini, et al., 2004; Gillespie, 2006). All four may be involved in the antigen presenting process involving T-lymphocytes. Environmental factors also appear to play a role in T1DM and may be responsible for the increasing incidence, although it is difficult to identify specifically which ones are most important.

Studies have also identified enteroviruses (Hyoty, 2002), rotavirus (Honeyman, 2000) and rubella (Ginsberg-Fellner et al., 1985) as possible pathogens involved in T1DM. Other factors include immunoregulatory defects, and formation of anti-islet antibodies against glutamic acid decarboxylase (Baekkeskov et al., 1989), a protein tyrosine phosphatase-like molecule (IA-2) (Lan et al., 1996) and insulin (Brusko et al., 2005).

The clinical presentation of T1DM is insidious, appearing rapidly at a stage when islet ß cell levels have fallen to a critically low level. The most common symptoms and presenting features of DM include polydipsia; drowsiness and fatigue; polyuria and bedwetting in children; polyphagia; vision changes (increased myopia); weight loss; sweet, fruity-smelling breath (due to ketone body production) and mood changes (Pepper 2006). These symptoms are the result of inadequate blood insulin, hyperglycemia, and dehydration.

1.1.3 Diagnosis of DM and Monitoring of Glycemia

DM is a disease of hyperglycemia and diagnosis of both T1DM and T2DM rely on demonstrating either a fasting or postmeal hyperglycemia (Canadian Diabetes Association Clinical Practice Guidelines, 2003). Normal blood glucose is typically less than 6.0 mmol/L in a fasting state. A fasting plasma glucose level ≥ 7.0 mmol/L (126 mg/dL); a random plasma glucose ≥ 11.1 mmol/L (200 mg/dL); or a glucose value ≥11.1 mmol/L (200 mg/dL) two hours after a 75g oral glucose load on more than one occasion are diagnostic criteria for DM. These diagnostic criteria are defined based on risk for

diabetic retinopathy (Englegau, 1997). Individuals with levels of glycemia that are intermediate between normal levels and levels that are consistent with DM are categorized as being in a prediabetic state and are at risk of developing DM later on (Canadian Diabetes Association Clinical Practice Guidelines, 2003).

Following the diagnosis of diabetes, long-term glycemic control is monitored using glycated hemoglobin (A1C). Normal adult hemoglobin A consists of two pairs of polypeptide chains, two α chains and two β chains. Long-term elevation of plasma glucose levels lead to small amounts of hemoglobin A being non-enzymatically glycated to form A1C. This occurs by glucose attaching to the N-terminal valine in each ß chain of hemoglobin (Valeri et al., 2004) and occurs through Shiff base formation followed by Amadori rearrangement to form the final stable glycation product (Figure 1.1). As the average life-span of a RBC is about 3 months, A1C levels serve as an estimate of the average glycemia over the previous 90 days. An elevated level of A1C greater than 6.0% is considered abnormal and is common in individuals with DM. As a therapeutic goal, however, levels of A1C <7.0% are considered to be consistent with "good glycemic control" and lower risk for microvascular and macrovascular complications (Botero and Wolfsdorf, 2005; Canadian Diabetes Association Clinical Practice Guidelines, 2003).

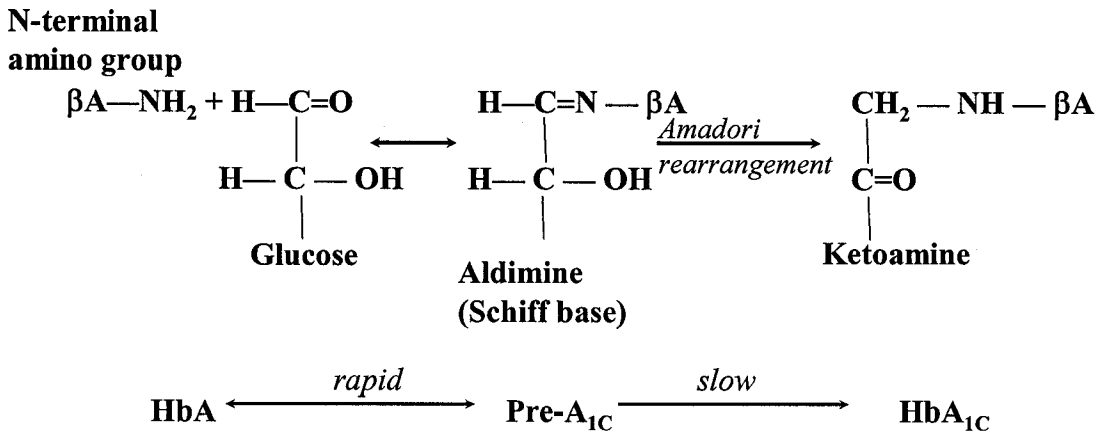

Figure 1.1 Formation of hemoglobin A1C. Hb A is hemoglobin A. βA-NH_2 refers to the N-terminal amino acid (valine) of the hemoglobin β-chains.

1.1.4 Treatment of T1DM

The control of T1DM involves both lifestyle adjustments and medical treatments. Insulin replacement is the cornerstone of therapy for T1DM (Talreja, 2005; Canadian Diabetes Association Clinical Practice Guidelines, 2003). The optimal treatment regimens involve nutritious diet and exercise, which allow the patient to maintain a healthy, active lifestyle. Maintaining glycemic control is accomplished by therapeutic use of insulin and can prevent or minimize long-term microvascular complications of DM (Habermann, 2006). Insulin must be administrated by subcutaneous injection (McCulloch, 2007). Many different insulin treatment regimes are used to control blood glucose levels. These vary in the speed and duration of a therapeutic effect and are variously referred to as rapid acting, short-acting, intermediate acting and long acting (Talreja, 2005; McCulloch, 2007). The best option depends upon a variety of individual factors (Habermann, 2006) and must be tempered with caution to avoid hypoglycemia.

Conventional (standard) insulin treatment and intensive insulin treatment are the two main types of insulin treatment plans. These differ in the types and dose of insulin used and the frequency of injections. In general, intensive insulin therapy involves more frequent insulin injections or use of an insulin pump. Intensive insulin treatment also requires more frequent monitoring of blood glucose. This type of therapy aims to more closely mimic insulin secretion by the pancreas and usually offers greater control of glycemia, (Habermann, 2006; Yki-Jarvinen, 1992), lower A1C, and therefore lower risk for complications (Habermann, 2006; Canadian Diabetes Association Clinical Practice

Guidelines, 2003). Conventional insulin treatment is an older regimen only recommended for selected patients (McCulloch, 2007).

1.1.5 Complications of T1DM

Poorly controlled T1DM carries an increased risk of developing both acute and chronic complications. Acute complications include diabetic ketoacidosis, hyperglycemic hyperosmolar coma, and hypoglycemic coma which can be life-threatening and, therefore, requires prompt treatment. The chronic complications are largely the effects of tissue damage occurring as a result of long term hyperglycemia and manifests as microvascular and macrovascular disease. Risk for the various complications are monitored by a variety of laboratory tests including A1C, serum lipid levels, and urine albumin measurements, as well as clinical tests for peripheral neuropathy, blood pressure measurements, and ophthalmologic tests (Botero and Wolfsdorf, 2005; Canadian Diabetes Association Clinical Practice Guidelines, 2003).

Both T1DM and T2DM are associated with increased risk of atherosclerosis and are associated with macrovascular disease like coronary artery disease (Wilson et al., 1998; McGill et al., 1998; Giannattasio et al., 1999; Garber et al., 2003), stroke, and peripheral vascular disease. Coronary artery disease is twice as common in patients with DM as compared with non-diabetic patients and imparts greater risk of angina and myocardial infarction (Garber et al., 2003). Microvascular disease, including retinopathy, nephropathy and peripheral neuropathy, also occurs in both T1DM and T2DM owing to

overlap in their pathophysiology (Weiman, 2005). Diabetic retinopathy, a complication affecting the retina (Habermann, 2006; Myers, 2005, Talreja, et al., 2005), is the most common cause of blindness among non-elderly adults in western countries. Diabetic nephropathy involves damage to the kidney caused by the deterioration of renal blood vessels, which can lead to chronic renal failure, eventually requiring dialysis. DM is the most common cause of chronic kidney failure worldwide. Diabetic neuropathy involves abnormal and decreased sensation, usually starting in the feet but eventually spreading elsewhere. When combined with vascular disease, this can lead to diabetic foot, which may cause necrosis, infection and gangrene. Diabetic foot is also the most common cause of amputation in western countries, usually involving toes and feet (Weiman et al., 2005; Myers, 2005; Talreja, et al., 2005). Other forms of diabetic neuropathy may present as mononeuritis or autonomic neuropathy (Talreja, et al., 2005).

1.1.6 Mechanisms of Complications

Several mechanisms have been proposed to explain how elevated glucose levels may cause microvascular complications. The four main mechanisms involve **1)** increased glucose flux through the polyol pathway (Engerman et al., 1994; Srivastava et al., 2005); **2)** increased glucose-induced activation of protein kinase C (PKC) isoforms (Koya and King, 1998); **3)** increased production of reactive oxygen species (ROS) (Giugliano et al., 1996); and **4)** increased formation of AGE residues (Vlassara and Palace, 2002; Goldin et al., 2006).

Increased flux through the polyol pathway can cause damage in two ways. High glucose concentrations favor the conversion of glucose to sorbitol by aldose reductase activation. This also results in consumption of NADPH and a decrease in the $NADPH/NADP^+$ ratio (Srivastava et al., 2005). Oxidative stress caused by depletion of NADPH, and the osmotic stress induced by accumulation of sorbitol, both can result in tissue dysfunction and damage, leading to various complications (Srivastava et al., 2005).

PKC consist of twelve isoforms with different structure and co-factor requirements and is involved in signal transduction pathways affecting many physiological processes related to vascular function (Godbout et al., 2002). One of the key signaling events occurring during hyperglycemia is activation of PKC (Koya and King, 1998) through increased release of diacylglycerol (DAG) as an intracellular second messenger. The mechanism of increased DAG synthesis involves elevated triose phosphates, dihydroxyacetone phosphate (DHAP) and glyceraldehydes-3-phosphate (G3P) concentrations during hyperglycemia. (Idris and Donnelly, 2006; Itani et al, 2002; Rolo and Palmeira, 2006). The damage resulting from PKC stimulation to the vascular tissue includes increased permeability, endothelial cell activation, altered blood flow, leukocyte adhesion and abnormal growth factor signaling, all of which contribute to several pathologies of diabetic complications (Brownlee, 2001).

Potential role of ROS in the development of DM complications has also been investigated (Baynes and Thorpe, 1999; Oberley 1988; and Ceriello et al. 2000). ROS, like superoxide and hydrogen peroxide, are constantly being generated as a consequence of aerobic metabolism. Nitric oxide, one of the reactive nitrogen species, is closely linked

to the ROS family and is generated by endothelial cells in response to stimuli like acetylcholine and insulin (Moncada et al. 1991). It plays an important role in regulation of vasomotor tone by stimulating cGMP formation and vasorelaxation. Nitric oxide can also rapidly react with superoxide to generate peroxynitrite, an extremely reactive oxidant (Cai and Harrison 2000), that can freely cross cell membranes (Curtin et al. 2002). Various metabolic changes occur during DM. Substrate flux through the mitochondria produce increasing levels of ROS (Baynes, 1991). This in turn can damage unsaturated fatty acids and proteins. The reaction of ROS with nitric oxide, to produce peroxynitrite can deplete nitric oxide, and cause the endothelial nitric oxide synthase (eNOS) to produce superoxide ion (Milstien and Katusic, 1999). Production of superoxide ion is important to generation of other ROS species that if not balanced by antioxidant defenses results in oxidative stress. Excessive ROS formation and increased vasoconstriction reduce blood flow and oxygen delivery. ROS decreases the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) resulting in increased levels of glyceraldehyde-3-phosphate (G3P), increasing DAG generation and PKC activation, as well as increasing production of AGEs (Reusch, 2003).

Finally, long term elevations of glucose and its metabolites promote formation of AGE residues which damage tissue proteins (Brownlee, 2001). This ultimately leads to damaged capillary basement membranes, vascular endothelium and other tissues, and the proliferation of vascular smooth muscle cells (VSMC) and platelet dysfunction- all contributing to the progression of DM complications. (Goldin et al., 2006; Vlassara and

Palace, 2002). This final mechanism is of special relevance to this thesis and will be discussed in greater detail later.

1.2 Methylglyoxal, Glyoxal and AGEs

1.2.1 Metabolism of Methylglyoxal and Glyoxal

The *in vivo* production of methylglyoxal and glyoxal can occur in all cell types, including VSMCs, and in blood plasma (Wu, 2006). Methylglyoxal (CH_3-CO-CH=O or $C_3H_4O_2$) (Figure 1.2) is a dicarbonyl formed as a byproduct of normal metabolism but has no known physiological function. It is also a highly reactive α-ketoaldehyde and can be produced from several sources by both enzymatic and non-enzymatic processes involved in glucose, lipid and protein metabolism. The major source of methylglyoxal involves the spontaneous transformation of triose phosphates from intermediates of glycolysis, including dihydroxyacetone phosphate (DHAP) and G3P (Thornalley, 1993b; Beisswenger et al., 2003) making it an intrinsic component of glucose metabolism via the glycolytic pathway (Figure 1.3). Under normal physiological conditions, glucose is converted to pyruvate via the glycolytic pathway forming only small amounts of methylglyoxal. GAPDH is a key enzyme in this process and is upregulated by insulin (Alexander et al., 1988). This enzyme is also believed to be modulated by a variety of other environmental and genetic factors, including T1DM (Beisswenger et al., 2003). Triose phosphates formed via the pentose phosphate pathway may also be a source of methylglyoxal (O'Brien et al., 2005). Factors that increase the availability of

methylglyoxal precursors such as increased plasma glucose (Thornalley 1988) or increases in fructose (Wang et al., 2006; Vasdev et al., 2003), glycine and threonine, and fatty acids may increase the formation of methylglyoxal.

Glyoxal (OCHCHO or $C_2H_2O_2$) is the smallest dialdehyde and is structurally related to methylglyoxal (Figure 1.2). Glyoxal's *in vivo* biosynthesis is also a byproduct of metabolism, but occurs mainly by oxidative degradation of glucose (Wells-Knecht, et al., 1995), lipid peroxidation (Shangari et al., 2003), oxidative degradation of DNA (Shangari et al., 2003), and oxidative degradation of fructosamine, a glucose-derived glycation product in proteins (Glomb and Monnier, 1995). Increased ROS, hyperglycemia, and glycated proteins containing fructosamine contribute to glyoxal formation in conditions like DM.

The balance between synthesis and catabolism of methylglyoxal and glyoxal are important in maintaining their intracellular concentration. Both methylglyoxal and glyoxal are degraded intracellularly by reaction with glutathione, via the glyoxalase pathway (Abordo et al., 1999) (Figure 1.4). The altered activity of glyoxalase I and glyoxalase II may affect the degradation of both methylglyoxal and glyoxal (Abordo et al., 1999). Furthermore, factors affecting the availability of reduced glutathione like oxidative stress or activities of glutathione peroxidase and glutathione reductase, or glutathione synthase can also have profound effects on methylglyoxal and glyoxal catabolism through the glyoxylase pathway (Ahmed et al., 2002).

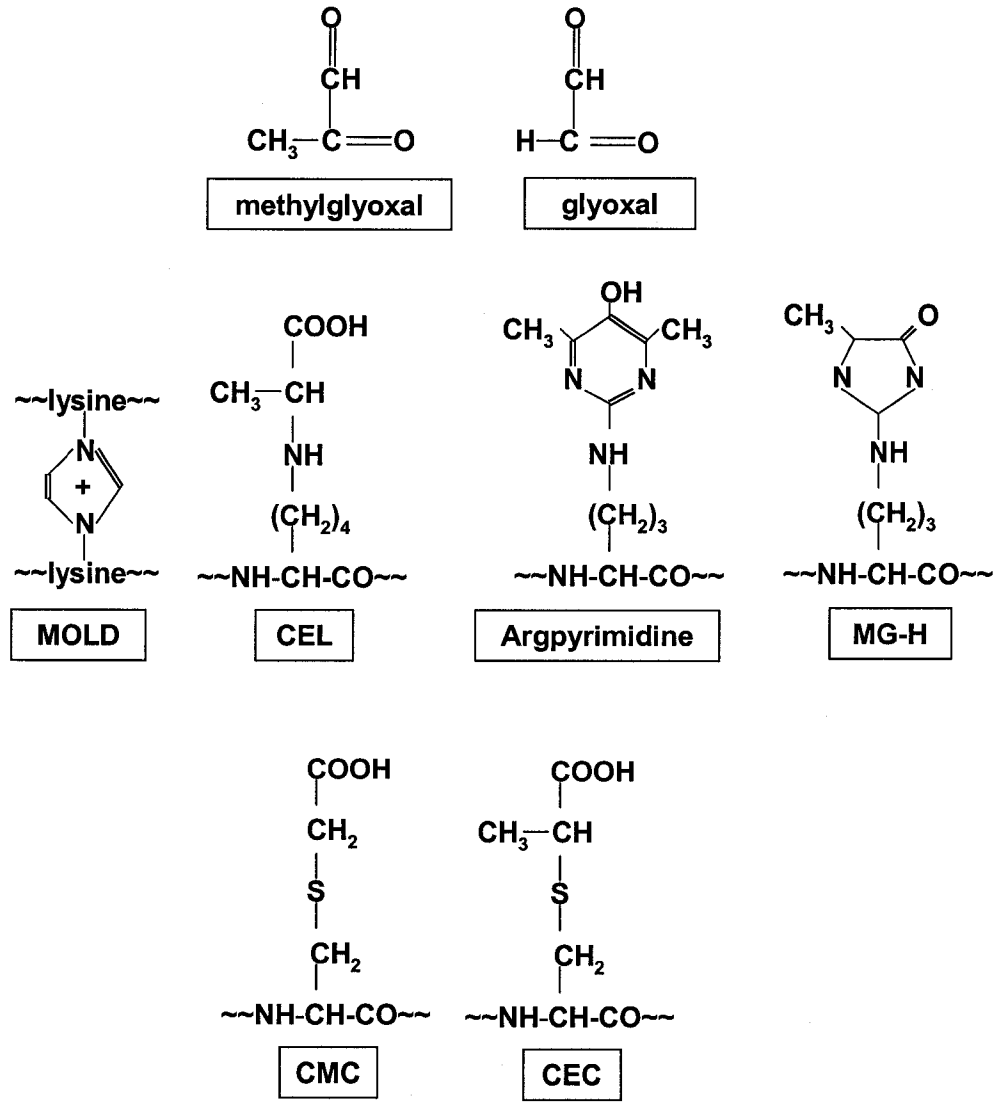

Figure 1.2 Structures of methylglyoxal, glyoxal, and some methylglyoxal and glyoxal derived advanced glycation end products (AGEs). Methylglyoxal-derived lysine dimer (MOLD), carboxyethyl lysine (CEL), argpyrimidine, methylglyoxal-derived hydroimidazolones (MG-H) and Carboxyethyl-cysteine (CEC) are all products of methylglyoxal-induced glycation. Carboxymethyl-cysteine (CMC) is believed to be a product of glyoxal-induced glycation.

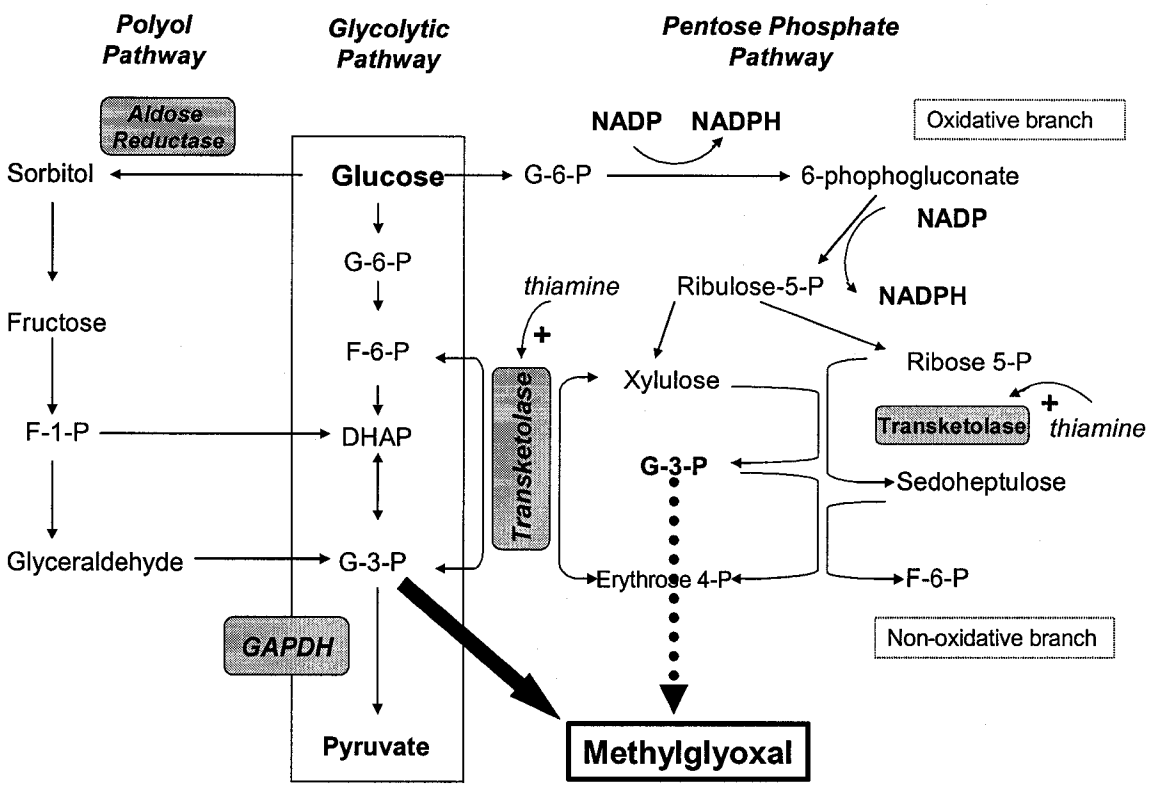

Figure 1.3 Relationship between the formation of methylglyoxal from glucose metabolism. The abbreviations are: DHAP is dihydroxyacetone phosphate; Erythrose 4-P is erythrose 4-phosphate; F-1-P is fructose-1-phosphate; F-6-P is fructose-6-phosphate; G-6-P is glucose-6-phosphate; G-3-P is glyceraldehydes-3-phosphate; GAPDH is glyceraldehyde-3-phosphate dehydrogenase; NADP is nicotinamide adenine dinucleotide phosphate; NADPH is reduced nicotinamide adenine dinucleotide phosphate; Ribose 5-P is ribose 5-phosphate; and Ribulose 5-P is ribulose 5-phosphate.

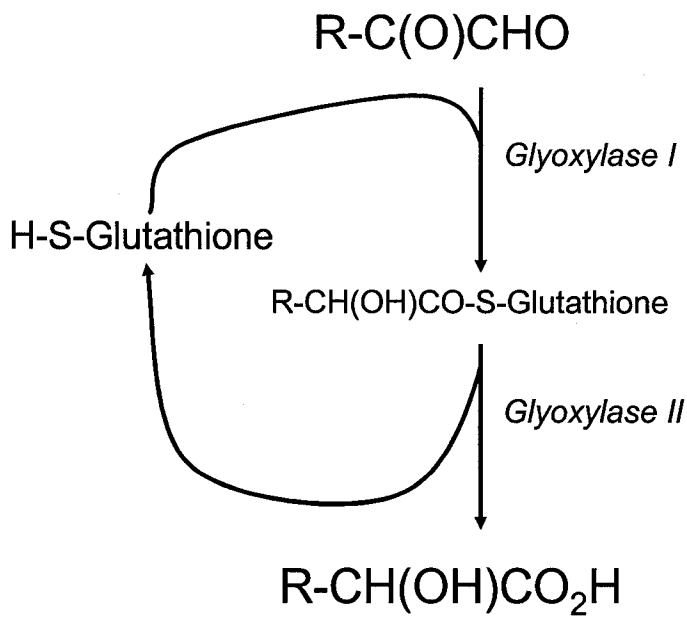

Figure 1.4 Metabolism of methylglyoxal and glyoxal by the glyoxalase system.

R is a methyl group in the case of methylglyoxal and hydrogen in the case of glyoxal.

1.2.2 Formation of Methylglyoxal and Glyoxal in T1DM

The production of methylglyoxal correlates with post-prandial glycemia in T1DM (Beisswenger et al., 2001). Plasma levels of methylglyoxal are higher in a group of combined adult T1DM and T2DM patients (Nemet et al., 2005). A number of processes may contribute to elevated methylglyoxal and glyoxal in T1DM. Insulin deficiency or inadequate insulin response, as well as altered glucose metabolism where the key enzyme of glycolytic pathway, GAPDH is down-regulated, can cause G3P to accumulate. This may lead to excess formation of methylglyoxal which also inhibits GAPDH and upregulates aldose reductase shunting glucose into the polyol pathway, resulting in accumulation of G3P (Kashiwagi et al., 1992). Accumulation of G3P, by both of these mechanisms, will result in excess formation of methylglyoxal in DM (Vander Jagt et al., 1993; Phillips et al., 1993).

In T1DM, insulin deficiency enhances lipolysis and may increase the generation of methylglyoxal during metabolism of acetone catalyzed by semicarbazide-sensitive amine oxidase (SSAO) or acetol mono-oxygenase (Casazza et al., 1984; Lyles and Chalmers, 1992). SSAO is found in high amounts within VSMCs and in plasma (Ekblom 1998), possibly originating from VSMC secretion. Low insulin levels in T1DM also induce excess protein catabolism. Methylglyoxal may also form from amino acetone, an intermediate formed during catabolism of glycine and threonine (Ray & Ray, 1987; Lyles and Chalmers 1992).

The concentrations of blood methylglyoxal and the activity of glyoxalase I and II are increased in T1DM and T2DM adults with complications (McLellan 1994). It has

been observed that in T1DM patients without retinopathy there is higher activity of glyoxalase II than those with retinopathy (Thornalley et al., 1989). This suggests a protective role for high glyoxylase II activity and a possible causal role for methylglyoxal in retinopathy. Oxidative stress also decreases the levels of glutathione required for the catabolism of methylglyoxal and glyoxal in the glyoxylase pathway. Thus, an imbalance between the formation of methylglyoxal and glyoxal, and its catabolism in T1DM results in their accumulation. It is, therefore, not surprising that methylglyoxal induced glycation is increased disproportionately in relation to the increase in glucose in DM (Ahmed and Thornalley, 2007).

1.2.3 Formation and Degradation of AGEs

Under normal physiological conditions, AGEs are formed by normal metabolism and aging as a result of oxidative and peroxidative stress. However, when glucose is elevated, or methylglyoxal or glyoxal are increased (due to increased production or decreased catabolism), AGEs may be formed in excess. The early glycation products formed by modification of N-terminal amino groups or lysyl chains by glucose via the Maillard reaction are reversible and require further oxidation to produce stable products like that involved in A1C formation. Both methylglyoxal and glyoxal are highly electrophilic and react non-enzymatically with free amino ($-NH_2$) or sulfhydryl (-SH) groups of lysine, arginine or cysteine of intra- and extracellular proteins to form AGEs (O'Brien et al., 2005) (Figure 1.5).

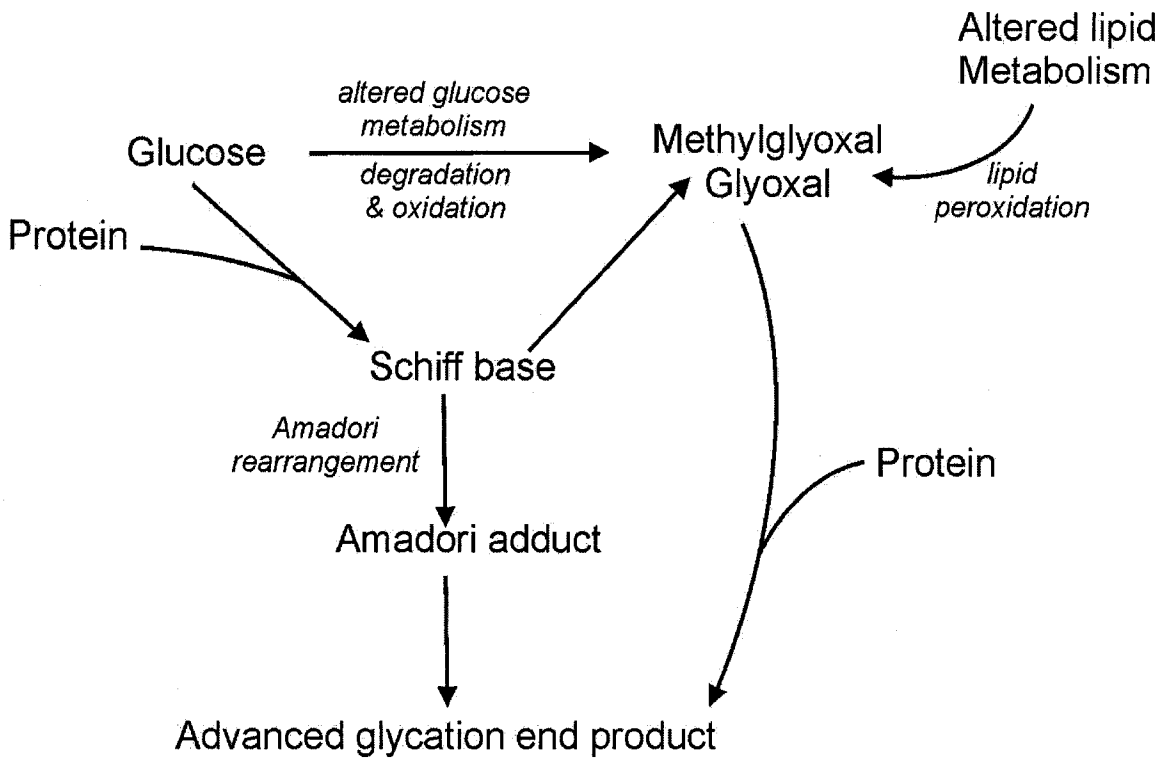

Figure 1.5 Formation of advanced glycation end products

The formation of AGEs is irreversible and stable (Wu, 2006; O'Brien et al., 2005). Their aldehyde precursors are up to 20,000 times more reactive than glucose in glycation processes (Thornalley 2005) and methylglyoxal is the most reactive of the AGE precursors (Kilhovd et al., 2003). These modifications to protein structure can directly alter protein function (Goldin et al., 2006). AGE residues can also act indirectly through cell surface receptors such as the receptor of AGEs (RAGEs) and scavenger receptors to alter tissue function (Jensen et al., 2005; Horiuchi et al., 2003).

AGE residues represent a chemically heterogenous group with over 30 different ones already described and possibly many more yet to be discovered. AGE residues can only be removed through proteolysis. The turn-over of AGE damaged proteins can be very slow depending on the protein and its location, but proteolysis results in the appearance of nonprotein-bound free AGE residues in biological fluids (Thornalley et al., 2003). Those appearing in the blood plasma may eventually be excreted in the urine if there is adequate renal function (Thornalley et al., 2003). AGE residues are also formed exogenously by heating, or cooking, sugars with fats or proteins (Koschinsky, 1997). The absorption of AGE residues from these thermally processed foods in the gastrointestinal tract may contribute to plasma free AGE residue concentrations (Ahmed and Thornalley, 2007). Free AGE residues re-absorbed from the kidney filtrate may also contribute to the plasma concentrations of free AGE residues (Ahmed and Thornalley, 2007). Methylglyoxal may bind free arginine in plasma but it is not known whether this contributes largely to free MGH-1 levels.

1.2.4 Methylglyoxal and Glyoxal Derived AGE Residues

A number of AGEs form from methylglyoxal and glyoxal (O'Brien et al., 2005). Methylglyoxal can produce a number of stable AGEs, including Nε-carboxyethyl-lysine (CEL), the methylglyoxal-derived hydroimidazolones (MG-H), argpyrimidine, and methylglyoxal-derived lysine dimer (MOLD) (Figure 1.2). The latter is formed by cross-linking two lysine residues of proteins which can also act to crosslink different proteins *in vivo*. CEL is also formed by the reaction of methylglyoxal with lysine. MG-H and argpyrimidine are formed by the reaction of methylglyoxal with arginine. The modification of arginine residues in proteins can be of particular functional relevance considering the high frequency with which arginine residues appear at ligand binding sites and active sites of enzymes (Thornalley, 2005). MG-H is a collection of at least three different isomers, MG-H1, MG-H2, and MG-H3 which differ from each other in stability (Ahmed et al., 2002). MG-H1 and MG-H2 appear to be most stable at physiological pH, whereas, MG-H3 is rapidly broken down (Ahmed et al., 2002). The MG-H1 isomer occurs as two epimers and is of great physiological relevance.

AGE residues can also form as a result of reactions involving glyoxal (O'Brien et al, 2005). For example, reaction of glyoxal with lysine forms glyoxal-lysine dimer (GOLD) and carboxymethyl lysine (CML); and reaction with arginine forms the glyoxal-derived hydroimidazolone (G-H1). Methylglyoxal and glyoxal are also known to react both reversibly and irreversibly with cysteine sulfhydryl (Zeng and Davies, 2005) forming carboxyethyl cysteine (CEC) and carboxymethyl cysteine (CMC), respectively, as the stable irreversible products (Zeng and Davies, 2005). All of these are present in

plasma and tissue proteins, but MG-H1 appears to dominate as the major methylglyoxal-derived AGE (Ahmed et al, 2005). Free MG-H1 also seems to be the main free AGE residue present in plasma, where levels of the others may be undetectable in healthy individuals (Ahmed et al, 2005). MG-H1 is increased in adults with T1DM (Ahmed et al., 2005).

1.2.5 Effects of Methylgyloxal, Glyoxal and AGE Residues

Methylglyoxal and glyoxal have been shown to cause detrimental effects to protein function. Whether this occurs via a non-AGE or AGE-related mechanism has not been confirmed. Numerous studies show that these aldehydes form AGEs changing the structure and function of proteins. The effects of AGEs may also be indirect, mediated through AGE receptors, like RAGEs or scavenger receptors.

1.2.5.1 Effects of methylglyoxal on gene transcription

Methylglyoxal-derived AGE residues can also be formed by reaction with the guanyl residues of RNA and DNA. Reaction of methylglyoxal with guanyl residues in DNA and RNA can lead to transcriptional abnormalities (Wu, 2006) including single strand breaks and teratogenic effects (Vlassara and Palace, 2002). Methylglyoxal may also directly regulate transcription of genes that are involved in acellular capillary formation causing endothelial cell death and vessel regression (Yao et al., 2006; Ramasamy et al., 2006). A mechanism has been proposed whereby methylglyoxal modification of mSin3A decreases mSin3A binding to the angiopoietin-2 promoter,

leading to an increase in angiopoietin-2 expression (Yao et al., 2006). Angiopoietin-2 and other proangiogenic factors play a key role in proliferative changes that characterize the advanced stages of diabetic retinopathy.

1.2.5.2 Effects of methylglyoxal on vascular calcium homeostasis

Methylglyoxal may contribute to enhanced vascular contractility or impaired endothelial-dependent vascular relaxation by altering calcium homeostasis in VSMCs and endothelial cells. Chronic treatment of WKY rats with oral methylglyoxal elevates intracellular calcium level in platelets (Vasdev et al. 1998b). Vascular calcification is a common feature in advanced atherosclerosis. High intracellular calcium levels in VSMC can increase vascular contractility, inducing further cardiovascular disease including angina and myocardial infarction (Yamagishi et al., 2007).

1.2.5.3 Effects of methylglyoxal on reactive oxygen species and oxidative stress

Endothelial cell function may also be affected through an inhibitory effect of methylglyoxal on nitric oxide synthesis, decreasing the bioavailability of nitric oxide and thereby increasing monocyte adhesion, platelet aggregation and VSMC proliferation (Wu, 2006). Methylglyoxal affects vascular function through its ability to generate ROS. Methylglyoxal is known to induce oxidative stress by a mechanism involving decreased levels of reduced glutathione, and lower glutathione reductase and glutathione peroxidase activities (Wu and Juurlink, 2002). Methylglyoxal also significantly increases superoxide, hydrogen peroxide production (Wu, 2006) and peroxynitrite production in VSMC (Chang

et al. 2005), impairing nitric oxide function. Blood cells are affected as well. For example methylglyoxal induces superoxide and hydrogen peroxide formation in neutrophils, potentially leading to kidney damage (Ward and McLeish 2004), and in the presence of thrombin, methylglyoxal induces a platelet peroxide accumulation and aggregation (Leoncini and Poggi 1996). The combined effect of increased ROS and decreased nitric oxide generation may include VSMC apoptosis, altered vascular tone, endothelial dysfunction and hypertension.

1.2.5.4 Effects of methylglyoxal on cell signaling and vascular proliferation

Methylglyoxal increases the activation of NF-κB p65, a transcription activator involved in inflammatory and proliferative vascular response, in cultured VSMC from aorta (Wu and Juurlink 2002) and mesenteric artery (Wu 2005). This may occur as a result of methylglyoxal-induced oxidative stress, as hydrogen peroxide also activates NF-κB p65 in spontaneously hypertensive rat VSMC (Wu and Juurlink 2002), and superoxide, peroxynitrite, and hydrogen peroxide can activate NF-κB in human endothelial cells (Canty et al. 1999; Ogata et al. 2000;Cooke and Davidage 2002). Hence, methylglyoxal may also be an important promoter of NF-κB activation, contributing to vascular proliferation during development of vascular disease.

Methylglyoxal can also affect cell signaling through interaction with components of the mitogen-activated protein kinases (MAPK) system, which includes a number of serine and threonine protein kinases that can induce cell proliferation. Methylglyoxal induces the expression of heparin-binding epidermal growth factor in rat aortic VSMC

(Che et al. 1997) and inhibits cellular response to human insulin-like growth factor-1 through a MEK/ERK-dependent pathway in cultured human embryonic kidney cell line and mouse fibroblast cell line (Du et al. 2003). Thus, methylglyoxal may cause abnormal cell growth.

There is strong evidence that the accumulation of methylglyoxal modifies cellular protein function, gene transcription and other cell-response-related factors, thereby altering vascular function and mediating vascular disease. While these effects have been ascribed to methylglyoxal it is likely that at least some of these are mediated through AGE formation and/or activation of specific AGE receptors. These events may be involved in the pathogenesis of microvascular and macrovascular disease in DM.

1.2.5.5 Effects of Glyoxal

Both methylglyoxal and glyoxal have mutagenic properties (Takahashi et al., 1989). Glyoxal has been shown to inhibit GAPDH and glutathione reductase *in vitro* (Morgan 2002). Also like methylgloxal, many of the effects of glyoxal appear to be mediated through formation of glyoxal AGEs. These effects may explain the increased susceptibility to hydrogen peroxide, increased ROS generation, reduced glutathine levels and decreased mitochondrial membrane potential caused by treatment with glyoxal (Shangari and O'Brien, 2004). Glyoxal showed site specific modification of ribonuclease by binding to arginine close to the enzyme active site (Cotham et al., 2004). The *in vivo* formation of GOLD has also been reported (Yamada et al, 2004; Lederer and Klaiber, 1999). Levels of other glyoxal AGE residues like CML and CMC are increased in tissue

proteins in response to oxidative stress and DM (Zeng and Davies, 2005; Ahmed and Thornalley, 2007; Mostafa et al., 2007). Both G-H1 and CML in plasma protein correlates with levels of A1C (Ahmed et al., 2005), and CML tends to accumulate in collagen with age (Baynes, 1991). The close relationship between fructosamine and glyoxal formation and hence the formation of glyoxal-derived AGE residues make glyoxal and glyoxal-derived AGE residues surrogate markers of DM (Ahmed and Thronalley, 2007). This is in contrast to AGE residues derived from methylglyoxal where there seems to be greater dependancy on glucose metabolism than glycemia per se.

1.2.5.6 Direct effects of AGE residues

The formation of methylglyoxal-derived AGEs may be a major mechanism by which methylglyoxal induced cell damage and dysfunction occurs (Goldin et al., 2006). Methylglyoxal as well as glyoxal-derived AGEs are known to accumulate with ageing. Formation of AGEs can alter function of cellular enzymes, receptors, carriers and structural proteins. For example AGE residues are known to cross-link human vitreous collagen potentially contributing to progression of retinopathy (Frye et al., 1998, Stitt et al., 2002). AGE modification of large matrix proteins like collagen, laminin and vitronectin alter their molecular charge, the three dimentional matrix assembly, and binding properties which has significant effects on the polyanionic nature and properties of the basement membrane (Vlassara and Palace, 2002). Treatment of human albumin with relatively low concentration of methylglyoxal is known to result in formation of MG-H residues that affect the drug binding function and esterase activity of albumin *in*

vitro (Ahmed et al., 2005 JBC). Heat shock protein 27 has been identified as a major methylglyoxal-modified protein in endothelial cells but argpyrimidine, rather than MG-H1, appears to be the main adduct in this case and may repress cytochrome c-mediated caspase activation (Sakamoto et al, 2002). Heat shock protein 27 is one of the chaperones that protects the cell against ischemic damage and inhibits cell death (Oya-Ito et al., 2006). AGE modification of intracellular basic fibroblast growth factor dramatically reduces the mitogenic activation of endothelial cell cytosol (Giardino et al., 1994) which may alter endothelial cell growth and basement membrane production.

1.2.5.7 Indirect effects of AGE residues

Circulating AGEs may interact with putative cell surface receptors for AGEs (Ahmed and Thornalley, 2007). These include the scavenger receptors, a specific receptor referred to as RAGE, and galectin 3. A number of intracellular AGE binding proteins have also been described (Ahmed and Thornalley, 2007). The scavenger receptors and galectin-3 recognizes protein highly modified with AGE residues. The scavenger receptor appears to be involved with clearance and degradation of AGE modified proteins by macrophages and macrophage-derived cells and may play a role in the pathogenesis of atherosclerosis (Nagai et al., 2007). Galectin-3 is also found on macrophages (Vlassara et al, 1995) and is believed to have a proinflammatory effect during the development of diabetic nephropathy (Kikuchi et al., 2004). RAGE is a membrane protein with wide tissue distribution and substrate-binding specificity. RAGE is expressed on endothelial cells, VSMCs, immune system cells, in the lung, liver, kidney and blood cells.

Extracellular methylglyoxal-modified protein can be removed by RAGE, internalized and subsequently degraded by proteolysis (Ramasamy et al., 2006).

Activation of RAGE by AGE binding is known to activate a multitude of cellular signaling pathways mediating a number of intracellular events which ultimately have implication for chronic disease. The activation of various signal transduction cascades are known to result in down-stream production of NF-kB and several other transcription factors (Li and Schmidt, 1997). NF-kB dependent gene expression affects cytokine, procoagulant, prothrombotic, and vasoconstrictive gene products including increased NADPH oxidase and iNOS expression (Wu et al, 2002). NF-kB increases expression of cellular adhesion molecules like intracellular adhesion molecule-1 (ICAM-1) which has been linked to general vascular disease (Barnes and Karin, 1997). Effects of RAGE on the suppression of glutathione and ascorbic acid levels contribute to oxidative stress and ROS accumulation (Lander et al., 1997; Bierhaus et al., 1997). Furthermore, the depletion of glutathione decreases glyoxalase I activity which compromises the ability to further control intracellular AGE formation via methylglyoxal and glyoxal. The binding of AGEs to RAGE on the endothelium also results in expression of inflammatory factors such as VCAM-1 and Tumor necrosis factor α (TNF-α) (Goldin et al., 2006).

1.2.6 Methylglyoxal, Glyoxal, AGEs and Diabetic Complications

Methylglyoxal, glyoxal and AGE formation are believed to play a causative role in the vascular complications of T1DM and T2DM (Ahmed and Thornally, 2007; Vlassara and Palace, 2002; O'Brien and Shangari, 2004). There is evidence in adults with T1DM and T1DM animal models that plasma methylglyoxal is elevated and that methylglyoxal- and glyoxal-derived AGEs are involved in the pathological changes in various tissues (Degenhardt et al., 2002; Karachalias et al, 2003; Beisswenger et al., 2003a, 2003b) resulting in microvascular and macrovascular complications of DM (Figure 1.6). Karachalais et al. (2003) showed that levels of CML, CEL, G-H1 and MG-H1 are variably increased in proteins of renal glomeruli, retina, sciatic nerve and plasma of streptozotocin-induced T1DM rats, consistent with a significant role of methylglyoxal and glyoxal in the development of nephropathy, retinopathy, and neuropathy. Increased levels of protein bound AGEs in humans with DM complications suggests a relationship between protein-bound AGE levels and complications. In humans with DM, the levels of specific methylglyoxal-AGEs in plasma proteins are increased and correlates with indices of complications. For example, the levels of serum protein bound argpyrimidine is increased 2 to 3 times in middle aged and elderly adult DM patients and correlates with levels of glycated hemoglobin (Wilker et al, 2001). Concentrations of plasma protein bound CEL and CML are increased in adult T1DM patients with decreased glomerular filtration rate and correlates with blood concentrations of markers of endothelial dysfunction (Lieuw-A-Fa et al, 2004). Plasma protein bound MG-H concentrations are also increased in adult T1DM patients (Ahmed et al., 2005 D) and elder T2DM patients

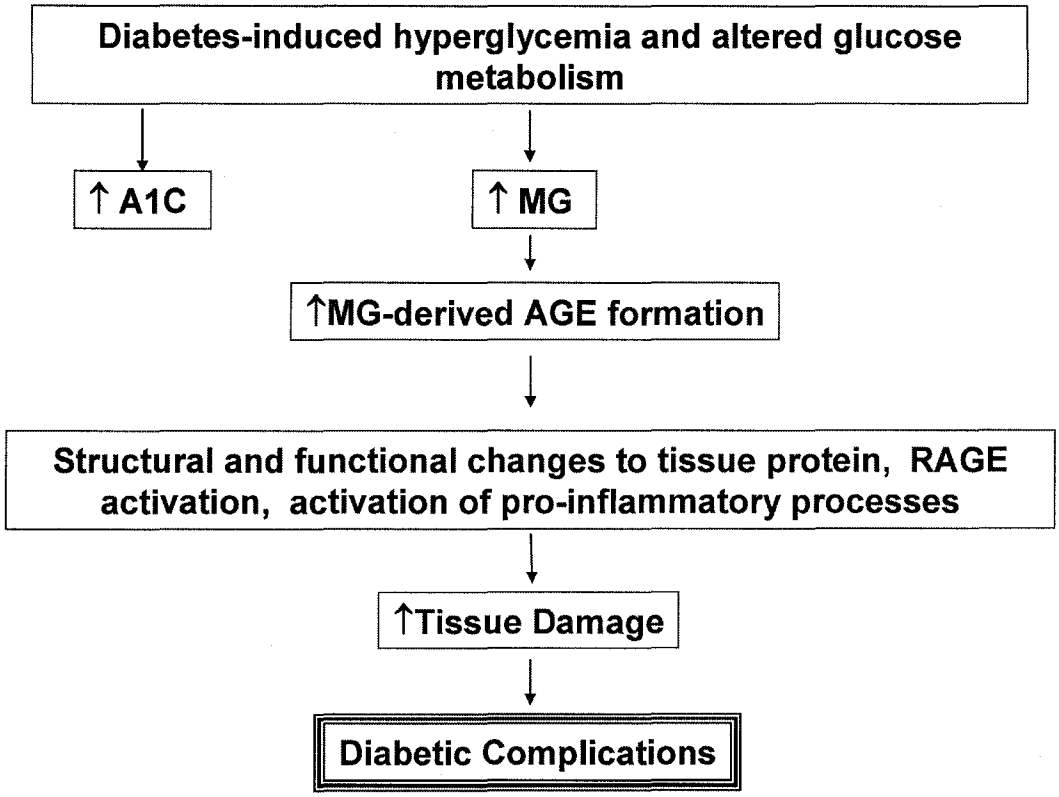

Figure 1.6 Proposed role of methylglyoxal and methylglyoxal-derived AGEs in DM. The abbreviations are: A1C is hemoglobin A1C; MG is methylglyoxal; RAGE is receptor for advanced glycation end products; AGEs is advanced glycation end products; and DM is diabetes mellitus.

and correlates with CML levels (Kilhovd et al, 2003). The glycation cross-linked MOLD is increased in adult T1DM patients (Ahmed et al., 2005), but none of the methylglyoxal derived AGEs apparently correlated with A1C levels in the adult DM patients (Ahmed et al., 2005; Kilhovd et al, 2003). The accumulation of AGE residues in tissues and in plasma protein is consistent with a role for AGEs in DM complications.

In animal studies, the injection of methylglyoxal (Berlanga et al., 2005) or AGE-modified protein can induce vascular damage similar to that observed in DM (Vlassara et al., 1992; Vlassara et al., 1995) including increased vascular permeability, expression of vascular adhesion molecules, and decreased nitric oxide generation. Furthermore the short-term administration of exogenous AGE in normal, non-diabetic animals was associated with increased production of collagen IV, a basement membrane component, and with other findings consistent with diabetic nephropathy (Yang et al., 1994; Vlassara et al., 1992). This suggests a causal role for methylglyoxal and methylglyoxal-AGEs in DM complications. In mice with streptozotocin-induced T1DM, treatment with aminoguanidine (an advanced glycation inhibitor) or ALT-711 (an AGE cross-link breaker) reduced both vascular AGE accumulation and atherosclerosis (Forbes et al., 2004). The accumulation of CEL and MG-H in renal glomeruli, retina, sciatic nerve, and plasma proteins (Babaei-Jadidi et al., 2003) precede the development of nephropathy in streptozotocin-induced T1DM in rats. Subsequent treatment of these animals with high doses of thiamine and benfotiamine therapy to increase metabolism of G3P through transketolase and the pentose phosphate shunt (and thereby prevent methylglyoxal formation) reduced the blood levels of methylglyoxal, MG-H and CEL, and prevented the

progression to retinopathy (Hammes et al, 2003). This was accomplished without any change in glycemia or A1C. Pyridoxamine inhibits the formation of AGE residues and also inhibits the development of retinopathy and neuropathy in streptozotocin-induced DM in rats (Degenhardt et al., 2002; Stitt et al., 2002). Taken together these studies are consistent with a causal role of methylglyoxal and methylglyoxal-derived AGE residue formation as independent and contributing factors in the development of DM vascular complications.

AGEs may also play a causal role in the pathological process leading to DM complications through effects mediated through RAGE. Increased RAGE expression in the endothelium of activated vessels is observed in DM humans (Ritthaler et al, 1995; Feng et al., 2005) and contributes to a chronic low grade inflammation (Stehouwer et al., 2002) and is believed to accelerate atherosclerosis (Naka et al., 2004) and hypertension (Schram et al., 2005). Binding of AGE residues to AGE receptors on neonatal rat mesangial cells *in vitro* results in overproduction of matrix proteins and induction of mesangial oxidative stress and PKC activation (Scivittaro et al., 2000), both of which are potential mechanisms of microvascular disease. Treatment of cultured human mesangial cells with an anti-AGE agent, N-acetylcysteine, inhibited vascular endothelium growth factor (VEGF) and MCP-1 secretion and apoptosis (Yamagishi et al., 2002). Overexpression of RAGE in DM mice increased albuminuria, serum creatinine, renal hypertrophy, mesangial expansion and glomerulosclerosis compared to non-diabetic littermates (Yamamoto et al., 2001). These changes were restored by blockade of RAGE

(Wendt et al., 2003). Furthermore, a diffuse upregulation of RAGE expression occurs in renal glomerular podocytes in patients with diabetic nephropathy (Tanji et al., 2000). These studies indicate a role of RAGE in diabetic nephropathy and possibly other complications (Ahmed et al, 2007).

1.2.7 Previous Methods for Measurement of Methylglyoxal, Glyoxal and AGEs

A number of methods have been published for quantification of methylglyoxal and other dicarbonyls with it. One of the first methods published to measure methylglyoxal in blood relied on derivatization with 1,2-diamino-4,5-dimethoxybenzene, followed by solid phase extraction (SPE), and finally reversed phase-HPLC chromatography and detection of the quinoxaline product by spectrophotometric or fluorescent properties (McLellan et al., 1992). Several other HPLC based techniques based on fluorometric or spectrophotometric detection have also been described (Ohmori et al., 1987; Cordeiro et al, 1996; Thornalley et al., 1999; Nemet et al., 2004; Chaplen et al., 1996, Espinosa-Mansilla et al., 1998). Most of these make use of aromatic diamino compounds as derivatizing agents. Recently, methods have been published based on the quantification of dicarbonyl adducts including methylglyoxal and glyoxal with 2,3-diaminonaphthalene (Odani et al., 1999) or o-phenylenediamine (Randell et al., 2005) using electrospray ionization liquid chromatography/mass spectrometry (ESI/LC/MS), or quantifying pentafluorobenzyl hydroxylamine adducts using gas chromatography/mass spectrometry (GC/MS) (Lapolla et al., 2003). Single phase LC/MS and GC/MS methods

lack the specificity of a LC/MS/MS method, and the solid phase extraction step included in a number of these methods may be cost prohibitive.

Relatively few methods have been published for measurement of methylglyoxal-derived AGE residues as a group (Ahmed et al., 2002; Ahmed et al., 2005). Pure standards are not commercially available for any of the methylglyoxal-derived AGE residues. Hence, any work in this area requires synthesis of the various AGE residues as a first step. Most of the quantitative methods available rely on LC-MS/MS (Mostafa et al., 2007; Teerlink et al., 2004 CC) or GC-MS (Petrovic et al., 2005) and measure only one or two different AGE residues. Only a few laboratories around the world have the capability to measure these. In general these methods are cumbersome. Thornalley et al (2003) recently published a method for measurement of a wide variety of free and bound AGE residues without derivatization. The method involves a rather complex column switching technique and the analysis time per sample was also relatively long (40 to 50 min).

1.3 Na^+/K^+-ATPase

1.3.1 Structure and Function of Na^+/K^+-ATPase

Na^+/K^+-ATPase (Sodium-potassium-activated adenosine triphosphatase) is present in the plasma membrane of all eukaryotic cells and helps maintain cell potential through maintainance of the electrochemical gradients of Na^+ and K^+. Na^+/K^+-ATPase

activity also contributes to the control of cellular pH, osmotic balance and therefore cell volume, as well as to the Na^+-coupled transport of nutrients such as amino acids and vitamins in all cells (Sherwood, 2001). In intestinal and renal tubular cells, it provides the energy for the uptake of glucose.

Na^+/K^+-ATPase catalyzes the hydrolysis of one mole of ATP to ADP. This energy is used to extrude three Na^+ ions from the cell and causes the uptake of two extracellular K^+ ions into the cell (Ganong, 2003; and Kaplan, 2002). Na^+/K^+-ATPase is not a single protein enzyme but is part of a super family consisting of more than 300 members (Kaplan, 2002). Na^+/K^+- ATPase consists of two subunits, α-subunit and β-subunit (Kaplan 2002; Jergensen, 2003; Ganong, 2003; Aperia, 2007) (Figure 1.7), each of which are heterogeneous consisting of several isoforms with different tissue specificity (Ganong, 2003; Levenson, 1994; Sweeney and Klip, 1998; Kaplan, 2002). The β-subunit of Na^+/K^+-ATPase is a glycoprotein and is involved in delivery and maintaining correct orientation of the α-subunit (Aperia, 2007, Ganong, 2003). The α-subunit of Na^+/K^+-ATPase has intracellular Na^+ and ATP binding sites and a phosphorylation site. The extracellular portion of this subunit has a K^+ and an ouabain-binding site (Ganong, 2003). Ouabain is an inhibitor of the enzyme. The mechanism of action is relatively straightforward. The α-subunit of unphosphorylated Na^+/K^+-ATPase binds to Na^+. This is followed by ATP binding and hydrolysis leading to phosphorylation of the pump (Ganong, 2003). Phosphorylation results in reduced affinity for Na^+ which is released into the extracellular fluid. Subsequent binding of K^+ results in dephosphorylated and transport of K^+ into the cytoplasm (Ganong, 2003). This process helps maintain the Na^+

and K^+ electrochemical gradients across plasma membrane and physiological function of the cell.

1.3.2 Regulation of Function of Na^+/K^+-ATPase

Regulation of Na^+/K^+ pump activity is mainly through the α subunit. An important short term regulation is the intracellular concentration of Na^+ (Sweeney and Klip, 1998; Kaplan, 2002; ganong, 2003). Elevated intracellular Na^+ concentrations increase the activity of Na^+/K^+ ATPase resulting in rapid removal of excess Na^+ and restore low steady-state concentrations (Sweeney and Klip, 1998; Kaplan, 2002; Ganong, 2003). Other short term regulation is through phosphorylation and dephosphorylation, translocation of subunits to the plasma membrane or by modification of Na^+ affinity (Ewart and Klip, 1995). Long term regulation of α1 subunit occurs through effects on gene transcription, translation and degradation of the protein (Ewart and Klip, 1995). Na^+/K^+-ATPase is upregulated by several hormones including aldosterone, thyroid hormone, endothelin, acetylcholine and insulin in muscle, fat, and kidney cells and erythrocyte (Ewart and Klip,1995; Baldini et al., 1986). Insulin stimulates the Na^+/K^+-ATPase by increasing intracellular Na^+ concentration (Brodsky JL. 1990), stimulating translocation of the pump subunits, promoting phosphorylation, increasing sensitivity of Na^+/K^+-ATPase to Na^+ (Feraille et al., 1994) and by increasing its biosynthesis (Sweeney and Klip, 1998).

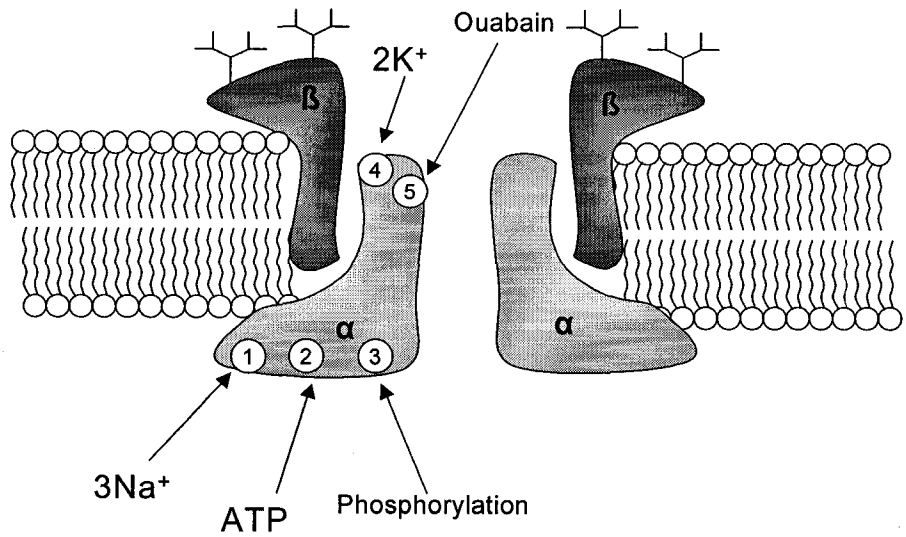

Figure 1.7 Structure of Na^+/K^+-ATPase in membrane. ATP is the abbreviation of adenosine triphosphate.

1.3.3 DM, Methylglyoxal and Erythrocyte Na^+/K^+-ATPase

Abnormalities in Na^+/K^+-ATPase activity are thought to be involved in development of heart disease, neuropathy, hypertension, and cataracts (Garner 1986 EER; Jannot et al., 1999). RBC membrane Na^+/K^+-ATPase can be separated from cytoplasmic components and its activity measured. Na^+/K^+-ATPase levels are low in adult T1DM and are strongly related to diabetic neuropathy (Jannot et al, 1999, Raccah et al 1996). Methylglyoxal inhibited Na^+/K^+-ATPase activity in RBC ghost cell membrane of normal human subjects likely occurs via the modification of sulfhydryl groups (Derham et al, 2003). In young complication-free TIDM patients there was no change in RBC Na^+/K^+-ATPase activity (Deak 2003). Rats showed a biphasic response with an increase in activity of this enzyme in medullary thick ascending limb of kidney 6 weeks after induction of T1DM, followed by a decrease at 12 weeks (Tsimaratos 2001). A decrease in Na^+/K^+-ATPase activity compromises microvascular blood flow by affecting microvascular regulation and decreasing RBC flexibility leading to an increase in blood viscosity. The DM-induced impairment in Na^+/K^+-ATPase activity is identical in RBC as that in neural tissue. RBC ATPase activity is related to nerve conduction velocity in the peroneal and the tibial nerve in patients with DM.

1.4 Research Proposal and Objectives

Elevations in methylglyoxal, glyoxal and AGEs may preceed and contribute to the pathogenesis of complications in DM. Other processes such as normal aging, impaired renal function, and other conditions can also contribute to the elevated levels of methylglyoxal, glyoxal and AGE residues found in plasma as well as in other tissues. In the initial phase of T1DM, the effect of these processes would be of minimal impact and the primary factors determining methylglyoxal and glyoxal levels would be the availability of substrate, primarily glucose, and individual differences in metabolism affecting methylglyoxal and glyoxal formation and catabolism by the glyoxylase system. Hence, correlation with A1C is expected since glycemia is a primary determinant of flux through pathways forming methylglyoxal and glyoxal. A contributory role for methylglyoxal and glyoxal in complications may be evident if there is a relationship between these compounds and the activity of a ubiquitous membrane enzyme like Na^+/K^+ATPase, which seems to be affected early in DM. The most profound effects of methylglyoxal in inducing complications may be at the intracellular level. AGE residues formed intracellularly will be released into the blood in the form of free AGE residues and finally filtered by the kidney. MG-H appears to be important since it is produced in relatively large amounts on both intracellular and extracellular protein. Demonstrating elevated levels of methylglyoxal and glyoxal and free AGE residues in blood of young T1DM patients may not only provide evidence for a role of these in the pathogenesis of DM complications, but offer potential clinical tests to identify T1DM patients at greatest

risk of complications by a diminished ability to respond to methylglyoxal and glyoxal production. This susceptibility to AGE formation would be manifested by increased blood levels of methylglyoxal and glyoxal and increased blood levels of free AGE residues like MG-H. These may, serve as predictors of future complications in T1DM independent of A1C.

Previous studies have shown increased concentrations of plasma methylglyoxal (Beisswenger et al., 2003; Nemet et al., 2005), glyoxal (Lapolla et al., 2003) and certain plasma protein bound AGEs and free AGE residues (Kilhovd et al., 2003; Fosmark et al., 2006) in T2DM patients or in adults with T1DM. The plasma free AGE residue that is present in largest amounts is the methylglyoxal-arginine adduct, MG-H1 (Ahmed 2005D). Although studies showing increased concentrations of the dicarbonyls and free and protein bound AGEs in adults with DM may indicate an association with DM complications, they do not necessarily indicate a direct role of the dicarbonyls and AGEs in causing complications. Elevations in dicarbonyls and AGE formation prior to clinical presentation with complications provide much better evidence for a causal role. There are no reports so far describing the concentrations of plasma methylglyoxal, glyoxal and AGEs in young patients of T1DM without complications.

Methylglyoxal and glyoxal may affect the activity of various enzymes (Morgan et al., 2002; Park et al., 2003; Lee et al., 2005; and Jia et al., 2006) by promoting AGE formation. A potential candidate for such modifications is the membrane enzyme, Na^+/K^+ ATPase (Derham et al., 2003). Alteration of Na^+/K^+ ATPase activity has been implicated in diabetic neuropathy (Raccah et al., 1996; and Djemli-Shipkolye A, et al., 2001) and

may play a role in other diabetic complications (Mimura et al., 1994; Tsimaratos et al., 2001; and Koc et al., 2003). To date there has been no investigations into the potential relationship between plasma glyoxal, methylglyoxal, or plasma free AGE levels and the activity of Na^+, K^+ ATPase.

In this research, I focused on the following:

1) Development of methods for measurement of methylglyoxal and glyoxal, and free AGE residues in human plasma. The study of methylglyoxal, glyoxal and methylglyoxal-derived AGEs presents many challenges. Owing to the high reactivity of methylglyoxal, effective capturing of the free methylglyoxal molecule for measurement is difficult. The methylglyoxal-derived AGEs, like MG-H, have only recently been described and as yet there are no robust or convenient methods for measurement of these. Moreover, the unavailability of pure samples of AGE residues amplifies the difficulties in designing and validating procedures to accurately quantify these. While the formation of methylglyoxal and methylglyoxal-derived AGEs may be a mechanism by which diabetic complications occur, the literature is relatively void of information on the levels of these found in young patients with T1DM before complications occur. This work will, therefore, involve synthesis of MG-H standards and allow development of a method to provide quantitative information on MG-H in plasma. Use of an LC-MS/MS procedure will increase specificity of measurement.

2) Establish the concentrations of methylglyoxal, glyoxal and methylglyoxal-derived AGE residues in complication-free young individuals with T1DM. Do increased levels of methylglyoxal and methylglyoxal-derived AGE residues precede

complications or do they occur secondary to other alterations that result in complications? Providing evidence for increased levels of methylglyoxal and methylglyoxal-derived AGE residues in complication-free young patients with T1DM may provide evidence to support the former. Demonstrating this may also help identify T1DM individuals at greatest risk of early complications independent of A1C values.

3) Determine the relationship of A1C, methylglyoxal, glyoxal and free methylglyoxal AGE residues with function of Na^+/K^+-ATPase activity. Information on the levels of RBC membrane Na^+/K^+ ATPase activity in T1DM without complications is scant. Moreover, the relationship between the levels of this enzyme and methylglyoxal, glyoxal, and methylglyoxal-AGEs is largely unknown. Levels of RBC membrane Na^+/K^+-ATPase activity in complication-free young patients with T1DM will be examined and compared with levels of glycemic control, and plasma methylglyoxal, glyoxal, and MG-H levels.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Study Subjects

Blood samples from study subjects were analyzed as part of this parallel case-control study. In order to examine the relationship between the levels of plasma methylglyoxal, glyoxal and specific free AGE residues in T1DM, four cohorts of up to 20 subjects aged 6 to 22 years with or without T1DM were recruited. Study subjects were recruited from patients presenting to the Diabetes Clinic at the Janeway Child Health Centre Health Care Corporation, St. John's, NL for routine check-up and A1C measurement. Nursing staff in the clinic approached patients for participation in the study and written informed consent was obtained (see **Appendix 1**). Ethics approval for the study was granted by the Human Investigation Committee of Memorial University, St. John's, Newfoundland and Labrador.

The four cohorts were constructed consisting of:

1. Subjects with normal levels of A1C (<6%) without biochemical evidence of T1DM as controls (n=18).
2. T1DM subjects with good glycemic control for at least 1 year and average A1C <8% (n = 20).

3. T1DM subjects with moderate glycemic control for at least 1 year and average A1C level 8 to 9% (n = 17).
4. T1DM subjects with poor glycemic control for at least 1 year and average A1C level > 9% (n = 19).

Clinical information including: duration of diabetes, type of insulin management, age, sex, and the presence of diabetic complications (Hypertension, Nephropathy, Retinopathy, and Neuropathy) was collected by a research nurse from chart review and documented (See **chart audit form Appendix 2**). Patients consenting to participate had a 2 ml aliquot of blood removed from their A1C sample for analysis of the biochemical parameters.

Outpatient blood samples (n=18) collected in EDTA-containing tubes from patients aged 6 to 21 years were used as controls. The samples were retrieved over a 2 to 3 week period by review of hematology sample logs, and age and sex of the individual was recorded. These samples were also analyzed for A1C and had A1C levels less than 6%.

A1C measurements were carried out using standardized clinical laboratory methodology on a G7 A1C analyzer (Tosoh) using an ion exchange HPLC procedure.

2.1.2 Chemicals and Reagents

Unless otherwise specified, chemicals and reagents were of commercial origin and were of the highest grade available.

Ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)amino-methane (Tris), methylglyoxal, glyoxal, 2,3-hexanedione, 2,3-diaminonaphthalene (2,3-DAN), formic acid, trichloroacetic acid (TCA), L-lysine, t-butyloxycarbonyl arginine ($N\alpha$-t-BOC arginine), t-butyloxycarbonyl lysine (N^{α}-*t*-BOC lysine), solid copper carbonate ($CuCO_3$), 2-bromopropionic acid, sodium acetate, sodium hydroxide (NaOH), nonafluoropentanoic acid (NFPA), trifluoroacetic acid (TFA), sodium chloride (NaCl), diethylenetriaminepenta acetic acid (DETPA), and heptafluoro butyric acid (HFBA), potassium chloride, magnesium chloride, ethyleneglycol bis (2-aminoethyl-ether) tetraacetic acid (EGTA), adenosine triphosphate (ATP), phosphoenolpyruvate (PEP), nicotinamide adenine dinucleotide (NADH), pyruvate kinase, lactate dehydrogenase, ouabain, adenosine 5'-triphosphatase (ATPase) and bovine serum albumin (BSA) were all purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Concentrated hydrochloric acid (HCl; 36.46%) was obtained from Fisher Scientific Company (Nepean, Ontario, Canada). Methanol, acetonitrile (Optima grade), ethyl acetate, and ammonium hydroxide were obtained from Fisher Scientific Company (New Jersey, USA). Sodium hydrogen phosphate was purchased from EM Science (MERCK, Darmstadt, Germany). L-Lysine-(4,4,5,5-D4) (d4-lysine) was purchased from Cambridge Isotope Laboratories Inc (Andover, MA, USA).

2.2 Method for Preparation of Blood Samples

Blood samples (about 2 ml) were centrifuged at 3000 rpm for 10 min. The upper plasma layer was removed and centrifuged at 13,000 rpm for 5 min to remove debris. The plasma was then aliquoted into at least two separate fresh microfuge tubes and stored at –70°C for the measurement of methylglyoxal, glyoxal and free AGE residues. A pooled plasma sample from patients without DM was also processed and used for calibration matrix.

To prepare red blood cell (RBC) membranes for the measurement of Na^+/K^+-ATPase activity, 200 µl of centrifuged blood cells, separated from plasma, was added to 1.5 ml of 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA and vortexed for 20 sec as previously described (Vasarhelyi B, 1997). This hemolyzed the blood and the resulting hemolysate was centrifuged at 14,000 rpm at 4°C for 5 min to give a RBC membrane pellet. The pellet was then washed four times with the same solution and then re-suspended each time with fresh solution. The final hemoglobin-free pellet was re-suspended in 200 µl of 10 mM Tris-HCl (pH 7.4) and stored at –70°C for measurement of protein and Na^+/K^+- ATPase activity.

2.3 Method for Measurement of Plasma Methylglyoxal and Glyoxal

To carry out the work in this thesis, a new method to measure methylglyoxal and glyoxal was developed. Characteristics of this method are described in detail in chapter 3.

2.3.1 Preparation of Plasma Samples

Plasma protein was precipitated by adding 500 µl of the plasma to 2 volumes of 12% trichloroacetic acid (TCA) and mixing by vortex. The mixture was then centrifuged at 5000 rpm for 5 min. The protein free supernatant was transferred to a fresh tube for methylglyoxal and glyoxal measurements.

2.3.2 Preparation of Calibration Standards

The calibration curve for methylglyoxal and glyoxal was prepared using supernatant from a plasma pool that had the protein removed by precipitation using 2 volumes of 12% TCA. The plasma used to prepare this pool was retrieved from non-DM patient blood samples. This was done to ensure that a common sample matrix was used for both samples and calibrators. To prepare calibration standards, 0 to 100 µl of 840 ng/ml methylglyoxal and 420 ng/ml glyoxal were added to tubes, followed by 400 µl of supernatant from TCA precipitated normal plasma. The concentrations used on the standard curve were corrected for the concentrations of methylglyoxal and glyoxal in normal plasma to allow the calibration curve to intersect zero.

2.3.3 Derivatization of Samples

Supernatant from TCA precipitated plasma samples were derivatized using 2,3-DAN before measurement. Briefly, 250 µl of 84 ng/ml 2,3-hexanedione (as internal standard) was added to tubes containing 400 µl of TCA precipitated plasma supernatant

from the T1DM groups, the control group, and standards, respectively. The final volume for each was adjusted to 1 ml using 10 mM phosphate buffer (pH 7.4) and the mixture was then incubated with 100 µl of the derivatizing agent 0.1% 2,3-DAN for 24 hours at 4°C. The long incubation time was critical for reproducible results. The 2,3-DAN derivative was then extracted into 4 ml of ethyl acetate by vortexing for 20 sec and centrifuging at 5000 rpm for 5 min to separate layers. The upper layer containing the 2,3-DAN derivatives was removed and dried under a stream of nitrogen gas. The dried extract was finally reconstituted with 200 µl of acetonitrile for high-pressure liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis.

2.3.4 Measurement of Methylglyoxal and Glyoxal by HPLC-MS/MS

All samples were analyzed on the Waters AllianceHT 2795- Micromass Quattro Ultima LC-MS/MS system. Five µl of sample was injected each time. The 2,3-DAN derivatives of methylglyoxal, glyoxal, and 2,3-hexanedione, were separated in an isocratic solvent system of aqueous 0.1% formic acid and acetonitrile (35:65, v/v) using a C8 column (Symmetry® C8 3.5 µm 2.1 x 100 mm, WAT058961, Water, Massachusetts, USA) at a flow rate of 0.30 ml/min at 25°C. In this system, the derivatives of methylglyoxal and glyoxal elute at about 1.7 min and 1.65 min, respectively, and the internal standard at 2.8 min. The run time was 5 min. The methylglyoxal-DAN product was determined by multiple reaction monitoring (MRM) of the transition 195>168, with collision energy (CE) set at 20; glyoxal-DAN product was monitored using the MRM

transition 181>154, with CE set at 20; and hexanedione-DAN product was monitored using the MRM transition 237>208, with CE set at 22. The calibration curve was constructed from the ratio of response of the area under the methylglyoxal-DAN peak, or glyoxal-DAN peak, to the area under the hexanedione-DAN peak in different standard solutions. Plasma methylglyoxal and glyoxal were expressed as nmol/L.

2.4 Method for Measurement of Free MG-H1 in Plasma

Attempts were made to synthesize four methylglyoxal derived AGEs and measure both free and bound AGE residues in plasma and plasma protein, respectively. This synthesis effort was successful in preparing sub-milligram quantities of argpyrimidine, MG-H, MOLD and CEL. The synthesis and method used to measure MG-H are described below. Information on the synthesis and performance in the same assay system for the other three AGE residues are described in Appendix 3.

2.4.1 Synthesis of MG-H

As pure standards for methylglyoxal-derived AGEs like MG-H were not commercially available, MG-H was synthesized in small amounts using a procedure previously described by Ahmed et al. (2002) for MG-H3. This procedure produces a mixture of MG-H isomers including MG-H1 and MG-H3. Briefly, 311 mg of t-BOC arginine (1 mmol) was dissolved in 60 ml of 200 mM sodium acetate buffer (pH 5.4) followed by adding 0.21 mL of methylglyoxal solution (1.2 mmol). The solution was

filter sterilized by passing through a 0.2μm filter and then incubated at 37°C for 7 days. The product was then lyophilized and extracted with methanol three times. The methanol extracts were evaporated to dryness and further purified by preparative HPLC using a reverse phase C18 column as previously described (Ahmed et al. 2002). Collected fractions corresponding to t-BOC-MG-H1 were lyophilized, dissolved in 0.5 M HCl and were allowed to stand at room temperature overnight to hydrolyze t-BOC groups. The solution was lyophilized once more and repurified by LC-MS/MS using a Gemini C18 semi-prep column (250x10mm; 5 μm; Phenomenex, USA) in a solvent system consisting of 0.1% TFA (aqueous) and methanol. Separation and identification of MG-H was accomplished by monitoring the MRM transition of 229.1>114 with CE set at 9 eV. The collected fraction was then vacuum dried and the final MG-H product was stored at –70°C.

2.4.2 Sample Preparation for Measurement of Free MG-H1 in Plasma

Plasma protein was precipitated by adding 300 μl of 20% TCA to an equal volume of plasma and stored on ice for 15 min. The mixture was then vortex mixed and centrifuged at 4°C at 3000 rpm for 5 min to separate the precipitate. The supernatant was removed to a 5 mL microcentrifuge tube and used for measurement of free MG-H1 in plasma.

2.4.3 Measurement of Free MG-H1 in Plasma

2.4.3.1 Preparation of stock solution and calibration standards

Stock solution was prepared by dissolving the purified MG-H1 sample in water to give an estimated final concentration of 40 mg/L for MG-H1. The standard curve was constructed by diluting the stock standard in plasma to final concentrations ranging from 0 to 16.8 mg/L for MG-H1. Calibration curve was corrected for endogenous free MG-H1 in plasma.

2.4.3.2 Extraction of samples for the measurement on HPLC

The supernatant of TCA precipitated plasma samples were prepared for analysis by first extracting on solid phase extraction (SPE) columns. Briefly, 20 µl of 1mg/ml d_4-lysine was added as an internal standard to 380 µl of the supernatant from all samples and prepared calibration standards. Samples were then diluted by adding 2 ml of water. The pH of samples was checked before loading to ensure approprate acidity for SPE. The SPE columns (Oasis MCX 1cc 30 mg, Waters) were prepared by adding 1 ml of methanol and vacuum drying for 10 min. This was followed by adding 1 ml of water to each SPE column to prepare the sorbent for extraction. Samples were loaded on to the columns, followed by washing with 1 ml of 0.1N HCl and 1 ml of methanol. The retained components from the loaded samples were eluted with 2 ml of 5% ammonium hydroxide in methanol and collected in fresh tubes. The elute were dried down under a nitrogen gas stream and resuspended in 200 µl of methanol/acetonitrile (25/75) for analysis.

2.4.3.3 Measurement of free MG-H1 in plasma by LC-MS/MS

All extracted samples were analyzed by LC-MS/MS on the Water Alliance HT 2795-Micromass Quattro Ultima LC-MS/MS system. Twenty µl of each sample was injected on a Hilic Sillica column (Atlantis® Hilic Silica, 3 µm, 2.1 x 50 mm, Waters Corporation, Massachusetts, USA). MG-H1 and d4-lysine were separated in a solvent system consisting of 200 mM formate buffer in methanol, water and acetonitrile at a flow rate of 0.5 ml/min at ambient temperature. During the 8 min chromatographic run, the composition of 200 mM formate buffer was held constant at 5%. The water content was increased from 10% to 55% during the first 2 min, returned back to 10% in the following 2 min, and then held constant at 10% for the remainder of the run. The content of acetonitrile decreased from 85% to 40% during the first 2 minutes, then increased back to 85% in the following 2 minutes, and finally held at 85% thereafter. MG-H1 and d4-lysine were determined by MRM. In this system, the MRM transition, CE, and retention time for the analytes were 229.1>114, 9 eV, and 2.58 min for MG-H1; and 151.1>88, 8 eV, and 2.7 min for d4-lysine, respectively.

The calibration curve was constructed from the response ratio of the area under the peaks of MG-H1 to the area under the d4-lysine peak in different standard concentrations. The concentrations used on the standard curve were corrected for the concentration of MG-H1 in normal plasma to allow the calibration curve to intersect zero.

The concentration of MG-H1 was determined according to the response ratio, and was expressed as mg of MG-H1 per L, assuming 100% purity of MG-H1 standard.

2.5 Method for Measurement of Na^+/K^+-ATPase Activity in Red Blood Cell Membrane

2.5.1 Preparation of Samples

The red blood cell membrane samples that were stored at –70°C were resuspended by sonication (Virsonic, Cell disrupter, Model 16-850, Gardiner, N.Y.) prior to measurement of protein and Na^+/K^+-ATPase activity.

2.5.2 Measurement of Protein Concentration

Protein concentration in the red blood cell membrane samples were measured using the Bio-Rad DC Protein Assay (Bio-Rad, Ontario, Canada), and BSA was used as a standard. The standard curve was prepared using 0 to 80 µl of 1.5 mg/ml BSA. Standards or 40 µl of each sample were transferred to test tubes, followed by 500 µl of Working Reagent A (200 µl of Reagent S added to 10 ml of Reagent A, an alkaline copper tartrate solution) and 4.0 ml of Reagent B (a dilute Folin Reagent) and incubated for 15 minutes. The protein concentration was measured by absorbance at 750 nm by spectrophotometer (Spectronic Genesys 5, Milton Roy) and calculated using the standard curve.

2.5.3 Measurement of Na^+/K^+ ATPase Activity by Spectrophotometry

Na^+/K^+ ATPase activity was measured in suspended RBC membrane samples as previously described (Vasarhelyi et al., 1997) but with some modification. Briefly, 760 µl of reagent 1 (100mM NaCl, 20 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM EGTA, 50 mM Tris-HCl pH 7.4, 1 mM ATP, 1mM phosphoenolpyruvate, 0.16 mM NADH, 5 kU/L pyruvate kinase, 12 kU/L lactate dehydrogenase) was incubated at 37°C for 5 min prior to adding a 40 µL aliquot of suspended RBC membranes. Decrease in absorbance was monitored at 340 nm by spectrophotometry (Spectronic Genesys 5, Milton Roy) over a 10 minute period. From the change in absorbance during this time period, rate 1(total ATPase activity) was obtained. A 50 µl aliquot of reagent 2 (10 mM Ouabain) was then added to inhibit the ouabain-sensitive ATPase activity. The change in absorbance after adding ouabain was monitored for the next 10 minutes, and rate 2 (ouabain-resistant ATPase activity) was obtained. Ouabain-sensitive Na^+/K^+ ATPase activity was calculated as the difference between the two slopes and adjusted for protein concentration. The final activity was recorded as nmol NADH oxidized/min/mg protein.

2.6 Statistical Analysis

Data were analyzed using SPSS for Windows (release 14.0). Results are expressed as mean ± standard deviation. Independent sample t-test was used to compare groups. When several different groups were compared, ANOVA was performed with correction for multiple comparisons by Bonferroni method. The relationship between

methylglyoxal, glyoxal, MG-H1, HbA1C, Na^+/K^+-ATPase activity, age, and duration of diabetes was analyzed using correlation analysis. Multiple linear regression analysis was also performed on HbA1C, methylglyoxal, glyoxal and MG-H1 to assess the ability of these variables to predict Na^+/K^+-ATPase activity. A p-value of less than 0.05 was considered statistically significant.

CHAPTER 3

DEVELOPMENT OF METHODS FOR MEASUREMENT OF METHYLGLYOXAL, GLYOXAL AND AGES IN PLASMA

3.1 Introduction

Although a few methods have been established to measure methylglyoxal, glyoxal and certain specific AGEs, we sought to develop more specific, and time- and cost-effective methods. This work presents many challenges: **1)** pure standards for many methylglyoxal-derived AGEs are not available and must be synthesized; **2)** a major problem with measuring alpha ketoaldehydes is their low concentration and high reactivity towards other endogenous compounds (Thornalley, 1996); **3)** the spontaneous formation of methylglyoxal from triose phosphates during the preparation procedures can be a problem (Mclellan et al 1992), which may be resolved by working under acidic conditions; **4)** low concentrations of some methylglyoxal-derived AGEs also presents a challenge, and the sensitivity of some to degradation by strong acid (Ahmed et al., 2002) prevents good recovery following protein acid hydrolysis procedures.

3.2 Development of Methods for Measurement of Methylglyoxal and Glyoxal in Plasma

3.2.1 LC-MS/MS Method for Measurement of Methylglyoxal and Glyoxal

To capture and stabilize methylglyoxal and glyoxal for measurement in plasma, we used 2, 3-DAN as a derivatizing agent and used 2,3-hexanedione as an internal standard for quantification purposes. Protein was removed by using TCA precipitation and the protein free supernatant was used for derivatization by 2,3-DAN. Optimum derivatization of the two alpha ketoaldehydes required an incubation time of at least 24 hours at 4 °C and calibrating standards were also prepared in the same sample matrix by diluting solution of pure methylglyoxal or glyoxal standards into TCA precipitated plasma supernatants. Figure 3.1 shows the products of derivatization of methylglyoxal, glyoxal and 2,3-hexanedione with 2,3-DAN. Formation of these products greatly increases the size and reduces the reactivity permitting more convenient measurement by chromatographic techniques.

A LC-MS/MS based method was developed for accurate quantification of methylglyoxal and glyoxal. Optimum conditions for the collision induced dissociation (CID) products of these were determined by continuous infusion of prepared samples (~1-10 μg/mL methanol) into a Waters Micromass Quattro Ultima tandem mass spectrometer and using positive ion electrospray as an ion source. Optimal tuning conditions for the parent ion were first determined using the M+1 ions: 195, 181, and 237 for methylglyoxal-DAN, glyoxal-DAN, and 2,3 hexanedione-DAN, respectively. Daughter ions were produced by CID by increasing the CE of argon gas until optimal amounts of selected daughter ions were produced. Figure 3.2 shows a CID profile for the methylglyoxal-DAN product. Optimum levels of the product ion of mass 168 were

produced when the methylglyoxal-DAN product is bombarded with argon gas at a CE of 20 eV. The transition of 195>168 also gave sufficient specificity for selective detection of the methylglyoxal-DAN product and this was subsequently used in all experiments for specific measurement of the methylglyoxal-DAN product. Glyoxal-DAN and 2,3-hexanedione-DAN were likewise optimally measured at the CE equal to 20 eV and 22 eV, and using the transitions 181>154 and 237>208, respectively, to ensure selectivity for these products (Figure 3.3 & 3.4).

For convenient measurement of methylglyoxal and glyoxal from biological samples a liquid chromatographic separation method was developed and coupled with MS/MS detection. Reversed phase chromatography performed on a C8 column (Symmetry® C8 3.5 μm 2.1 x 100 mm, WAT058961) using a simple isocratic solvent system consisting of 0.1% formic acid in water and acetonitrile (35:65, v/v) was finally adopted to accomplish separation. Figure 3.5 shows a typical chromatogram achieved for 2,3-DAN derivatization products of methylglyoxal, glyoxal, and 2,3-hexanedione in a serum sample by this LC-MS/MS method. Apart from the peaks of interest there was no evidence of potentially interfering substances at the region of the chromatogram where the analytes eluted. As all blood plasma has detectable levels of methylglyoxal and glyoxal it was necessary to correct calibration curves for this endogenous methylglyoxal and glyoxal. Figure 3.6 and Figure 3.7 shows typical calibration curves for methylglyoxal and for glyoxal, respectively. Both were linear over the range of concentration examined.

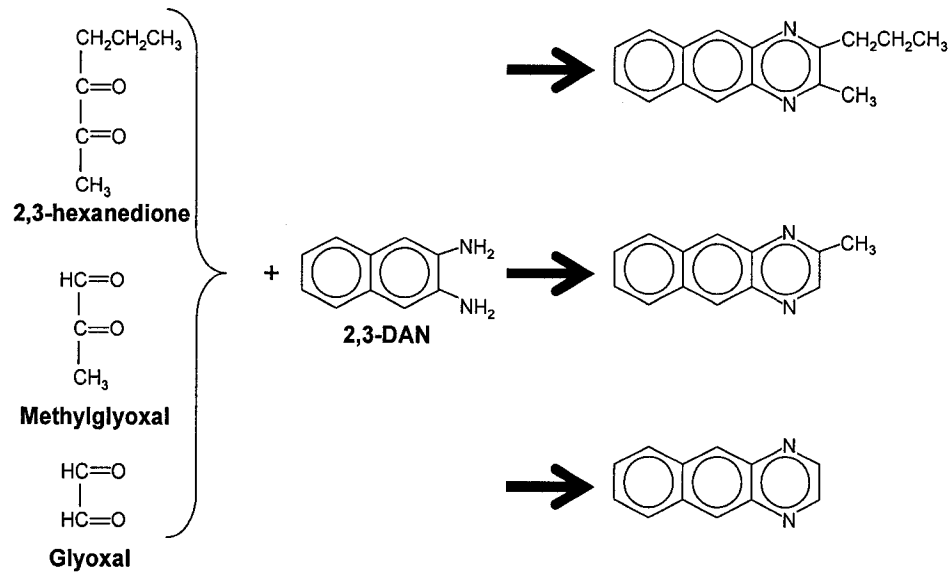

Figure 3.1 The products of derivitization of methylglyoxal, glyoxal and 2,3-hexanedione with 2,3-diaminonapthalene (DAN). The products are 2,3-hexanedione-DAN, methylglyoxal-DAN, and glyoxal-DAN products from top to bottom.

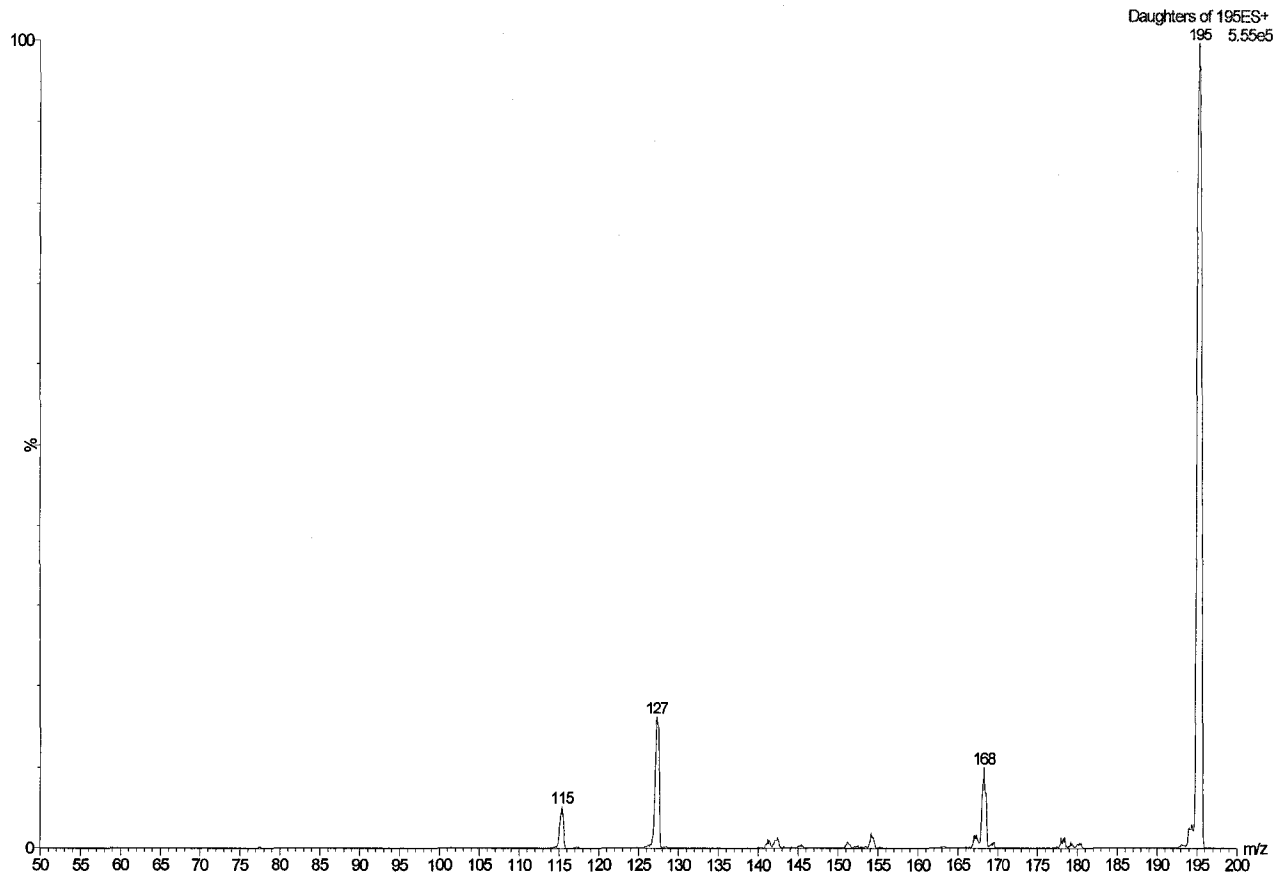

Figure 3.2 CID profile for the methylglyoxal-DAN product. Spectrum was produced at a CE of 20 eV. Capillary voltage was set at 3.0 kV; cone voltage: 90 V; source temperature 100°C; and desolvation gas flow was set of 461 L/hr. Protonated molecular ion (M+1) corresponds to the peak identified as 195. Positively charged ions 168, 127, and 115 represent daughter ions produced from the protonated molecular ion. Size of the peaks represents relative amounts. The abbreviations are: CID is collision induced dissociation; DAN is diaminonaphthalene; and CE is collision energy.

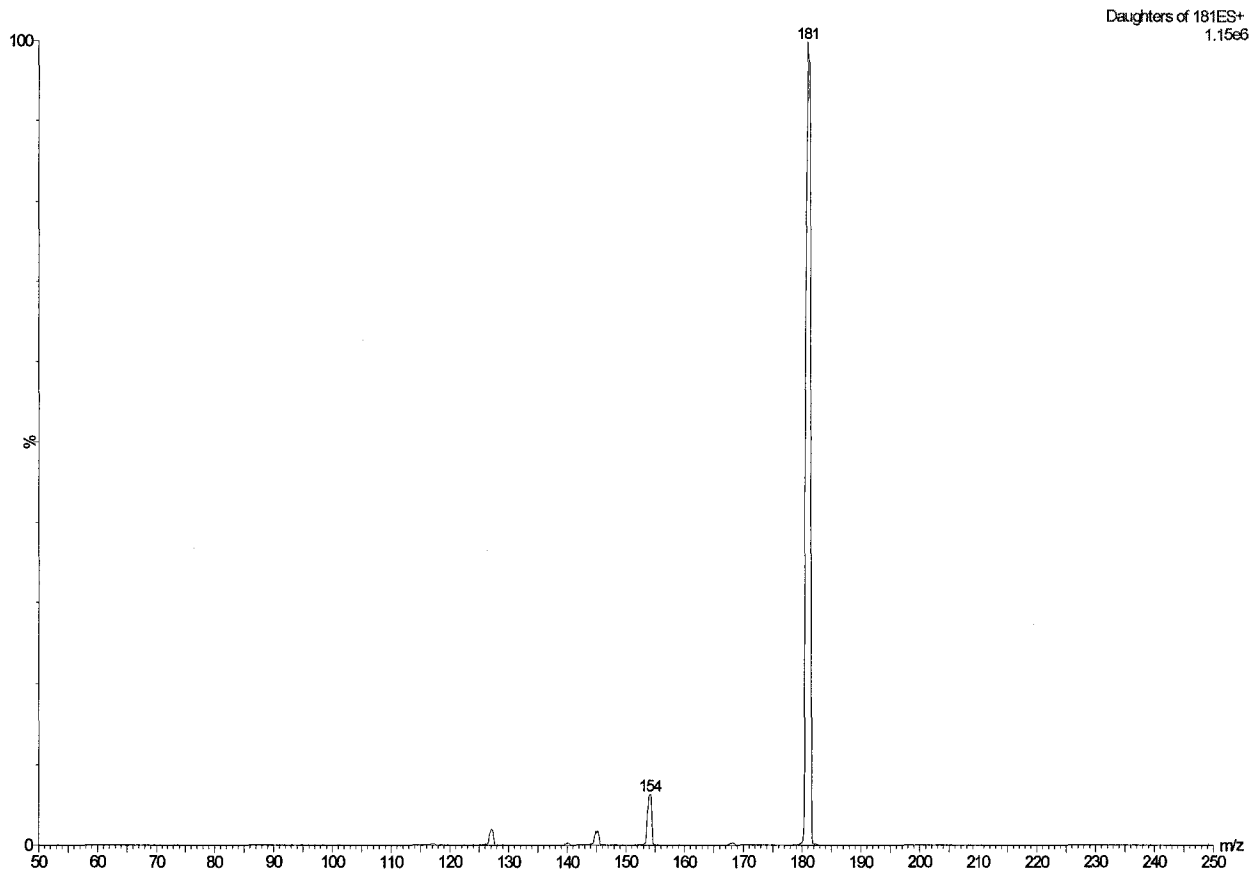

Figure 3.3 CID profile for the glyoxal-DAN product. Spectrum was produced at a CE of 20 eV. Capillary voltage was set at 3.0 kV; cone voltage was 90 V; source temperature was 100°C, and desolvation gas flow was 461L/hr. Protonated molecular ion (M+1) corresponds to the peak identified as 181. Positively charged ions 154 represent the daughter ion produced from the protonated molecular ion. Size of the peaks represents relative amounts. The abbreviations are: CID is collision induced dissociation; DAN is diaminonaphthalene; and CE is collision energy.

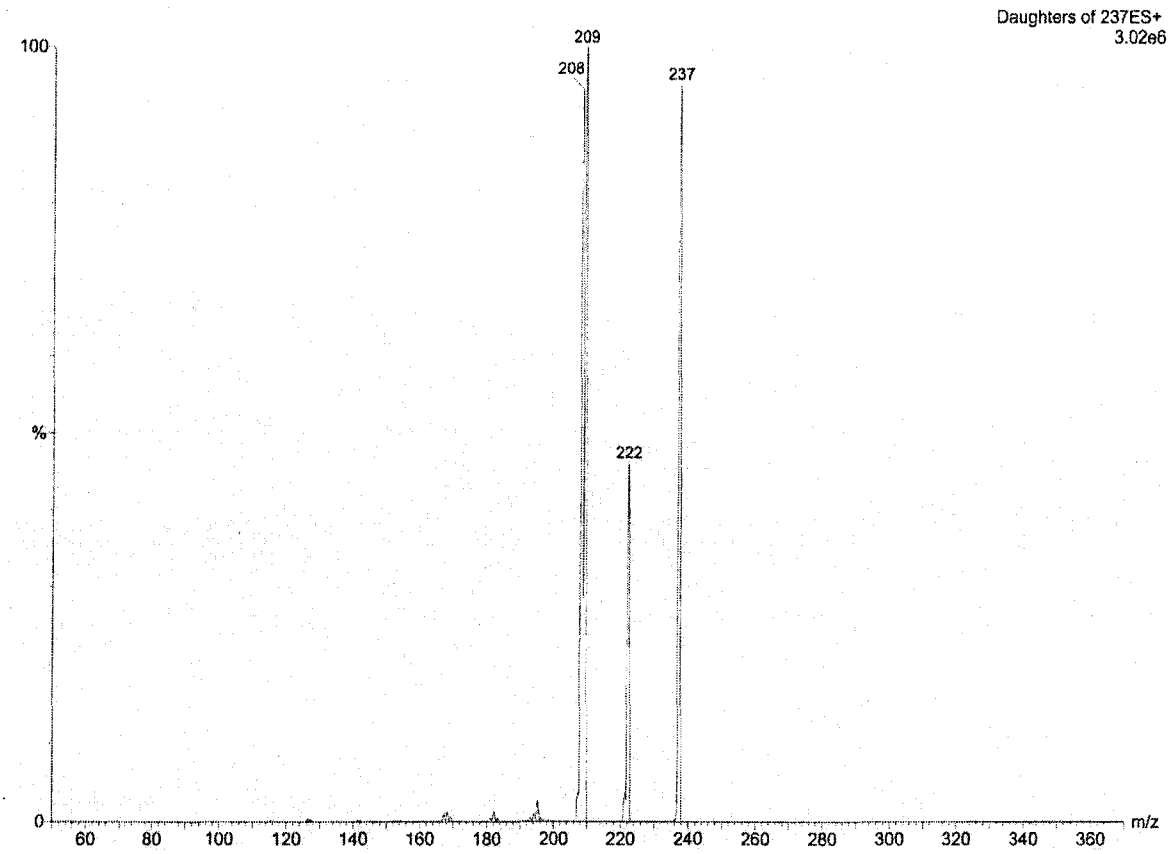

Figure 3.4 CID profile for the 2,3 hexanedione-DAN product. Spectrum was produced at a collision energy of 22 eV. Capillary voltage was set at 3.0 kV; Cone voltage: 90 V; Source temperature 100°C; and desolvation gas flow was set of 461 L/hr. Protonated molecular ion (M+1) corresponds to the peak identified as 237. Positively charged ions 208, 209 and 222 represent the daughter ions produced from the protonated molecular ion. Size of the peaks represents relative amounts. Abbreviations are: CID is collision induced dissociation; DAN is diaminonaphthalene; and CE is collision energy.

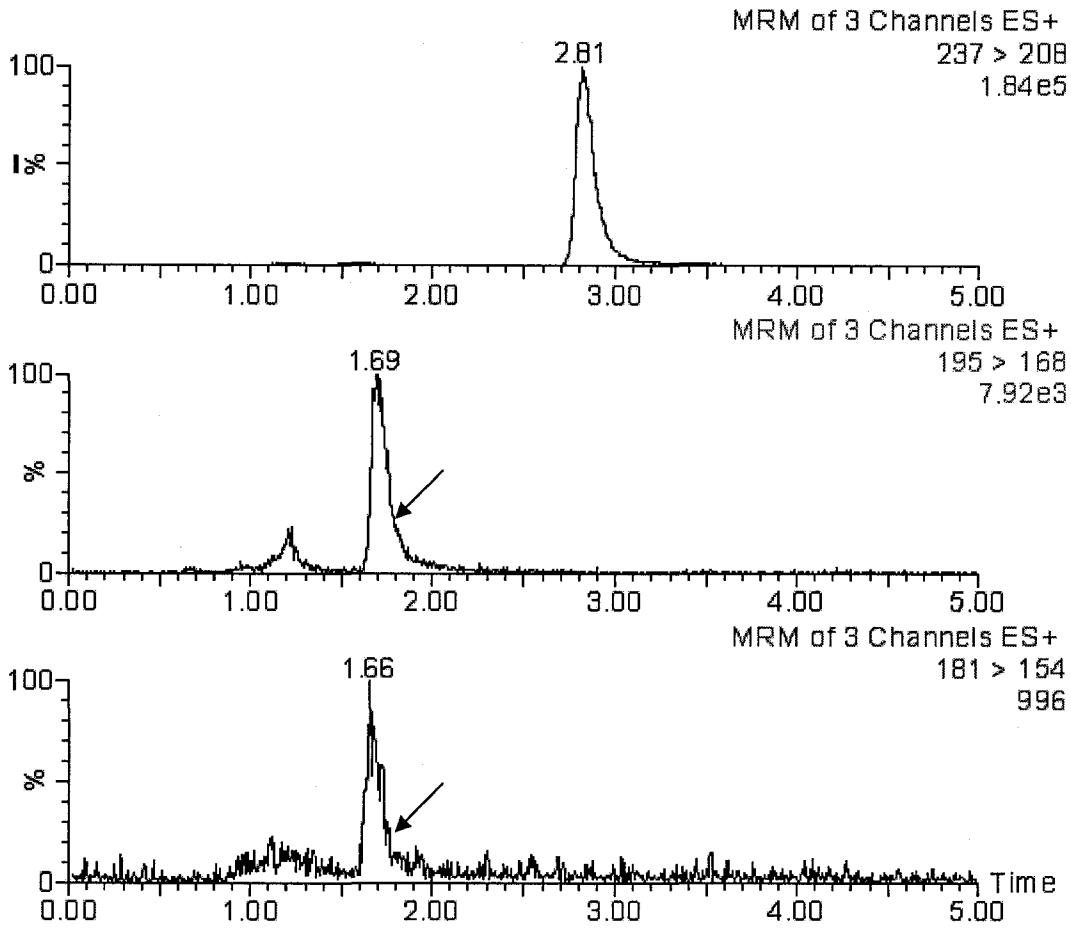

Figure 3.5 A typical LC-MS/MS chromatograph of methylglyoxal, glyoxal, and 2,3-hexanedione derivatized with 2,3-DAN from a normal plasma sample. Levels of methylglyoxal and glyoxal measured in this sample were 0.52 ng and 0.26 ng, respectively. The profiles are 2,3-hexanedione, methylglyoxal and glyoxal from top to bottom. The unit of time is minutes. The abbreviations are: LC-MS/MS is liquid chromatography-tandem mass spectrometry; and DAN is Diaminonaphthalene.

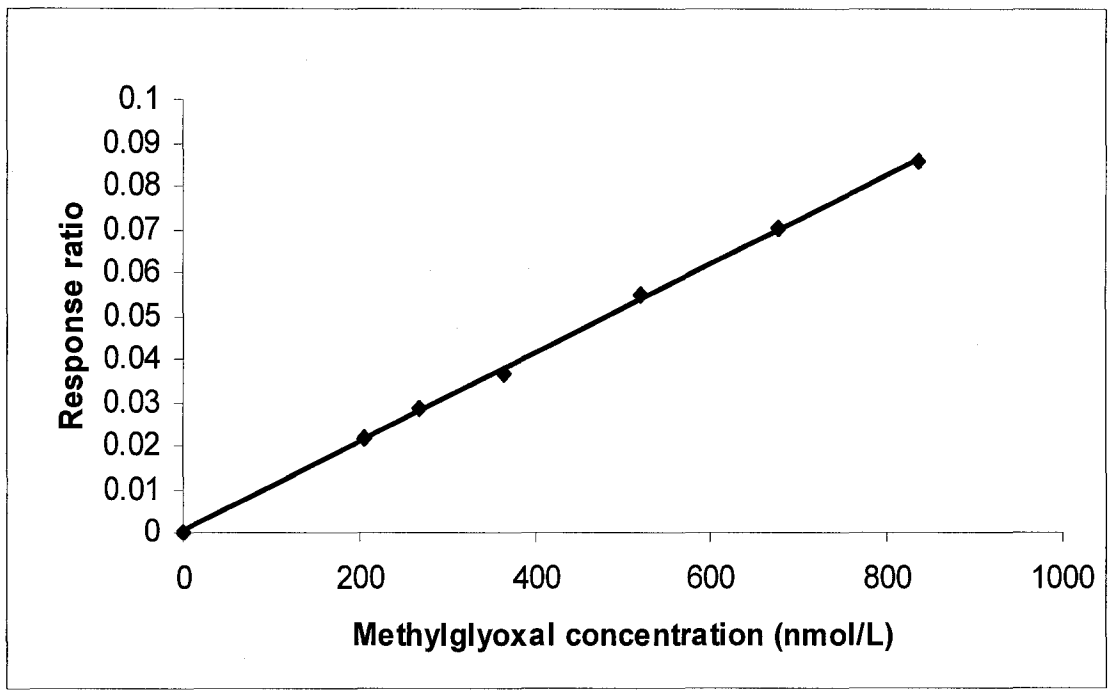

Figure 3.6 Calibration curve for methylglyoxal in serum. The response ratio was calculated as the area under the curve (AUC) for methylglyoxal divided by the AUC for the internal standard.

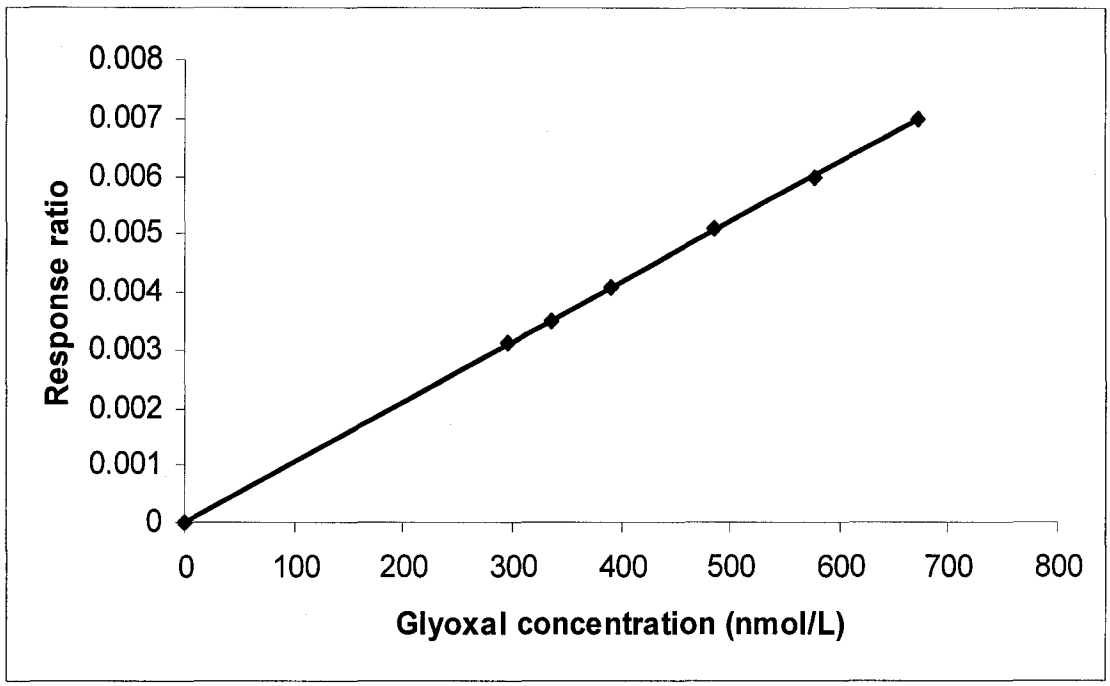

Figure 3.7 Calibration curve for glyoxal in serum. The response ratio was calculated as the area under the curve (AUC) for glyoxal divided by the AUC for the internal standard.

3.2.2 Discussion

This method represents the first developed using LC-MS/MS to measure methylglyoxal and glyoxal in plasma and makes use of 2,3-DAN as a derivatizing agent. Most previously described methods used HPLC and fluorescence detection, spectrophotometer detection, or single ion monitoring mass spectrometry to measure methylglyoxal in cell culture, plasma or urine. Essentially all of these relied on formation of diamino aromatic products for capture and detection of the dicarbonyls. In particular o-phenylenediamine (Chaplen et al., 1996; Randell et al., 2005; Ohmori et al. 1987), and 1,2-diamino-4,5-dimethoxybenzene (McLellan et al., 1992, Nemet et al, 2004) was previously used for blood samples. In our early studies we attempted to increase the analytical specificity for methylglyoxal using o-phenylenediamine derivatives and LC-MS/MS. However, because of poor sensitivity due to inadequate fragmentation of this product in the collision chamber these efforts were abandoned. Odani et al. (1999) used 2,3-DAN and 3,4-hexanedione as internal standard to measure methylglyoxal, glyoxal and 3-deoxyglucosone using an electrospray ionization LC/MS technique with chromatography on a C18 column. This method had relatively long chromatographic times in excess of 35 minutes. Because of the greater potential of 2,3-DAN derivatives for MS/MS analysis we used this as a derivatizing agent. This new method is an improvement in that it provides greater reproductility and takes advantage of the increased specificity of tandem mass spectrometry with less likelihood of interference from products with the same mass (isobaric compounds).

Analyte analogues labeled by stable isotopes are the best internal standard for MS/MS because they have similar chromatograph and fragmentation characteristics as the analyte itself and thereby aid in correcting for sample specific ion suppression effects that can affect the accuracy of any determination (Annesley, 2003 CC). Stable isotope labeled analogues of methylglyoxal or glyoxal are not commercially available so instead 2,3-hexanedione was used as an internal standard because of its dicarbonyl structure. It was added to the protein free supernatant after precipitation of protein and helped correct for variable losses of derivatization products during subsequent extraction steps. All products are relatively similar structurally and are expected to have similar physical properties and extraction efficiency.

This new method for measurement of methylglyoxal and glyoxal has the following advantages: **1)** Improved selectivity owing to use of tandem mass spectrometry; **2)** Shorter analysis times using 2,3 DAN as a derivatizing agent; **3)** Measurement of both glyoxal and methylglyoxal in a single analytical run. This method also has the potential to measure tissue and intracellular glyoxal and methylglyoxal concentration as it makes use of the rather robust process of TCA precipitation to remove proteins and involves liquid-liquid extraction of the final derivatized products for analysis. It is has not yet been determined if deoxyglucosone, the other major reactive dicarbonyl involved in formation of AGEs, can also be measured in the same chromatographic run. The ability to measure deoxyglycosone would allow measurement of the three most important dicarbonyl AGE precursors present *in vivo*.

3.3 Development of Methods for Measurement of AGEs in Plasma

3.3.1 LC-MS/MS Method for Measurement of AGEs

A LC-MS/MS procedure was developed with the goal of analyzing levels of 4 different methylglyoxal derived AGE residues present in free form in plasma and released from protein by acid hydrolysis. These four, including MG-H (Ahmed et al., 2002), argpyrimidine (Shipanova et al., 1997), CEL (Ahmed et al., 1997) and MOLD (Nagaraja et al., 1996), were first synthesized by procedures described in the literature. However, only MG-H1 was successfully measured in this work and its measurement is described below. The published procedures allowed us to prepare adequate amounts of MG-H1 for a semi-quantitative assessment of the levels present in plasma. The relatively small amounts made during this work, however, was not sufficient to allow rigorous assessment of the purity of the preparation. Figure 3.8 shows CID mass spectra produced by a daughter scan of products of MG-H1. The identity was confirmed in comparison with CID characteristics previously described (Thornalley et al., 2003)

MG-H1 and other methylglyoxal-derived AGE residues were separated on a Waters High Performance Liquid Chromatography system (2795 separators module equipped with a temperature controller) and a Micromass Quattro Ultima PT for ESI/MS/MS. Various sample components were resolved on a Atlantis HILIC Silica 3 μm (2.1 x 50mm) HPLC Column in an water gradient (10% to 55%) against 10 mM Formate Buffer and 5% Methanol in Acetonitrile over the first 2 minutes followed with isocratic

conditions in the high water solvent for the next 2 minutes. Flow rate was held constant at 0.5 ml/min and the column maintained at ambient temperature. The MS method used positive ion electrospray with capillary spray voltage held at 3.5 kV and cone voltage of 35 V. The elution of AGE residues were monitored by MS/MS at ionization source temperature of 120 °C; Desolvation gas temperature 350 °C; and desolvation gas flow of 550 L/hr. MS parameters for amino acids and specific AGEs in our system are indicated in Table 3.1. Figure 3.9 shows the chromatogram for a mixture of synthesized standards of the AGEs separated in this system.

A solid phase extraction (SPE) procedure on MCX columns were used to help clean up biological samples prior to analyses. For measurement of the free non protein-bound MG-H1 the plasma protein was first precipitated by 20% TCA and this supernatant was mixed with d4-lysine as an internal standard prior to SPE and measurement of MG-H1.

3.3.1.1 Chromatographic properties of MG-H1

Figure 3.10 shows a typical chromatogram of free MG-H1 residues from a plasma sample from a healthy volunteer and the same sample but with an amount of purified MG-H1 added immediately prior to SPE. In the spiked sample MG-H1 appears at elution time of about 2.6 minutes. This is similar to the chromatogram obtained for a highly purified sample of MG-H1. Free non-protein bound MG-H1 levels are detectable in the plasma of the volunteer.

Table 3.1 MS parameters for amino acids and specific AGEs. The abbreviations are: MS is mass spectrometry; AGEs is advanced glycation end products; MOLD is methylglyoxal-derived lysine dimer; CEL is carboxyethyl lysine; and MG-H is methylglyoxal-derived hydroimidazolones.

Compound	Retention Time	Transitions	Collision Energy
Arginine	2.55	175>116	7.0
Lysine	2.61	147>84	8.0
Argpyrimidine	2.21	255.2>192.2	13.0
MG-H1	2.60	229.1>114	9.0
MOLD	3.48	341.3>212.2	15.0
CEL	2.77	219>130	8.0

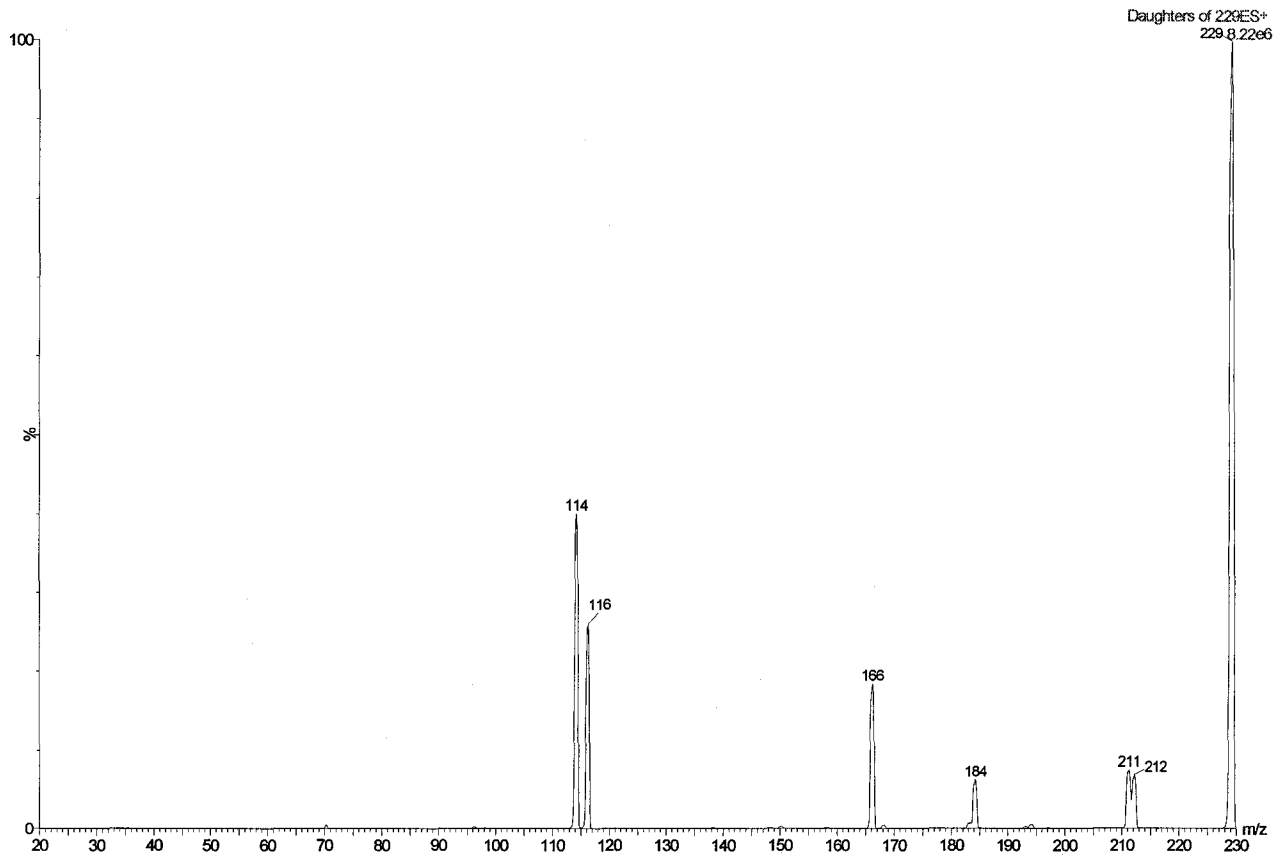

Figure 3.8 CID profile for the MG-H. Mass spectra were produced at a CE of 9 eV. Capillary voltage was held at 3.5 kV, cone voltage was set of 35 V, source temperature was 120°C, desolvation gas flow was set at 500 (L/hr). Protonated molecular ion (M+1) corresponds to the peak identified as 229. Positively charged ions 114, 116, 166, 184, 211, 212 represent the daughter ions produced from the protonated molecular ion. Size of the peaks represents relative amounts. The abbreviations are: CID is collision induced dissociation; CE is collision energy; and MG-H is methylglyoxal-derived hydroimidazolone.

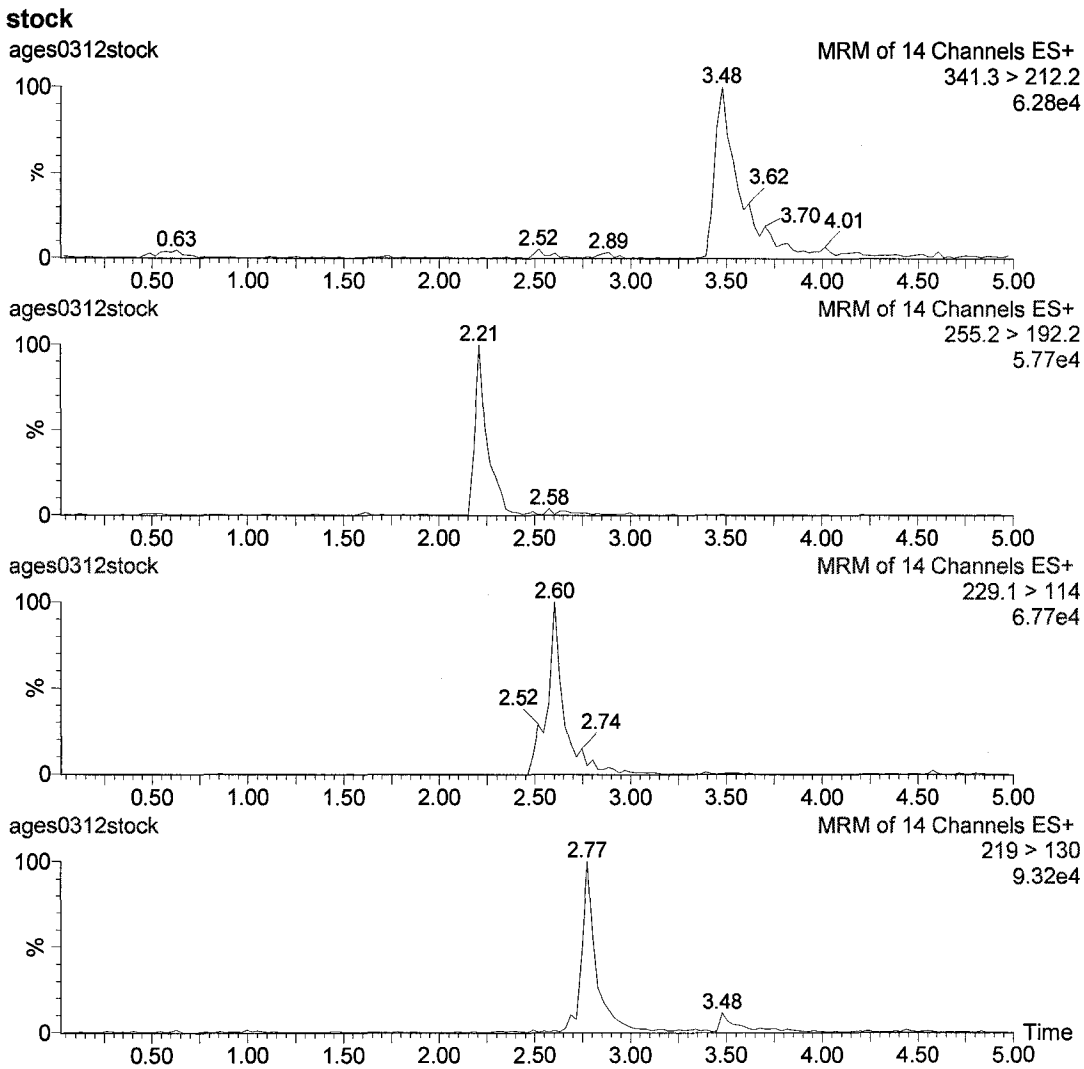

Figure 3.9 Chromatogram of a pure sample of AGEs. The profiles are MOLD, argpyrimidine, MG-H and CEL from top to bottom. The level of each AGEs measured in this sample was 2 μg. The unit for time is minutes. The abbreviations are: AGEs is advanced glycation end-products; MOLD is methylglyoxal-derived lysine dimer; MG-H is methylglyoxal-derived hydroimidazolone; and CEL is carboxyethyl-lysine.

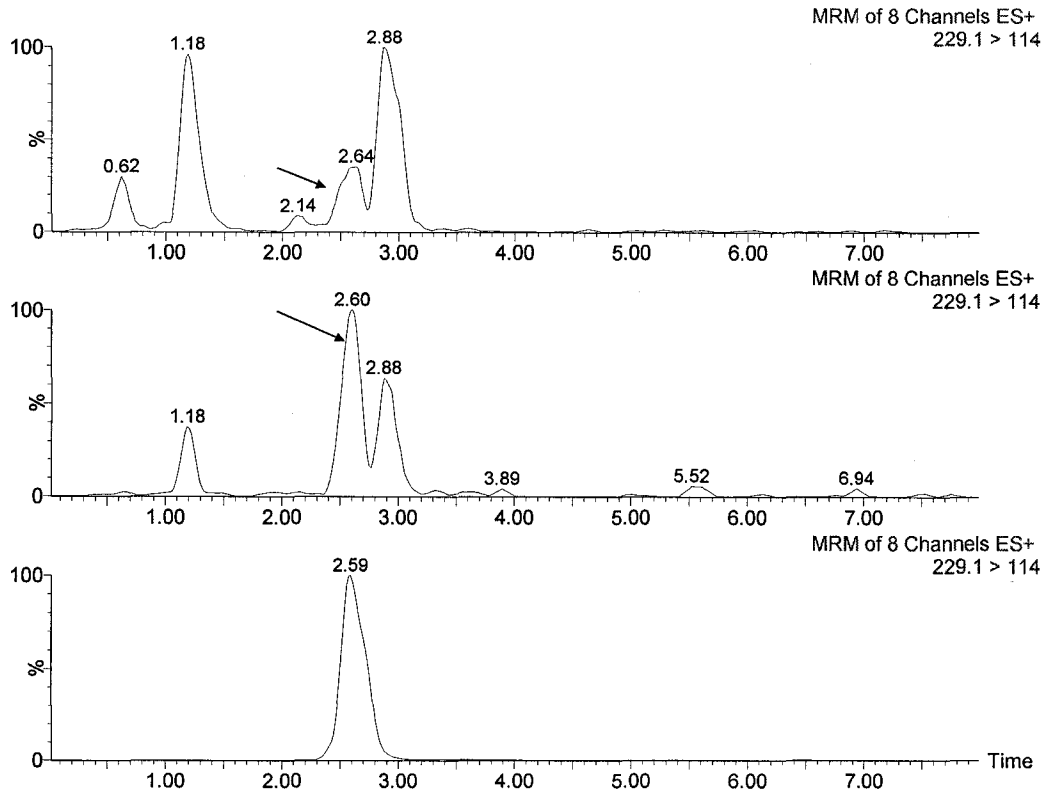

Figure 3.10 A typical LC-MS/MS chromatograph of free MG-H1 from a plasma sample from a healthy volunteer and a similar sample spiked with an amount of purified MG-H1 immediately prior to SPE. Arrow points to MG-H1 peak. From top to bottom profiles are plasma sample from a health volunteer; plasma sample spiked with MG-H1; and a pure MG-H1 standard. The unit for time is minutes. The abbreviations are: LC-MS/MS is liquid chromatography-tandem mass spectrometry; MG-H1 is methylglyoxal-derived hydroimidazolone; and SPE is solid phase extraction.

3.3.1.2 Standard curve for MG-H1

Figures 3.11 show a linear calibration curve for MG-H1. Increasing amounts of MG-H1 were recovered with increasing amounts of the purified MG-H1 added to samples prior to SPE. This indicates that AGE residues were being retained by the SPE column and appropriately released in a concentration dependent manner. The low recovery of other endogenously produced AGEs was a problem and prevented measurement of these in the biological samples.

3.3.2. Discussion for Development of Methods on AGEs

The goal of this work was to develop a relatively simple procedure for measurement of several methylglyoxal-derived AGE residues by LC-MS/MS. While the LC-MS/MS components of the method do allow separation and measurement of the four free methylglyoxal-derived AGE residues, the sample preparation was inadequate to provide adequate amounts of these AGE residues to measure. The chromatographic method described uses a single HPLC column with a total analysis time of about 8 minutes (See Methods Section for details) to separate and measure the four MG-derived AGEs. This offered the potential for a significant improvement in analytical time compared to a previously described method with capability of measuring all four (Thornalley et al., 2003) which required run times of about 50 minutes. However, only MG-H1 was recovered from the precipitated samples for measurement. Another HPLC based method for measurement of MG-H1 has also been published (Ahmed et al. 2002)

but requires 6-aminoquinolyl-N-hydroxysuccinidmdyl carbonate (ACQ) derivatization and chromatographic run times in excess of 250 minutes. This is not practical for the analysis of large numbers of samples.

The method described here offers sufficient sensitivity to measure MG-H1 that is free in plasma as well as that released by hydrolysis of protein. Free MG-derived AGE concentrations range from 10^{-9} to 10^{-6} M in plasma. Levels of MG-H1 have been previously been reported at about 50 nmol/L in plasma on healthy individuals (Ahmed et al., 2005), exceeding the plasma concentration of CEL, Argpyrimidine, and MOLD, the latter two being undetectable in free form. The free AGE plasma component most likely represents that released from intracellular sources following intracellular hydrolysis of modified proteins. These free AGE residues are effectively removed by the kidney and excreted into the urine. A portion of these may also come from the diet (Thornalley et al, 2003). The majority of plasma AGEs are bound to plasma protein and can be released by acid or enzyme-mediated hydrolysis (Ahmed et al., 2002). Our work using acid hydrolysis gave unsatisfactory results when analyzed by LC-MS-MS. Acid hydrolysis has been reported to result in excessive loss of hydroimidazolone AGE residues like MG-H1 (Ahmed et al., 2002). A step-wise enzymatic hydrolysis procedure has been successfully used by others to prepare protein bound AGEs for LC-MS/MS analysis (Ahmed et al., 2002). Nevertheless, LC-MS/MS still offers the best method for measurement of methylglyoxal-derived AGE residues. Attempts to measure these by other techniques have significant limitations for providing quantitative data (Ahmed and Thornalley, 2007).

Quantitative analysis of AGEs requires availability of mg quantities of pure standards. These are not commercially available but must be synthesized and purified to high purity to use in quantitative assay methods. Synthesis procedures have been published for all of the MG-derived AGEs. We were successful in the synthesis of only very small quantities of the various standards using instrumentation available to us. Thus, determination of purity and accurate and precise mass of the final MG-H1 product was not possible. We made the assumption that MG-H1 standard was 100% pure and expressed sample plasma concentrations as mg/L based on this assumption.

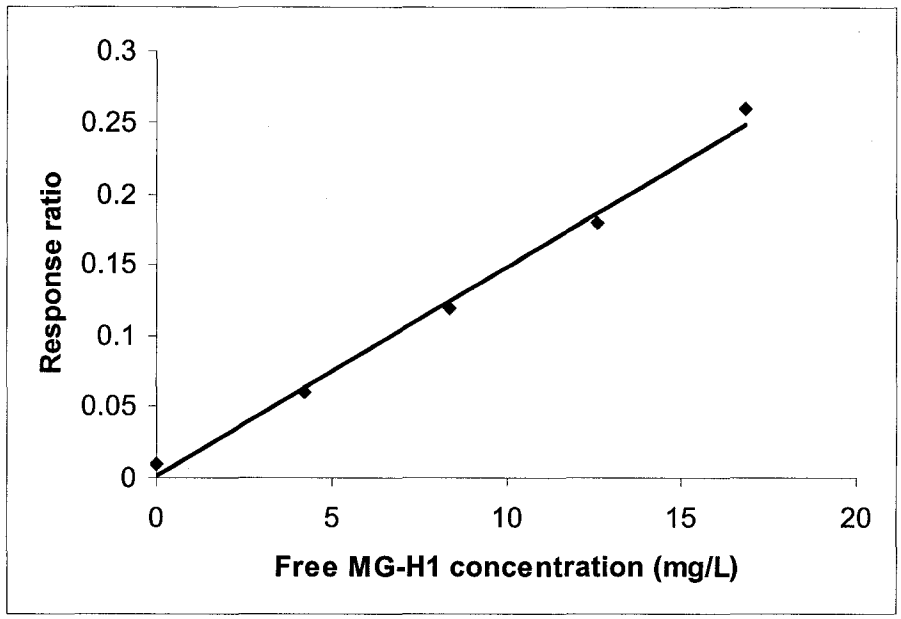

Figure 3.11 A linear calibration curve for free MG-H1 in serum. The response ratio was calculated as the area under the curve (AUC) for MG-H1 divided by the AUC for the internal standard. MG-H1 is the abbreviation of methylglyoxal-derived hydroimidazolone.

CHAPTER 4

STUDIES ON METHYLGLYOXAL, GLYOXAL, AGES AND NA^+/K^+-ATPASE ACTIVITY IN T1DM

4.1 Results

4.1.1 Study Subject Characteristics

Fifty six young complication-free T1DM patients and 18 age matched controls without diabetes were recruited to study the relationships between methylglyoxal, glyoxal and plasma free MG-H1 levels and early risk of complications as determined by A1C concentrations. The relationship of these with Na^+/K^+-ATPase activity, a potential target of methylglyoxal or glyoxal damage, was also examined. Table 4.1 summarizes data from controls and T1DM subjects recruited. Given the young age of the control subjects and the normal A1C values there is very little chance that an undiagnosed T1DM or T2DM patient exists in this group, although sample collection was anonymous. There was no difference in the mean age, or in the proportion of males and females between the T1DM and control groups. The plasma A1C (%) was higher in the T1DM group (8.5 ± 1.3) as compared to the control group (5.0 ± 0.3). All of the T1DM patients recruited as part of this study, except for one subject who had been diagnosed with T1DM 1.2 years prior, had their diagnosis for at least 2 years. They were all receiving insulin treatment and none of them had diagnosed microvascular complications.

Table 4.1 Characteristics of controls and T1DM subjects. Values are expressed as mean ± standard deviation and those in brackets are the range of values within each category. * indicates that values are significantly different from the control group by student t-test ($p<0.05$). α indicates that values are significantly different from the control group by ANOVA with multiple comparison correction using the Bonferroni method. Normal range for hemoglobin A1C (A1C) is 4 – 6%. Good glycemic control was defined as mean A1C <8 for one year; Moderate glycemic control as mean A1C 8 to 9; and Poor glycemic control as mean A1C >9. The abbreviation N/A represents not applicable.

	Control	**T1DM (whole group)**	**Good control**	**Moderate control**	**Poor control**
Numbers	18	56	20	17	19
Age (years)	14.6 ± 4.6 (6-22)	15.0 ± 4.2 (6-21)	13.3 ± 4.4 (6-21)	15.3 ± 5.1 (8-21)	16.2 ± 2.8 (11-20)
Sex (female, male)	10F, 8M	28F, 28M	10F, 10M	9F, 8M	9F, 10M
Duration of DM (years)	N/A	7.5 ± 4.2 (1.2-20)	6.6 ± 3.7 (1.2-14)	8.8 ± 4.8 (3.5-20)	7.2 ± 4.3 (2-18)
A1C (%)	5.0 ± 0.3 (4.6-5.4)	8.5 ± 1.3 * (6.1-12.3)	7.5 ± 0.7 $^{\alpha}$ (6.1-9.1)	8.5 ± 0.5 $^{\alpha}$ (7.7-9.8)	9.5 ± 1.4 $^{\alpha}$ (6.7-12.3)
Insulin Therapy (number/group)					
Conventional (2 injections/day)	N/A	9	1	4	4
Multiple (3 injections/day)	N/A	13	2	3	8
Multiple (4 injections/day)	N/A	14	3	5	6
Insulin pump	N/A	20	14	5	1

4.1.2 Levels of Methylglyoxal, Glyoxal, Free MG-H1 and Na^+/K^+-ATPase Activity in T1DM

Levels of plasma methylglyoxal, glyoxal, plasma free MG-H1 and RBC membrane Na^+/K^+-ATPase activity were determined in both control and T1DM patients (Table 4.2). The mean plasma methylglyoxal (nmol/L) and glyoxal level (nmol/L), respectively, were higher in the T1DM group as a whole (842 ± 238, 1052 ± 515) versus the controls (439 ± 90, 328 ± 208) (Figure 4.1 & 4.2). Plasma free MG-H1 (mg/L) was also higher in the DM group (2.7 ± 1.1) versus the control group (1.7 ± 0.9) (Figure 4.3). RBC membrane Na^+/K^+-ATPase activity (nmol NADH oxidized/min/mg protein) was elevated in the DM group (4.47 ± 0.98) compared to the control group (2.16 ± 0.59).

Figure 4.4 shows Na^+/K^+-ATPase activity in controls and T1DM patients separated according to glycemic control. Individuals in the T1DM group were separated into two subgroups, those with a duration of diabetes of less than or equal to 7 years, and those with a duration greater than 7 years (based on median value for duration of diabetes). There was no difference in the level of Na^+/K^+- ATPase activity between these two subgroups.

Table 4.2 Levels of plasma methylglyoxal, glyoxal, free MG-H1 according to the levels of glycemic control in study subjects. Good glycemic control was defined as A1C <8; Moderate glycemic control as A1C 8 to 9; and Poor glycemic control as A1C >9. Values are mean ± standard deviation. * indicates significantly different from control group by student t test. α indicates significantly different from control group by ANOVA with multiple comparison correction using Bonferroni method. The abbreviations are: MG-H1 is methylglyoxal-derived hydroimidazolone; and NADH is reduced nicotinamide adenine dinucleotide.

	Control	**T1DM (whole group)**	**Good control**	**Moderate control**	**Poor control**
Plasma methylglyoxal (nmol/L)	439 ± 90 (278-597)	842 ± 238 * (431-1403)	751 ± 236 $^{\alpha}$ (431-1403)	819 ± 153 $^{\alpha}$ (653-1125)	944 ± 250 $^{\alpha}$ (583-1375)
Plasma glyoxal (nmol/L)	328 ± 208 (34-638)	1052 ±515 * (379-2396)	724 ± 396 (379-1414)	1224 ±552 $^{\alpha}$ (707-2206)	1241 ± 552 $^{\alpha}$ (586-2396)
Plasma free MG-H1 (mg/L)	1.7 ± 0.9 (0.7-4.0)	2.7 ± 1.1 * (1.4-5.9)	2.8 ± 1.1 (1.4-5.0)	2.6 ± 1.2 (1.4-5.9)	2.5 ± 0.9 (1.6-5.0)
Na^+/K^+-ATPase activity (nmol (NADH oxidized /min/mg protein)	2.2 ± 0.6 (1.1-3.1)	4.5 ± 1.0 * (2.4-7.6)	4.0 ± 0.8 $^{\alpha}$ (2.4-5.3)	4.2 ± 0.7 $^{\alpha}$ (2.9-5.3)	5.1 ± 1.0 $^{\alpha}$ (3.4-7.6)

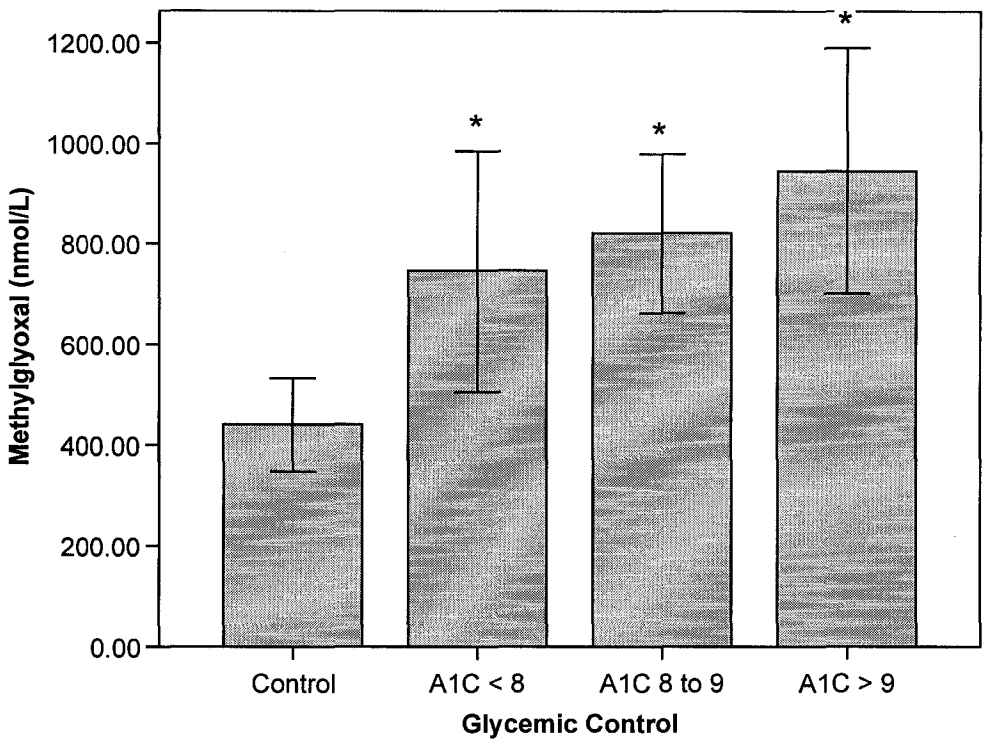

Figure 4.1 Levels of plasma methylglyoxal in controls and T1DM subjects separated according to the level of glycemic control. Good glycemic control was defined as A1C <8; Moderate glycemic control as A1C 8 to 9; and Poor glycemic control as A1C >9. Results are mean ± standard deviation. * indicates significantly different from control group by ANOVA with multiple comparison correction by Bonferroni method. The abbreviations are: T1DM is Type 1 diabetes mellitus; and A1C is hemoglobin A1C.

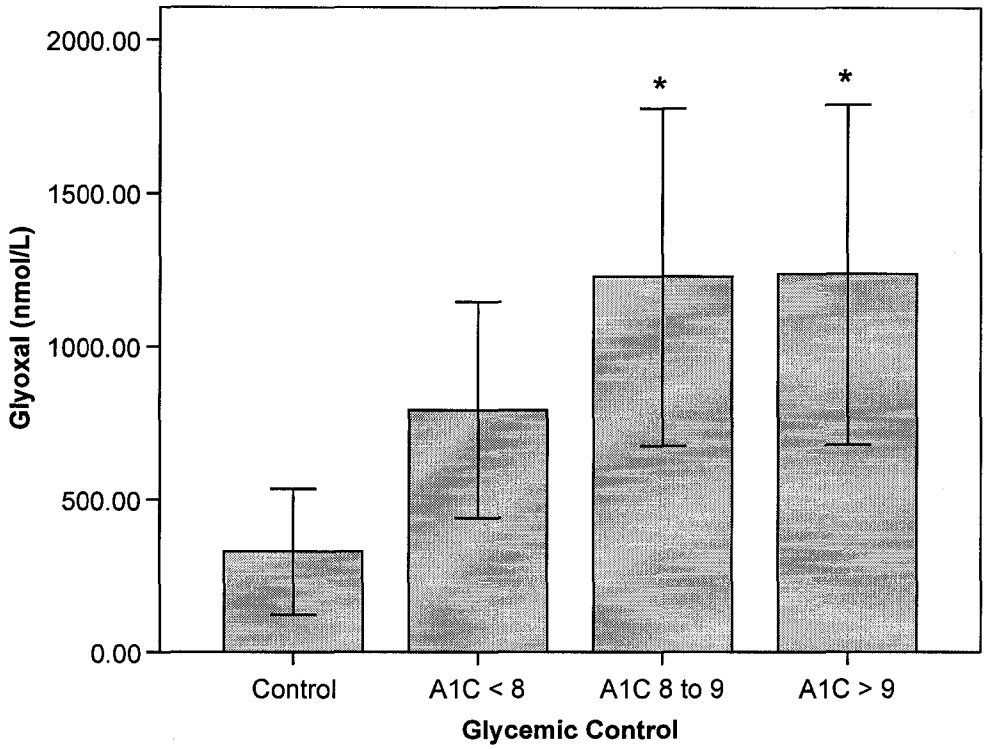

Figure 4.2 Levels of plasma glyoxal in controls and T1DM subjects separated according to the level of glycemic control. Good glycemic control was defined as A1C <8; Moderate glycemic control as A1C 8 to 9; and Poor glycemic control as A1C >9. The results are mean ± standard deviation. * indicates significantly different from control group by ANOVA with multiple comparison correction by Bonferroni method. The abbreviations are: T1DM is Type 1 diabetes mellitus; and A1C is hemoglobin A1C.

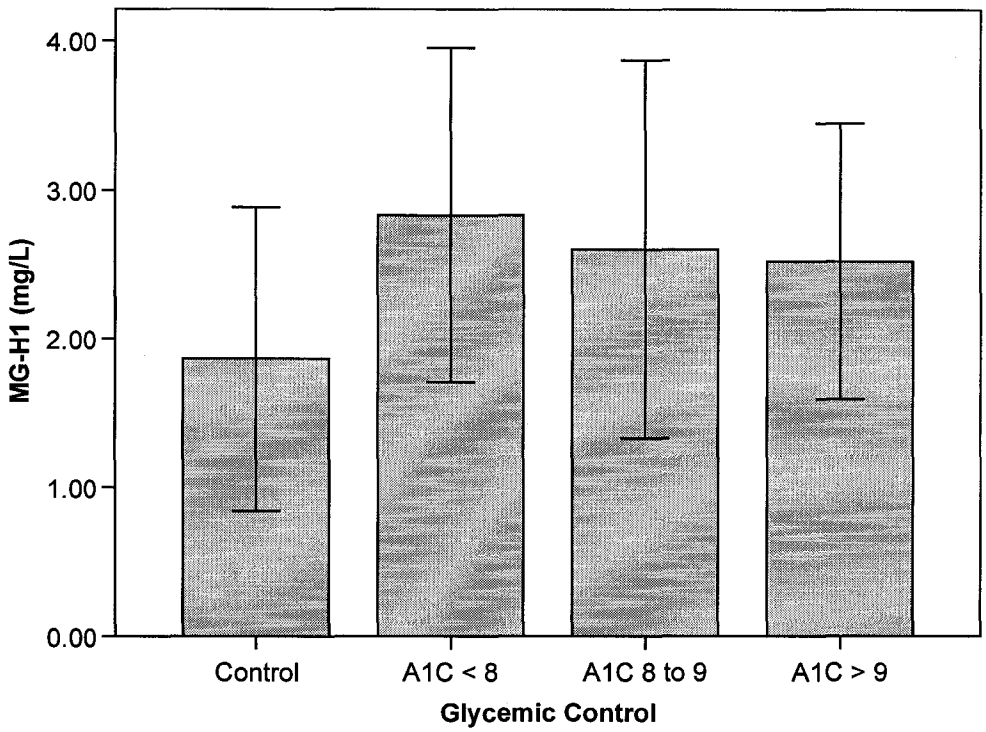

Figure 4.3 Levels of plasma free MG-H1 in controls and T1DM subjects separated according to the level of glycemic control. Good glycemic control was defined as A1C <8; Moderate glycemic control as A1C 8 to 9; and Poor glycemic control as A1C >9. The results are mean ± standard deviation. Values were not significantly different between groups as assessed by ANOVA with multiple comparison correction by Bonferroni method. The abbreviations are: MG-H1 is methylglyoxal-derived hydroimidazolone; T1DM is Type 1 diabetes mellitus; and A1C is hemoglobin A1C.

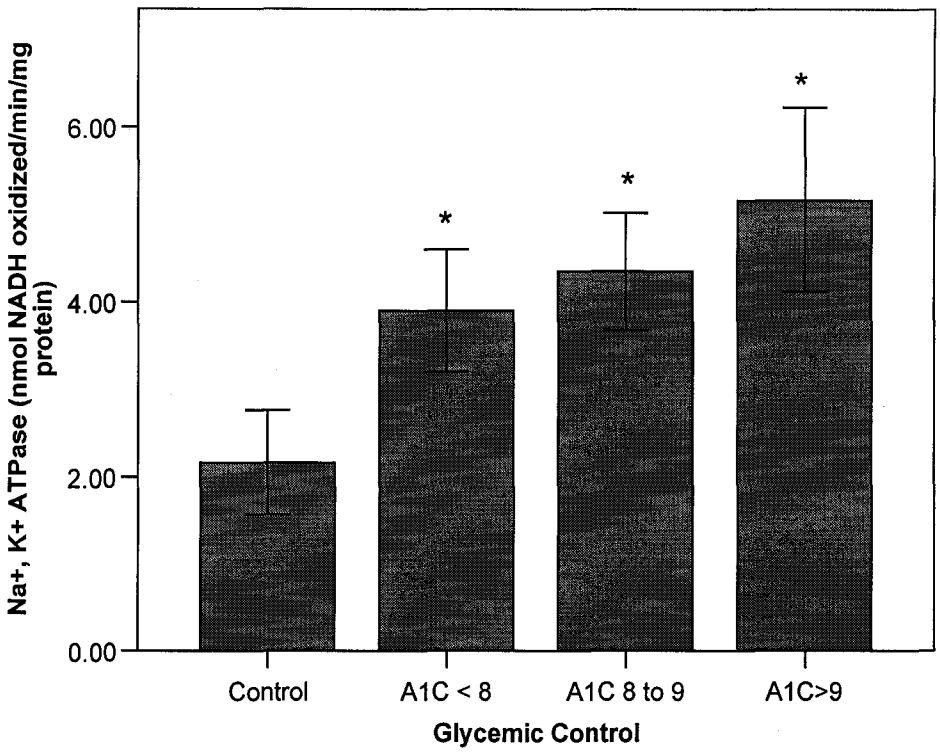

Figure 4.4 Erythrocyte membrane Na^+/K^+-ATPase activity in controls and T1DM subjects separated according to level of glycemic control. Good glycemic control was defined as A1C <8; Moderate glycemic control as A1C 8 to 9; and Poor glycemic control as A1C >9. The results are mean ± standard deviation. * indicates significantly different from control group by ANOVA with multiple comparison correction by Bonferroni method. T1DM is the abbreviation of Type 1 diabetes mellitus.

4.1.3 Relationships between Plasma Methylglyoxal, Glyoxal, MG-H1 and A1C in T1DM

Pearson correlation analysis was carried out to determine if there was a relationship of methylglyoxal, glyoxal and MG-H1 with A1C and with each other. A1C correlated with both plasma methylglyoxal ($r = 0.635$, $p<0.001$) and plasma glyoxal ($r = 0.523$, $p<0.001$) levels but not with MG-H1. Plasma methylglyoxal and glyoxal correlated strongly with each other ($r = 0.705$, $p<0.001$). MG-H1 levels correlated weakly with methylglyoxal concentrations ($r = 0.265$, $p=0.05$) but not with glyoxal concentrations.

4.1.4 Relationships of Plasma Methylglyoxal, Glyoxal, and A1C with Na^+/K^+-ATPase Activity in RBC Membrane in T1DM.

Correlation analysis of all subjects revealed that Na^+/K^+ ATPase activity was strongly correlated with methylglyoxal ($r = 0.449$, $p<0.001$), glyoxal ($r = 0.554$, $p<0.001$), and A1C ($r = 0.730$, $p<0.001$) (Figure 4.5) (Table 4.3). Multiple linear regression analysis showed that A1C was a strong predictor of Na^+/K^+-ATPase activity ($R^2=0.582$, $p<0.001$), and in the presence of this information, the information on methylglyoxal and glyoxal did not add significantly to the predictability of Na^+/K^+-ATPase activity. Correlation analysis within each group (T1DM and controls) revealed no additional or stronger relationships.

Table 4.3 Relationships assessed by correlation analysis. The abbreviations are: NADH is reduced nicotinamide adenine dinucleotide; and MG-H is Methylglyoxal-derived hydroimidazolone.

Variable	Variable	r value	p value
Hemoglobin A1C (%)	Methylglyoxal (nmol/L)	0.635	0.001
Hemoglobin A1C (%)	Glyoxal (nmol/L)	0.523	0.001
Methylglyoxal (nmol/L)	Glyoxal (nmol/L)	0.705	0.001
Na^+/K^+-ATPase activity (nmol NADH oxidized /min/mg protein)	Hemoglobin A1C (%)	0.730	0.001
Na^+/K^+-ATPase activity (nmol NADH oxidized /min/mg protein)	Methylglyoxal (nmol/L)	0.449	0.001
Na^+/K^+-ATPase activity (nmol NADH oxidized /min/mg protein)	Glyoxal (nmol/L)	0.554	0.001
Free MG-H1 (mg/L)	Methylglyoxal (nmol/L)	0.265	0.05

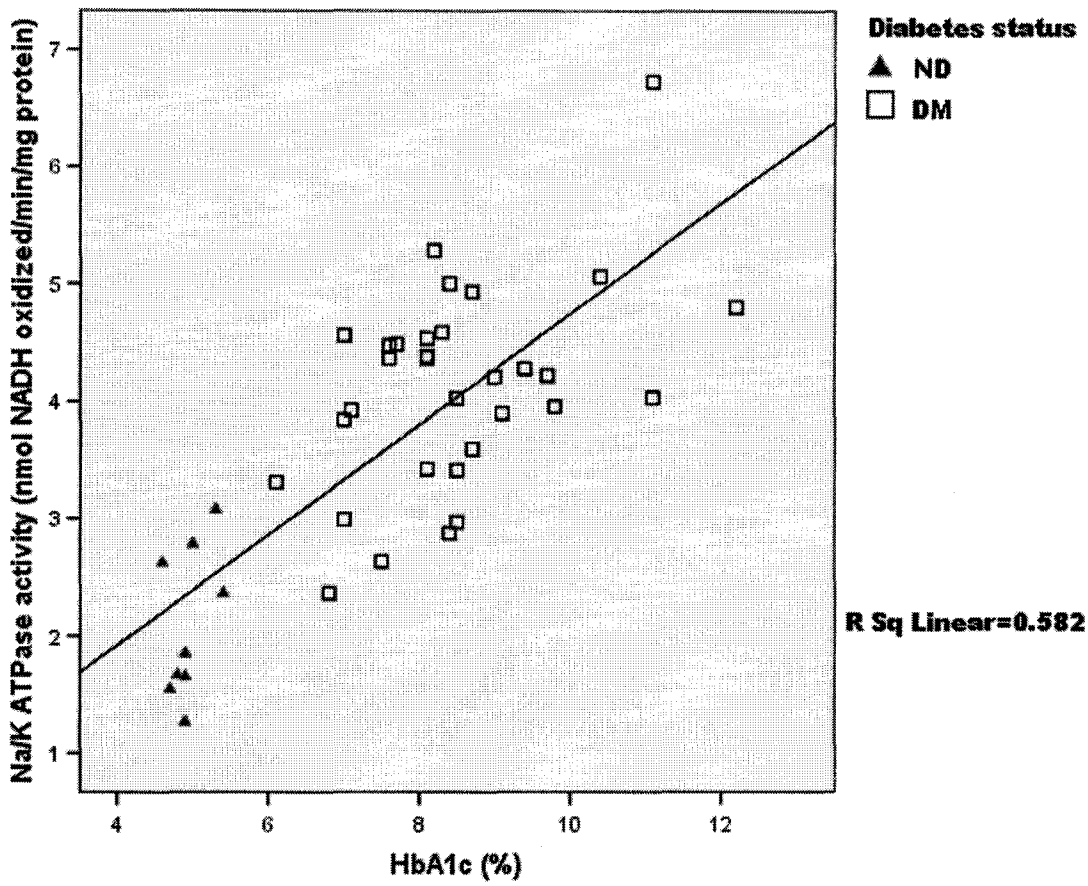

Figure 4.5 Linear regression of hemoglobin A1C with Na^+/K^+-ATPase activity.

(ND: non-diabetes controls; DM: patients with Type 1 diabetes)

4.2 Discussion

This work demonstrates for the first time that plasma levels of both methylglyoxal and glyoxal, and the free plasma methylglyoxal-arginine adduct MG-H1 are elevated in young patients with T1DM in the absence of diabetic complications. As these patients had no documented renal disease, it is likely that these elevations are not due to decreased excretion by kidney. There is little published data on levels of plasma methylglyoxal and glyoxal, or plasma free MG-H1 residues in DM. Blood methylglyoxal levels have been shown to be elevated in a group of individuals with insulin-dependent and non-insulin dependent diabetes with complications compared with controls (McLellan et al., 1994). When compared with normoglycemic individuals, a combined group of mainly adult patients (ages 15 to 82 years) with T1DM and T2DM with complications showed significantly elevated levels of methylglyoxal in plasma, but showed no significant difference when whole blood levels were used (Nemet et al., 2005). Plasma methylglyoxal and glyoxal were higher in older adult individuals with T2DM (Odani et al., 1999), and remained higher than normal controls even after an intervention which significantly improved glycemic control (Lapolla et al., 2003). Although all T1DM subjects in the present study were receiving insulin therapy, the average A1C level was still significantly higher than that of the normal control group indicating a general lack of glycemic control. As high glucose is one source of elevated methylglyoxal and glyoxal, lack of glycemic control may partially explain their elevated levels. The high correlation between A1C and plasma levels of these aldehydes substantiates this. The high

correlation between methylglyoxal and glyoxal also suggests that they arise from the same source. As methylglyoxal- and glyoxal-derived AGEs are implicated in complications of diabetes (Hammes et al., 1991; Misselwitz et al., 2002; Lieuw-A-Fa et al., 2004; and Hwang et al., 2005), this finding underscores the need for stringent glycemic control in patients with T1DM.

Although levels of methylglyoxal and glyoxal are elevated in patients in the T1DM group, they have yet to develop demonstrable complications. However, the presence of increased glyoxal likely indicates that they are already experiencing oxidative stress. Likewise, other sub-clinical changes may be occurring. For instance, Elhadd et al. (1999) demonstrated an increase in markers of oxidative stress and endothelial dysfunction in young patients without clinical diabetic angiopathy. It may be that the magnitude of elevations in aldehydes seen in our study must be sustained for a longer interval in order for detrimental effects to become clinically apparent. As uncontrolled diabetes progresses, and tissue AGEs continue to accumulate, patients may begin to experience complications. We have shown, in an older group of T1DM and T2DM patients, that the cysteine AGEs, CEC and CMC, derived from methylglyoxal and glyoxal respectively, are elevated in plasma and are associated with diabetic nephropathy (Mostafa et al., 2007).

The levels of methylglyoxal in plasma also correlated weakly with the levels of free plasma MG-H1, whereas the levels of glycemia as determined by A1C did not. This is not surprising considering that MG-H1 is a MG-derived AGE. Increased levels of free MG-H1 observed in individuals with diabetes, however, is not merely the result of short

term changes in methylglyoxal levels but also the result of intracellular proteolysis of intracellular and extracellular proteins modified by methylglyoxal over longer periods of time depending on the half-life of the protein. In the present study, levels of MG-H1 were 60% higher in T1DM than in controls. Others have reported a more substantial difference in free MG-H1 levels of 7 to 10 times higher when older T1DM and T2DM individuals are examined (Ahmed et al., 2005) or when healthy controls are compared with renal failure patients on dialysis where differences are greater than 10 times (Thornalley et al., 2003). It is possible that increasing age and duration of DM are significant factors in determining the accumulation of AGEs in proteins and therefore the amount of free MG-H1 residue produced once these are hydrolysed. The impact of renal impairment in some older individuals may be another factor (Thornalley et al., 2003). Nevertheless, higher free MG-H1 levels in complication free young T1DM patients suggests that even at this very early stage in the disease process, significant amounts of methylglyoxal mediated damage is occurring.

The activity of a ubiquitous membrane enzyme, Na^+/K^+-ATPase, in RBC was also measured in this research. Na^+/K^+-ATPase regulates active transport of sodium and potassium across plasma membranes, and alteration in activity of this enzyme has been implicated in diabetic neuropathy (Raccah et al., 1996; Djemli-Shipkolye et al., 2001) and may be involved in other diabetic complications (Mimura et al., 1994; Tsimaratos et al., 2001; Koc et al., 2003). Previous studies have shown a decrease in RBC Na^+/K^+-ATPase activity in TIDM patients ranging in age from late teens to 70 years of age, and with and without diabetic complications (Raccah et al., 1996; Baldini et al., 1989; Besch

et al., 1995; Finotti and Palatini, 1986; and Jannot et al., 2002). Methylglyoxal, glyoxal, and AGEs are known to cause inhibition of enzymes such as glyceraldehyde-3-phosphate dehydrogenase, nitric oxide synthase, and glutathione peroxidase and reductase (Morgan et al., 2002; Park et al., 2003; Lee et al., 2005; Xu et al., 2003) causing changes characteristic of diabetes including altered glucose metabolism, endothelial dysfunction and oxidative stress (Rodriguez-Manas et al., 2003; Elhadd et al., 1999). We anticipated that if methylglyoxal and glyoxal levels were high in T1DM, then RBC membrane Na^+/K^+-ATPase activity would be decreased. However, we found that the activity of this enzyme was significantly higher in DM group patients versus patients in the ND group. Another study in a similar group of complication-free children and adolescents with T1DM showed no difference in Na^+/K^+-ATPase activity in RBC's compared to healthy non-diabetic controls (Deak et al., 2003). This may be due to the shorter duration of diabetes (3-10 years) and better glycemic control of these study subjects as compared to subjects in other reported studies. In a study of streptozotocin-induced diabetic rats, Tsimarato et al. (2001) showed that Na^+/K^+-ATPase activity in medullary thick ascending limb of kidney was increased in rats at 6 weeks, but decreased at 12 weeks, after induction of diabetes, when compared to normal control rats. Collectively these studies suggest that there may be a biphasic time-dependent response in Na^+/K^+-ATPase activity in diabetes.

Diabetes is associated with oxidative stress (Jay et al., 2006), and it has been suggested that low levels of oxidative stress stimulate membrane ion transport, whereas with sustained and increasing levels of oxidative stress, oxidative membrane damage

results in inhibition of these functions (Stark, 2005). A recent study showed that AGEs enhanced sodium channel messenger RNA and sodium uptake in renal cortical collecting duct cells in a time- and dose-dependent manner (Chang et al., 2007). Additionally, hyperglycemia has been shown to increase intracellular sodium concentrations in normal human red blood cells (Barbagallo et al., 1993). These processes lead to increased levels of intracellular sodium that may activate membrane Na^+/K^+-ATPase in a compensatory manner in order to maintain normal intracellular sodium levels. This may explain the elevated levels of membrane Na^+/K^+-ATPase activity demonstrated in the present study. The high correlation of A1C, methylglyoxal and glyoxal with Na^+/K^+-ATPase activity, and the results of the regression analysis indicating that A1C levels are good predictors of enzyme activity, point to a role for glucose, methylgyoxal and glyoxal in the membrane alteration. As hyperglycemia, aldehyde and AGE levels, and/or oxidative stress increase with time, alterations to the lipid environment or to Na^+/K^+-ATPase itself may eventually result in inhibition of the enzyme with a decrease in activity (Djemli-Shipkoye et al., 2001; Baldini et al., 1989).

Although speculative, the foregoing explanation would seem reasonable, and substantiates the concept of a biphasic response. In order to test this concept, we divided data from the DM group into two subgroups based on median duration of diabetes as described in the methods section. There was no difference between the levels of Na^+/K^+-ATPase activity between these two subgroups. Thus, the reason(s) for these divergent reports of enzyme activity remains to be elucidated. Whatever the case, the elevated levels of Na^+/K^+-ATPase activity demonstrated in the present study appear to indicate

that membrane function is altered leading to changes in sodium/potassium handling. Na^+/K^+-ATPase is present in membranes throughout the body, and alteration in the activity of this enzyme lends itself to widespread impact. More direct effects on Na^+/K^+ ATPase activity are also possible due to the wide variety of metabolic abnormalities occuring during DM. As this change is occurring prior to development of complications, levels of Na^+/K^+-ATPase activity may foretell future renal, vascular or neurological complications.

The findings of the present study have implications for clinical management of diabetes. The results of this research strengthen current thinking about the need to structure management to maintain stringent glycemic control, starting at diagnosis of diabetes. In the event that glucose is difficult to control for physiologic or behavioral reasons, supplemental or increased dietary antioxidants may be useful in preventing damage caused by hyperglycemia- or AGE-induced oxidative stress. Also, an agent such as lipoic acid, that has been shown to decrease AGEs in animals (Vasdev et al., 2000; Midaoui et al., 2003), and is already in use in DM in humans for treatment of neuropathy (Foster, 2007), may be considered to aid in the prevention of AGE-induced complications.

We acknowledge the following limitations of this study. Although we measured two of the most likely aldehydes involved in AGEs formation and diabetic complications, other aldehydes produced from glucose catabolism and lipid peroxidation may also be involved in these processes, and may offer valuable information with regards to sub-

clinical alterations. As discussed in Chapter 3, the inability to synthesize large quantities of MG-H1 did not allow us to know with certainty the purity of our standard.

4.3 Conclusion

In young, complication-free patients with T1DM, plasma methylglyoxal and glyoxal levels are elevated, and are strongly associated with elevated hemoglobin A1C, a measure of glycemic control. Free plasma MG-H1 residues are also higher in T1DM patients compared with non-DM controls and correlated with MG levels but not mean glycemia as determined by A1C. These findings underscore the importance of stringent glycemic control in T1DM as methylglyoxal- and glyoxal-derived AGEs are implicated in complications of DM. These young patients with DM also show an increase in the activity of the ubiquitous membrane enzyme, Na^+/K^+-ATPase, indicating early membrane alteration which may also presage future complications of diabetes.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

In this research, we were successful in developing novel LC-MS/MS methods to measure plasma methylglyoxal and glyoxal, and plasma free MG-H1 residues which were used to characterize concentrations of these parameters found in young complication free T1DM patients compared to non-DM controls. RBC membrane Na^+/K^+-ATPase activity was also measured in the complication-free T1DM compared with non-DM controls. The complication-free T1DM patients had higher levels methylglyoxal, glyoxal and free MG-H, as well as erythrocyte membrane Na^+/K^+-ATPase activity. The higher levels of Na^+/K^+-ATPase activity in complication-free T1DM may suggest altered function as a result of hyperglycemia at an early stage in disease. The elevations in methylglyoxal and glyoxal may at least partially be related to glycemia as measured by A1C. Higher levels of free MG-H1 may indicate increased AGE formation at this very early stage and is consistent with a causal role in the pathogenesis of complications. Intensive insulin therapy may significantly reduce free MG-H1 formation. Taken together these results are consistent with a role for methylglyoxal and glyoxal, and methylglyoxal induced AGE formation early on in T1DM and may indicate a role in the pathogenesis of complications. Establishing a stronger link to a causal role in DM complications will require a prospective study. Many of the T1DM subjects studied in this work will eventually develop microvascular complications. It is tempting to speculate that those with highest levels of MG-H1 will be

at greatest risk for developing early complications. A prospective clinical study with measurement of AGE residues like plasma free MG-H1 in complication free T1DM patients and then routine follow-up with examinations for evidence of complications may provide the necessary data to place markers like MG-H1 into routine clinical use as potentially modifiable risk factors for DM complications.

MG-H1 is one of several MG derived AGEs that may exist as a free residue or bound in protein. The amounts of MG-H1 synthesized for these studies were inadequate to provide accurate quantitative information on actual MG-H1 concentrations free in plasma. With synthesis and purification of larger amounts of pure standards of AGE residues like MG-H1, argpyrimidine, CEL or MOLD, or even glyoxal-derived AGE residues, we can begin to explore and quantify other blood-based markers of AGE formation and correlate these with A1C in this patient group. Successful synthesis of relatively large amounts of these AGE residues will be of great benefit to further study on AGEs in animal models as well. Future work in this area may also expand into measurement and evaluation of free AGE residues in urine or protein-bound AGEs in plasma or tissue as potential markers of AGE-induced damage and risk for DM complications.

In this study, the relationship between A1C, methylglyoxal, glyoxal, free MG-H1 with type of insulin treatment in T1DM was also examined. Although it did not achieve statistical significance, preliminary analysis suggested that a relationship may exist between the type of insulin therapy and the level of free plasma AGEs. A study using a larger sample size of patients on conventional therapy is warranted to further examine

this relationship, as the results of this may have clinical significance in choosing the type of insulin therapy.

Although two of the most likely aldehydes involved in AGEs formation and diabetic complications, methylglyoxal and glyoxal, were measured, other aldehydes produced from glucose catabolism and lipid peroxidation may also be involved and may offer valuable information with regards to sub-clinical alterations. The level of other aldehydes may be explored in future study.

CHAPTER 6

REFERENCE

Abordo EA, Minhas HS and Thornalley PJ. Accumulation of a-Oxoaldehydes during Oxidative Stress. A Role in Cytotoxicity. Biochemical Pharmacology. 1999; 58: 641–648.

Ahmed MU, Frye EB, Degenhardt TP, Thorpe SR, and Baynes JW. N^{ε}-(Carboxyethyl)lysine, a product of the chemical modification of porteins by methylglyoxal, increase with age in human lens proteins. Biochem. J. 1997; 324: 565-570.

Ahmed N, Argirov OK, Minhas HS, Cordeiro CA, and Thornalley PJ. Assay of advanced glycation endproducts (AGEs): surveying AGEs by chromatographic assay with derivatization by 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate and application to Nε-epsilon-carboxymethyl-lysine and Nε-epsilon-(1-carboxyethyl)lysine-modified albumin. Biochem J. 2002; 364: 1-14.

Ahmed N, Babaei-Jadidi R, Howell SK, Thornalley PJ and Beisswenger PJ. Glycated and oxidized protein degradation products are indicators of fasting and postprandial hyperglycemia in diabetes. Diabetes Care. 2005; 28: 2465-2471.

Ahmed N, Dobler D, Dean M, and Thornalley PJ. Peptide Mapping Identifies Hotspot Site of Modification in Human Serum Albumin by Methylglyoxal Involved in Ligand Binding and Esterase Activity. The Journal of Biological Chemistry. 2005; 280(7): 5724–5732.

Ahmed N and Thornalley PJ. Advanced glycation endproducts: what is their relevance to diabetic complications? Diabetes, Obesity and Metabolism. 2007; 9: 233-245.

Alexander MC, Lomanto M, Nasrin N, and Ramaika C. Insulin stimulates glyceraldehyde-3-phosphate dehydrogenase gene expression through CIS-acting DNA sequences. Proc Natl Acad Sci. 1988; 85: 5092-6.

Annesley TM. Ion suppression in mass spectrometry. Clin Chem. 2003; 49(7): 1041-4. Review.

Aperia A. New roles for an old enzyme: Na, K-ATPase emerges as an interesting drug target. Journal of Internal Medicine. 2007: 261; 44-52

Babaei-jadidi R, Karachalias N, Ahmed N, Battah S, and Thornalley PJ. Prevention of incipient diabetic nephropathy by high-dose thiamine and benfotiamine. Diabetes. 2003; 52: 2110-20.

Baekkeskov S, Warnock G, Christie M, et al. Revelation of specificity of 64K autoantibodies in IDDM serums by high-resolution 2-D gel electrophoresis. Unambiguous identification of 64K target antigen. Diabetes. 1989; 38: 1133-41.

Baldini P, Incerpi S, Pascale E, Rinaldi C, Verna R and Luly P. Insulin effects on human red blood cells. Molecular and Cellular Endocrinology. 1986; 46: 93-102.

Baldini P, Incerpi S, Lambert-Gardini S, Spinedi A, Luly P. Membrane lipid alterations and Na^+-pumping activity in erythrocytes from IDDM and NIDDM subjects. Diabetes. 1989; 38: 825-831

Barbagallo M, Gupta RK, Resnick LM. Independent effects of hyperinsulinemia and hyperglycemia on intracellular sodium in normal human red cells. AJH. 1993; 6: 264-267.

Barnes PJ, Karin M. Nuclear factor-κB-α pivotal transcription factor in chronic inflammatory diseases. N Engl J Med. 1997; 336: 1066-1071.

Baynes JW. Role of oxidative stress in development of complications in diabetec. Diabetes. 1991; 40: 405–412

Baynes JW. Thorpe SR. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. Diabetes. 1999; 48: 1–9

Beisswenger PJ, Howell SK, O'Dell RM, Wood ME, and Touchette AD, Szwergold BS. Alpha-Dicarbonyls increase in the postprandial period and reflect the degree of hyperglycemia. Diabetes Care. 2001; 24: 726-32.

Beisswenger PJ, Howell SK, Nelson RG, Mauer M, kand Szwergold BS. Alpha-oxoaldehyde metabolism and diabetic complications. Biochem Soc Trans. 2003; 31: 1358-63.

Beisswenger PJ, Howell SK, Smith K, and Szwergold BS. Glyceraldehyde-3-hposphate dehydrogenase activity as an independent modifier of methylglyoxal levels in diabetes. Biochim Biophys acta. 2003; 1637: 98-106.

Bennett ST, Lucassen AM,Gough SC, Powell EE, Undlien DE, Pritchard LE, Merriman ME, Kawaguchi Y, Dronsfield MJ, Pociot F Susceptibility to human type 1 diabetes at

IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. Nat Genet. 1995; 9(3): 284-92.

Berlanga J, Cibrian D, Guillen I et al., Methylglyoxal administration induces diabetes-like microvascular changes and perturbs the healing process of skin wounds. Clin Sci. 2005; 109: 83-95.

Besch W, Blucher H, Bettin D, Wolf E, Michaelis D, Kohnert KD. Erythrocyte sodium-lithium countertransport, adenosine triphosphatase activity and sodium-potassium fluxes in insulin-dependent diabetes. Int J Clin Lab Res. 1995; 25: 104-109

Bierhaus A, Chevion S, Chevion M, Hofmann M, Quehenberger P, Illmer T, Luther T, Wahl P, Tritschler H, Muller M, Ziegler R, Nawroth PP. Advanced glycation endproduct (AGEs) induced activation of NF-κB is suppressed by α-lipoic acid in cultured endothelial cells. Diabetes. 1997; 46: 1481-1490.

Botero D and Wolfsdorf JI. Diabetes Mellitus in Children and Adolescents.(REVIEW) Archives of Medical Research. 2005; 36: 281–290

Bottini N, Musumeci L, Alonso A, et al. A functional variant of lymphoid tyrosine phosphatase is associated with type 1 diabetes. Nat Genet. 2004; 36: 337-8.

Brodsky JL. Characterization of the (Na^+ (+) K^+)-ATPase from 3T3-F442A fibroblasts and adipocytes. Isozymes and insulin sensitivity. J Biol Chem. 1990; 265: 10458-10465,

Brownlee M: Biochemistry and molecular cell biology of diabetes complications. Nature. 2001; 414: 813 –820.

Brusko TM, Wasserfall CH, Clare-Salzler MJ, Schatz DA, Atkinson MA. Functional defects and the influence of age on the frequency of CD4+ CD25+ T-cells in type 1 diabetes. Diabetes. 2005; 54: 1407-14.

Cai H and Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ. Res. 2000; 87: 840-844.

Canadian Diabetes Association 2003 Clinical Practice Guidelines for the Prevention and Management of Diabetes in Canada. 2003; 27: 1-163

Canty TG, Jr., Boyle EM, Jr., Fair A, Morgan EN, Verrier ED, and Pohlman TH. Oxidative stress induces NF-kappaB nuclear translocation without degradation of IkappaBalpha. Circulation. 1999; 100: II361-II364.

Casazza JP, Felve, ME, and Veech RL. The metabolism of acetone in rat. J. Biol. Chem. 1984; 259: 231-236.

Ceriello A, Morocutti A, Mercuri F et al. Defective intracellular antioxidant enzyme production in type 1 diabetic patients with nephropathy. Diabetes. 2000; 49: 2170–2177

Champe PC, Harvey RA, and Ferrier DR. Lippincott's Illustrated Reviews: Biochemistry, 3rd Edition, 2005; 25: 335-346.

Chang T, Wang R, and Wu L. Methylglyoxal-induced nitric oxide and peroxynitrite production in vascular smooth muscle cells. Free Radie. Biol. Med. 2005; 38: 286-293.

Chang CT, WU MS, Tian YC, Chen KH, Yu CC Liao CH, Hung CC, Yang CW. Enhancement of epithelial sodium channel expression in renal cortical collecting duct cells by advanced glycation end products. Nephrol Dian Transplant. 2007; 22: 722-731

Chaplen FWR, E. Fahl WE, and Cameron DC. Method for Determination of Free Intracellular and Extracellular Methylglyoxal in Animal Cells Grown in Culture. Analytical Biochemistry. 1996; 238: 171-178.

Che W, Asahi M, Takahashi M, Kaneto H, Okado A, Higashiyama S, and Taniguchi N. Selective induction of heparin-binding epidermal growth factor-like growth factor by methylglyoxal and 3-deoxyglucosone in rat aortic smooth muscle cells. The involvement of reactive oxygen species formation and a possible implication for atherogenesis in diabetes. J. Biol. Chem. 1997; 272: 18453-18459.

Cooke CL, and Davidge ST. Peroxynitrite increases iNOS through NF-kappaB and decreases prostacyclin synthase in endothelial cells. Am. J. Physiol. Cell Physiol. 2002; 282: C395-C402.

Cordeiro C and Freire AP. Methylglyoxal Assay in Cells as 2-Methylquinoxaline Using 1,2-Diaminobenzene as Derivatizing Reagent. Analytical Biochemistry. 1996; 234: 221–224.

Cotham WE, Metz TO, Ferguson PL, Brock JW. Hinton DJ, Thorpe SR, Baynes JW, Ames JM. Proteomic analysis of arginine adducts on glyoxal-modified ribonuclease. Mol Cell Proteomics. 2004; 3: 1145-1153.

Cudworth AG, Woodrow JC, HLA system and diabetes mellitus, Diabetes. 1975; 24: 345-9.

Curtin JF, Donovan M and Cotter TG. Regulation and measurement of oxidative stress in apoptosis. J. Immunol. Methods. 2002; 265: 49-72.

Dawson KG, Gomes D, Gerstein H, Blanchard JF, Kahler KH. The economic costs of diabetes in Canada, 1998. Diabetes Care. 2002; 25 (8): 1303-7.

Deak B, Dobos M, Kocsis I, Krikovszky D, Tordai A, Madacsy L, Tulassay T, Vasarhelyi B. HbA_{1c} levels and erythrocyte transport functions in complication-free type 1 diabetic children and adolescents. Acta Diabetol. 2003; 40: 9-13

Degenhardt TP, Alderson NL, Arrington D, Beattie RJ, Basgen JM, Steffes MW, et al. Pyridoxamine inhibits early renal disease and dyslipidemia in strptozotocin-diabetic rat. Kidney Int. 2002; 61: 939-950.

Derham BK, Ellory JC, Bron AJ and Harding JJ. The molecular chaperone a-crystallin incorporated into red cell ghosts protects membrane Na/K-ATPase against glycation and oxidative stress. Eur. J. Biochem. 2003; 270, 2605–2611.

Devary Y, Gottlieb RA, Smeal T, and Karin M. The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell, 1992; 71: 1081-1091.

Djemli-Shipkolye A, Coste T, Raccah D, Vague P, Pieroni G, Gerbi A. Na,K-ATPase alterations in diabetic rats: Relationship with lipid metabolism and nerve physiological parameters. Cell Mol Biol. 2001; 47: 297-304

Du J, Cai S, Suzuki H, Akhand AA, Ma X, Takagi Y, et al. Involvement of MEKKl/ERK/P21Wafl/Cipl signal transduction pathway in inhibition of IGF-I-mediated cell growth response by methylglyoxal. J. Cell. Biochem. 2003; 88: 1235-1246.

Ekblom J. Potential therapeutic value of drugs inhibiting semicarbazide-sensitive amine oxidase: vascular cytoprotection in diabetes mellitus. Pharmacol. Res. 1998; 37: 87-92.

Elhadd TA, Kennedy G, Hill A, McLaren M, Newton RW, Greene SA, Belch JJF. Abnormal markers of endothelial cell activation and oxidative stress in children, adolescents and young adults with Type 1 diabetes with no clinical vascular disease. Diabetes Metab Res Rev. 1999; 15: 405-411

Engerman RL, Kern TS and Larson ME. Nerve conduction and aldose reductase inhibition during 5 years of diabetes or galactosaemia in dogs, Diabetologia. 1994; 37: 141–144.

Englegau MM, Thompson TJ, Herman WH, Boyle JP, Aubert RE, Kenny SJ, Badran A, Sous ES and Ali MA. Comparison of fasting and 2-hour glucose and HbA1c levels for diagnosing diabetes. Diagnostic criteria and performance revisited. Diabetes Care. 1997; 20(5): 785-9.

Espinosa-Mansilla A, Duran-Meras I, and Salinas F. High-Performance Liquid Chromatographic–Fluorometric Determination of Glyoxal, Methylglyoxal, and Diacetyl in Urine by Prederivatization to Pteridinic Rings. Analytical Biochemistry. 1998; 255: 263–273.

EURODIAB ACE Study Group. Variation and trends in incidence of childhood diabetes in Europe. Lancet. 2000; 355: 873-6.

Ewart HS, Klip A. Hormonal regulation of the Na^+-K^+-ATPase:Mechanisms underlying rapid and sustained changes in pump activity. Am J phsiol. 1995; 269: C295-C311.

Feng L, Matsumoto C, Schwartz A, Schmidt AM, Stern DM. Pile-Spellman J Chromic vascular inflammation in patients with type 2 diabetes: endothelial biopsy and RT-PCR analysis. Diabetes Care. 2005; 28: 379-384.

Feraille E, Carranza ML, Rousselot M, and Favre H. Insulin enhances sodium sensitivity of Na-K-ATPase in isolated rat proximal convoluted tubule. Am. J. Physiol. 1994; 267: F55-F62.

Finotti P, Palatini P. Reduction of erythrocyte (Na^+-K^+)ATPase activity in Type 1 (insulin-dependent) diabetic subjects and its activation by homologous plasma. Diabetologia. 1986; 29:623-628.

Forbes JM, Yee LT, Thallas V, Lassila M, Candido R, Jandeleit-Dahm KA, Thomas MC, Burns WC, Deemer EK, Thorpe SM, Cooper ME, and Allen TJ. Advanced glycation end product interventions reduce diabetes-accelerated atherosclerosis. Diabetes. 2004; 53: 1813-23.

Forbes JM, Soldatos G, and Thomas MC. Below the Radar: Advanced Glycation End Products that Detour "around the side" Is HbA1c not an accurate enough predictor of long term progression and glycaemic control in diabetes?
Clin Biochem Rev. 2005; 26: I 123-134

Fosmark DS, Torjesen PA, Kilhovd BK, Berg TJ, Sandvik L, Hanssen KF, Agardh C and Agardh E. Increased serum levels of the specific advanced glycation end product methylglyoxal-derived hydroimidazolone are associated with retinopathy in patients with type 2 diabetes mellitus. Metabolism Clinical and Experimental. 2006; 55: 232-236.

Foster TS. Efficacy and safety of α-lipoic acid supplementation in the treatment of symptomatic diabetic neuropathy. Diabetes Edu. 2007; 33: 111-117.

Foulis AK, McGill M, Farquharson MA. Insulitis in type I (insulin-dependent) diabetes mellitus in man – macrophages, lymphocytes, and interferon-gamma containing cells. J Pathol. 1991; 165: 97-103.

Frye EB, Degenhardt TP, and Thorpe SR. Role of maillard reaction in aging of tissue proteins. J Biol Chem 1998; 273: 18714-18719.

Ganong W.F. Review of Medical Physiology. 2003; 21th edition. 357-358.

Garber AJ. Cardiovascular complications of diabetes: prevention and management. Clin Cornerstone. 2003; 5(2): 22-37.

Garner MH, Spector A. ATP hydrolysis kinetics of Na,K-ATPase in cataract. Exp Eye Res. 1986; 42(4): 339-48.

Geiss LS, Herman WH, Smith PJ, National Diabetes Data Group. Diabetes in America. Bethesda, Md: National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases. 1995: 233–257.

Giannattasio C, Failla M, Piperno A, Grappiolo A, Gamba P, Paleari F, Mancia G. Early impairment of large artery structure and function in type I diabetes mellitus.Diabetologia. 1999; 42(8): 987-94

Giardino I, Edelstein D, and Brownlee M. Nonenzymatic Glycosylation In Vitro and in Bovine Endothelial Cells Alters Basic Fibroblast Growth Factor Activity, A Model for Intracellular Glycosylation in Diabetes. J. Clin. Invest. 1994; 94: 110-117.

Gillespie K.M. Type 1 diabetes: pathogenesis and prevention. CMAJ 2006; 175(2): 165-170.

Ginsberg-Fellner F, Witt ME, Gedum B, et al. Diabetes mellitus and qutoimmunity in patients with the congenital rubella syndrme. Rev infect Dis. 1985; 7(Suppl 1): S170-6.

Giugliano D, Ceriello A and Paolisso G. Oxidative stress and diabetic vascular complications. Diabetes Care. 1996;19: 257–267.

Glomb MA and Monnier VM. Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. J. Biol. Chem. 1995; 276: 10017-10025.

Godbout JP, Pesavento J, Hartman ME, Manson SR, and Freund GG. Methylglyoxal enhances cisplatin-induced cytotoxicity by activating protein kinase Cδ. The journal of biological chemistry. 2002; 277(4): 2554-2561.

Goldin A, Beckman JA, Schmidt AM, creager MA. Advanced Glycation End Products Sparking the Development of Diabetic Vascular Injury. Circulation. 2006;114:597-605.

Graier WF, Grubenthal I, Dittrich P, Wascher TC, Kostner GM: Intracellular mechanism of high D-glucose-induced modulation of vascular cell proliferation. Eur J Pharmacol. 1995; 294: 221 –229.

Groop P-H, Forsblom C. and Thomas M.C. Mechanisms of Disease: pathway-selective insulin resistance and microvascular complications of diabetes. Nature Clinical Practice Endocrinol Metab. 2005; 1(2): 100-110.

Grosskopf I, Baroukh N, Lee SJ, Kamari Y, Harats D, Rubin EM, Pennacchio LA, and Cooper AD. Apolipoprotein A-V deficiency results in marked hypertriglyceridemia attributable to decreased lipolysis of triglyceride-rich lipoproteins and removal of their remnants. Arterioscler Thromb Vasc Biol. 2005; 25(12): 2573-9.

Habermann T.M. Mayo Clinic Internal Medicine Review 2006-2007. 2006; 7th edition: 228-235.

Hammes HP, Martin S, Federlin K, Geisen K and Brownlee M. Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. Proc. Natl. Acad. Sci. U.S.A. 1991; 88: 11555–11558.

Hammes HP, Du X, Edelstein D, Tajuchi T, Matsumura T, Ju Q, Lin J, Bierhaus A, Nawroth P, Hannak D, Neumaier M, Bergfeid R, Giardino I, and BrownLee M. Benfoltiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retiopathy. Nature Medicine. 2003; 9(3): 294-299.

Hirata K, Kubo K: Relationship between blood levels of N-carboxymethyl-lysine and pentosidine and the severity of microangiopathy in Type 2 diabetes. Endocrine J. 2004; 51: 537-544.

Honeyman MC, Coulson BS, Stone NL, et al. Association between rotavirus infection and pancreatic islet autoimmunity in children at risk of developing type 1 diabetes. Diabetes. 2000; 49: 1319-24.

Horiuchi S, Sakamoto Y, Sakai M: Scavenger receptors for oxidized and glycated proteins. Amino Acids. 2003; 25: 283-292.

Hwang JS, Shin CH, Yang SW. Clinical implications of N epsilon-(carboxymethyl)lysine, advanced glycation end product, in children and adolescents with type 1 diabetes. Diabetes Obes Metab. 2005; 7(3): 263-7.

Hyoty H. Enterovirus infections and type 1 diabetes. Ann Med. 2002; 34: 138-47.

Idris I and Donnelly R. Protein kinase C beta inhibition: A novel therapeutic strategy for diabetic microangiopathy. Diab Vasc Dis Res. 2006; 3(3):172-8

Itani SI et al. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol protein kinase C, and IκB-α. Diabetes. 2002; 51: 2005-2011.

Jannot MF, Raccah D, Dufayet de la Tour D, Coste T, Gouvernet J, and Vague P. Relationship between neuropathy, hypertension and red blood cell Na/K ATPase in patients with insulin-dependent diabetes mellitus. Diabetes. 1999; 25(1): 35-42.

Jannot MF, Raccah D, Dufayet de la Tour D, Coste T, Vague P. Genetic and environment regulation of NA/K adenosine triphosphatase activity in diabetic patients. Metabolism. 2002; 51: 284-291.

Jay D, Hitomi H, Griendling KK : Oxidative stress and diabetic cardiovascular complications. 2006; 41: 183-192.

Jensen LJN, Ostergaard J, Flyvbjerg A; AGE-RAGE and AGE cross-link interaction: Important players in the pathogenesis of diabetic kidney disease. Horm Metab Res. 2005; 37: S26-S34.

Jia X, Olson DJH, Ross ARS, Wu L. Structural and functional changes in human insulin induced by methylglyoxal. FASEB J. 2006; 20: E871-E879

Jorgensen PL, H°akansson KO, and Karlish SJD. Structure andmechanism of Na,K-ATPase.Functional Sites and Their Interactions. Annu. Rev. Physiol. 2003; 65: 817–49

Kaplan JH. Biochemistry of Na, K-ATPase. Annu. Rew. Biochem. 2002. 71: 511-35.

Karachalias N, Babaei-Jadidi R, Ahmed N, and Thornalley PJ. Accumulation of fructosyl-lysine and advanced glycation end products in the kidney, retina and peripheral nerve of streptozotocin-induced diabetic rats. Biochem Soc Trans. 2003; 31: 1423-5.

Kashiwagi A, Obata T, Suzaki M, Takagi Y, Kida Y, Ogawa T, *et al.* Increase in cardiac muscle fructose content in streptozotocin-induced diabetic rats. Metabolism 1992; 41: 1041-1046.

Kikuchi Y, Kobayashi S, Hemmi N et al. Galectin-3-positive cell infiltration in human diabetic nephropathy. Nephrol Dial Transplant. 2004; 19: 602-607.

Kilhovd BK, Giardino I, Torjesen PA, Birkeland KI, Berg TJ, Thornalley PJ, Brownlee M, and Hanssen KF. Increased serum levels of the specific AGE-compound methylglyoxal-derived hydroimidazolone in patients with type 2 diabetes. Metabolism. 2003; 52: 163-167.

Knip M. Environmental triggers and determinants of beta-cell autoimmunity and type 1 diabetes. Rev Endocr Metab Disord. 2003; 4: 213-23.

Koc B, Erten V, Yilmaz MI, Sonmez A, Kocar IH. The relationship between red blood cell NA/K-ATPase activities and diabetic complications in patients with type 2 diabetes mellitus. Endocrine. 2003; 21: 273-278

Koschinsky T, He CJ, Mitsuhashi T, Bucala R, Liu C, Buenting C, Heitmann K, Vlassara H (1997). Orally absorbed reactive glycation products (glycotoxins): an environmental risk factor in diabetic nephropathy. Proceedings of the national academy of science (USA) 1997; 94 (12): 6474-6479.

Koya D and King GL. Protein kinase C activation and the development of diabetic complications, Diabetes.1998; 47: 859–866.

Lander HM, Taurus JM, Ogiste JS, Hori O, Moss RA, Schmidt AM. Activation of the receptor for advanced glycation end products triggers a p21 (ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. J Biol Chem. 1997; 272: 17810-17814.

Lan MS, Wasserfall C, Maclaren NK et al. IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. Proc Natl Acad Sci U S A. 1996; 93(13): 6367-70.

Lapolla A, Flamini R, Vedova AD, Senesi A, Reitano R, Fedele D, Basso E, Seraglia R, Traldi P: Glyoxal and methylglyoxal levels in diabetic patients: Quantitive determination by a new GC/MS method. Clin Chem Lab Med. 2003; 41: 1166-1173.

Lederer MO, Klaiber RG. Cross-linking of proteins by Maillard processes: Characterization and detection of lysine-arginine cross-links derived from glyoxal and N^{α}-hippuryllysine. Bioorg Med Chem. 1999; 7: 2499-2507.

Lee HJ, Howell SK, Sanford RJ, Beisswenger PJ. Methylglyoxal can modify GAPDH activity and structure. Ann NY Acad Sci. 2005; 1043: 135-145.

Leoncini. G., and Poggi, M. Effects of methylglyoxal on platelet hydrogen peroxide accumulation, aggregation and release reaction. Cell Biochem. Funct. 1996; 14: 89-95.

Levenson R. Isoforms of the Na,K-ATPase: Family members in search of function. Rev physiol Biochem Pharma. 1994; 123: 1-45.

Li J, Schmidt AM. Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. J Biol Chem.1997; 272(1): 6498-6506.

Lieuw-A-Fa ML, Van Hinsbergh VW, Teerlink T, Barto R, Twisk J, Stehouwer CD, and Schalkwijk CG. Increased levels of N(epsilon)-(carboxymethyl)lysine and N(epsilon)-(carboxyethyl)lysine in type 1 diabetic patients with impaired renal function: correlation with markers of endothelial dysfunction. Nephrol Dial Transplant. 2004; 19: 631-6.

Lyles GA, and Chalmers J. 1992. The metabolism of aminoacetone to methylglyoxal by semicarbazide-sensitive amine oxidase in human umbilical artery. Biochem. Pharmacol. 1992; 43: 1409-1414.

McCulloch D.K. Patient information: Insulin treatment in type 1 diabetes. UpToDate. 2007. http://patients.uptodate.com

McGill HC Jr, McMahan CA. Determinants of atherosclerosis in the young: Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group. Am J Cardiol. 1998; 82: 30T-36T.

McLellan AC, Phillips SA and Thornalley PJ. The assay of methylglyoxal in biological systems byderivatization with 1,2-diamino-4,5-dimethoxybenzene. Analytical Biochemistry. 1992; 206(1): 17-23

McLellan AC, Thornalley PJ, Benn J, and Sonksen PH. Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. Clin. Sci. (Lond.), 1994; 87: 21-29.

Midaoui AEL, Elimadi A, Wu L, Haddad PS, de Champlain J. Lipoic acid prevents hypertension, hyperglycemia and the increase in heart mitochondrial superoxide production. Am J Hypertens 2003; 16: 173-9.

Milstien S, Katusic Z. Oxidation of tetrahydrobiopterin by peroxynitrite. Biochem Biophys Res Commun.1999; 263: 681-684.

Mimura M, Makino H, Kanatsuka A, Asai T, Yoshida S. Reduction of erythrocyte (Na(+)-K)ATPase activity in type 2 (non-insulin-dependent) diabetic patients with microalbuminuria. Horm Met Res. 1994; 26: 33-38

Misselwitz J, Franke S, Kauf E, John U, Stein G : Advanced glycation end products in children with chronic renal failure and type 1 diabetes. Pediatr Nephrol. 2002; 17: 316-321.

Moncada S, Palmer RM, and Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol. Rev. 1991; 43: 109-142.

Morgan PE, Dean RT, Davies MJ. Inactivation of cellular enzymes by carbonyls and protein-bound glycation/glycoxidation products. Arch Biochem Biophys. 2002; 403: 259-269.

Mostafa AA, Randell EW, Vasdev SC, Gill VD, Han Y, Gadag V, Raouf AA, El Said H (2007) Plasma protein advanced glycation end products, carboxymethyl cysteine and carboxyethyl cysteine, are elevated and related to nephropathy in patients with diabetes. Mol Cell Biochem. 2007; DOI 10.1007/s11010-007-9422-9.

Myers AR. Disorders of Glucose Homeostasis.Medicine (Lippincott Williams & Wilkins). 2005; 5th edition: 514-532.

Nagai R, Fujiwara Y, Mera K, and Otagiri M. Investigation of pathways of advanced glycation end-products accumulation in macrophages. Mol. Nutr. Food Res. 2007; 51: 462 – 467.

Nagaraja RH, Shipanova IN and Faust FM. Protein cross-linking by the Maillard reaction. Isolation, characterization, and in vivo detection of a lysine-lysine cross-link derived from methylglyoxal. J. Biol. Chem. 1996; 271: 19338-19345.

Naka Y, Bucciarelli LG, Wendt T, Lee LK, Rong LL, Ramasamy R, Yan SF, and Schmidt AM. RAGE axis: Animal models and novel insights into the vascular complications of diabetes. Arterioscler Thromb Vasc Biol. 2004; 24: 1342-9.

Nemet I, Varga-Defterdarovic L, and Turkb Z. Preparation and quantification of methylglyoxal in human plasma using reverse-phase high-performance liquid chromatography. Clinical Biochemistry. 2004; 37: 875– 881.

Nemet I, Turk Z, Duvenjak L, Car N, and Varga-Defterdarovic L. Humoral methylglyoxal level reflects glycemic fluctuatioln. Clinical biochemistry. 2005; 38: 379-383.

Newhook LA, Curtis J, Hagerty D, Grant M, Paterson AD, Crummel C, Bridger T, Parfrey P. High incidence of childhood type 1 diabetes in the Avalon Peninsula, Newfoundland, Canada. Diabetes Care. 2004; 27(4): 885-8.

Oberley LW. Free radicals and diabetes. Free Radic Biol Med. 1988; 5:113–124.

O'Brien PJ and Shangari N. The cytotoxic mechanism of glyoxal invoiles oxidative stress. Biochemical Pharmacology. 2004; 68: 1433-1442.

O'Brien PJ, Siraki AG, Shangari N: Aldehyde sources, metabolism, molecular toxicity mechanisms, and possible effects on human health. Critical Rev Toxicol. 2005; 35: 609-662.

Odani H, Shinzato T, Matsumoto Y, Usami J, Maeda K. Increase in three α_1 β-dicarbonyl compound levels un human uremic plasma : Specific *in vivo* determination on intermediates in advanced maillard reaction. Biochem Biophys Res Comm. 1999; 256: 89-93.

Ogata. N, Yamamoto H, Kugiyama K, Yasue H, and Miyamoto E. Involvement of protein kinase C in superoxide anion-induced activation of nuclear factor-kappa B in human endothelial cells. Cardiovasc. Res. 2000; 45: 513-521.

Ohmori S, Mori M, Kawase M, Tsuboi S. Determination of methylglyoxal as 2-methylquinoxaline by high-performance liquid chromatography and its application to biological samples. J Chromatogr. 1987; 414(1): 149-55.

Oya-Ito T, Liu BF, and Nagaraj RH. Effect of Methylglyoxal Modification and Phosphorylation on the Chaperone and Anti-Apoptotic Properties of Heat Shock Protein 27. Journal of Cellular Biochemistry. 2006; 99: 279–291.

Park YS, Koh YH, Takahashi M, Miyamoto Y, Suzuki K, Dohmae N, Takio K, Honke K, Taniguchi N. Identification of the binding site of methylglyoxal on glutathione peroxidase: Methylglyoxal inhibits glutathione peroxidase activity via binding to glutathione binding sites Arg 184 and 185. Free Rad Res. 2003; 37: 205-211.

Pepper G. Diabetes and Endocrine. Health Centre On Line. 2006

Petrovic R, Futas J, Chandoga J, Jakus V. Rapid and simple method for determination of Nepsilon-(carboxymethyl)lysine and Nepsilon-(carboxyethyl)lysine in urine using gas chromatography/mass spectrometry. Biomed Chromatogr. 2005; 19(9): 649-54.

Phillips SA, Mirlees D, and Thornalley PJ. Modification of the glyoxalase system in streptozotocin-induced diabetic rats. Effect of the aldose reductase inhibitor statil. Biochem Pharmacol 1993; 46: 805-811.

Phillips SA, Thornalley PJ. The formation of methylgyoxal from triose phosphates. Investigation using a specific assay for methylglyoxal. Eur J Biochem. 1993; 212: 101-105

Raccah D, Fabreguettes C, Azulay JP, Vague P. Erythrocyte NA^+-K^+-ATPase activity, metabolic control, and neuropathy in IDDM patients. Diabetes Care. 1996; 19:564-568

Ramasamy R, Yan SY, and Schmidt AM. Methylglyoxal Comes of AGE. Cell. 2006; 124: 258-260.

Randell EW, Vasdev S, Gill V. Measurement of methylglyoxal in rat tissues by electrospray ionization mass spectrometry and liquid chromatography. J Pharmacol Toxicol Methods. 2005; 51(2): 153-7

Ray, S. & Ray, M. J. Biol. Chem. 1983; 258: 3461-3462

Ray, M. & Ray, S. J. Biol. Chem. 1987; 262: 5974-5977

Reber F, Kasper M, Siegner A, Kniep E, Seigel G, Funk PH. Alteration of the intracellular pH and apoptosis induction in a retinal cell line by the AGE-inducing agent glyoxal. Graefes Arch Clin Exp Ophthalmol. 2002; 240: 1022-32.

Regan TJ. Congestive heart failure in the diabetic. Annu Rev Med. 1983; 34: 161–168.

Reusch JEB. Diabetes, microvascular complications, and cardiovascular complications: what is it about glucose? J. Clin. Invest. 2003; 112: 986–988.

Risch N. Assessing the Role of HLA-linked and Unlinked Determinants of Disease. Am. J. Hum. Genet. 1987; 40: 1-14.

Ritthaler U, Deng Y, Zhang Y, Greten J, Abel M, Sido B, Allenberg J, Otto G, Roth H, Bierhaus A. Expression of receptors for advanced glycation end products in peripheral occlusive vascular disease. Am J Pathol. 1995; 146: 688-694.

Rodriguez-Manas L, Angulo J, Vallejo S, Peiro C, Sanchez-Ferrer A, Cercas E, Lopez-Doriga P, Sanchez-Ferrer CF. Early and intermediate Amadori glycosylation adducts, oxidative stress, and endothelial dysfunction in the streptozotocin-induced diabetic rats vasculature. Diabetologia 2003; 46: 556-566

Roep BO. The role of T-cells in the pathogenesis of Type 1 diabetes: From cause to cure. (Review) Diabetologia. 2003; 46: 305–321

Rolo AP and Palmeira CM. Diabetes and mitochondrial function: Role of hyperglycemia and oxidative stress. Toxicology and Applied Pharmacology. 2006; 212(2): 167-178.

Rubler S, Dlugash J, Yuceoglu YZ, Kumral T, Branwood AW, Grishman A. New type of cardiomyopathy associated with diabetic glomerulosclerosis. Am J Cardiol. 1972; 30: 595–602.

Sakamoto H, Mashima T, Yamamoto K, and TsuruoT. Modulation of Heat-shock Protein 27 (Hsp27) Anti-apoptotic Activity by Methylglyoxal Modification. The hournal of Biological Chemistry. 2002; 277(48): 45770–45775.

Schram MT, Schalkwijk CG, Bootsma AH, Fuller JH, Chaturvedi N, Stehouwer CDA. Advanced Glycation End Products Are Associated With Pulse Pressure in Type 1 Diabetes: The EURODIAB Prospective Complications Study. 2005; 46; 232-237.

Scivittaro V, Ganz MB, Weiss MF. AGEs induce oxidative stress and activate protein kinase C-beta(II) in neonatal mesangial cells. Am J Physiol Renal Physiol. 2000; 278: 676-683.

Shangari N, Bruce WR, Poon R, O'Brien PJ. Toxicity of glyoxals—Role of oxidative stress, metabolic detoxification and thiamine deficiency. Biochem Soc Trans. 2003; 31: 1390-1393.

Shangari N, O'Brien PJ. The cytotoxicity mechanism of glyoxal involves oxidative stress. Biochem Pharmacol. 2004; 668: 1433-1442.

Sherwood L. Human physiology from cells to systems. 2001.

Shipanova IN, Glomb MA, and Nagaraj RH. Protein modifiction by methylglyoxal: chemical nature and synthetic mechanism of a major fluorescent adduct. Arch. Biochem. Biophys. 1997; 344: 29-36.

Smyth D, Cooper JD, Collins JE, et al. Replication of an association between the lymphoid tyrosine phosphatease locus (LYP/PTPN22) with type 1 diabetes, and evidence for its role as a general autoimmunity locus. Diabetes. 2004; 53: 3020-3.

Snieder H, Sawtell PA, Ross L, Walker J, Spector TD, and Leslie RDG. HbA1c Levels Are Genetically Determined Even in Type 1 Diabetes, Evidence From Healthy and Diabetic Twins. Diabetes. 2001; 50: 2858-2863.

Soulis-Liparota T, Cooper M, Papazoglou D, Clarke B, and Jerums G. Retardation by aminoguanidine of development of albuminuria, mesangial expansion, and tissue fluorescence in streptozocin-induced diabetic rat. Diabetes. 1991; 40: 1328–1334.

Srivastava SK, Ramana KV, and Bhatnagar A. Role of aldose reductase and oxidative damage in diabetes and the consequent potential for therapeatic options. Endocrine Reviews. 2005; 26(3): 380-392.

Stark G. Functional consequences of oxidative membrane damage, J Membrane Biol 2005; 205: 1-16

Stehouwer CDA, Gall MA, Twisk JWR, Knudsen E, Emeis JJ, Parving H-H. Increased urinary albumin excretion, endothelial dysfunction, and chronic low-grade inflammation in type 2 diabetes. Diabetes. 2002; 51: 1157-1165.

Stitt A, Gardiner TA, Anderson NL, Canning P, Frizzell N, Duffy N, et al. The AGE inhibitor pyridoxamine inhibits development of retinopathy in experimental diabetes. Diabetes. 2002; 51: 2826-2832.

Suhail M, and Rizvi SI. Red cell membrane (N^++K^+)-ATPase in diabetes mellitus. Biochemical and Biophysical research communications. 1987; 146 (1): 179-186.

Susic D, Varagic J, Ahn J, and Frohlich ED. Crosslink breakers: a new approach to cardiovascular therapy. Curr Opin Cardiol. 2004; 19: 336-40.

Sweeney G and Klip A. Regulation of the Na^+/K^+-ATPase by insulin: Why and how? Molecular and Cellular Biochemistry. 1998; 182: 121-133.

Takahashi E, and Berk BC. MAP kinases and vascular smooth muscle function. Acta Physiol. Scand. 1998; 164: 611-621.

Talreja DR, Talreja RR, Talreja RS. Diabetes Mellitus. Internal Medicine (Lippincott William & Wilkins). 2005; 24: 159-181.

Tanji N, Markowitz GS, Fu C, Kislinger T, Taguchi A, Pischetsrieder M, Stern D, Schmidt AM, D'Agati VD: Expression of advanced glycation end products and their cellular receptor RAGE in diabetic nephropathy and nondiabetic renal disease. J Am Soc Nephrol. 2000; 11: 1656-1666.

Teerlink T, Barto R, Ten Brink HJ, and Schalkwijk CG. Measurement of N-epsilon-(carboxymethyl)lysine and N-epsilon-(carboxyethyl)lysine in human plasma protein by stable-isotope-dilution tandem mass spectrometry. Clin Chem. 2004; 50: 1222-8.

Thornalley PJ. Modification of the glyoxalase system in human red blood cells by glucose in vitro. Biochem. J. 1988; 254: 751-755.

Thornalley PJ, Hooper NI, Jennings PE, Florkowski CM, Jones AF, Lunec J, Barnett AH. The human red blood cell glyoxalase system in diabetes mellitus. Diabetes Res Clin Pract. 1989; 7(2): 115-20.

Thornalley PJ. The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. Biochem. J. 1990; 269: 1-11.

Thornalley PJ. The glyoxylase system in health and disease. Molec. Aspects. Med. 1993; 14: 287-371

Thornalley PJ. Modification of the glyoxalase system in disease processes and prospects for therapeutic strategies. Biochem Soc trans. 1993; 21; 531-534.

Thornalley PJ. Pharmacology of Methylglyoxal: Formation, Modification of Proteins and Nucleic Acids, and Enzymatic Detoxification-A Role in Pathogenesis and ntiproliferative Chemotherapy. Gen. Pharmac. 1996; 27(4): 565-573.

Thornalley PJ. Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs. Cell Mol Biol. 1998; 44: 1013-1023.

Thornalley PJ. Glutathione-dependent detoxification of α-oxoaldehydes by the glyoxalase system: involvement in disease mechanisms and antiproliferative activity of glyoxalase I inhibitors. Chemico-Biological interactions. 1998; 111-112: 137-151.

Thornalley PJ, Langborg AM, Minhas HS. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. Biochem J. 1999; 344: 109-116.

Thornalley PJ. Glycation in diabetic neuropathy: characteristics, consequences, causes, and therapeutic options. Int. Rev. Neurobiol. 2002; 50: 37-57.

Thornalley PJ, Battah S, Ahmed N, Karachalias N, Agalou S, Babaei-Jadidi R, Dawnay A: Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. Biochem J. 2003; 375: 581-592.

Thornalley PJ. Dicarbonyl intermediates in the maillard reaction. Ann NY Acad Sci. 2005; 1043: 111-117.

Todd JA. Genetic analysis of type 1 diabetes using whole genome approaches. Proc Natl Acad Sci U S A. 1995; 92: 8560-6.

Tressel T, Thompson R, Zieske LR, Menendez MI, and Davis L. Interaction between L-threonine dehydrogenase and aminoacetone synthetase and mechanism of aminoacetone production. J. Biol. Chem. 1986; 261: 16428-16437.

Trick GL, Edwards P, Desai U, Berkowitz BA. Early supernormal retinal oxygenation response in patients with diabetes. Invest Ophthalmol Vis Sci. 2006; 47(4): 1612-9.

Tsimaratos M, Coste TC, Djemli-Shipkolye A, Daniel L, Shipkolye F, Vague P, Raccah D. Evidence of time-dependent changes in renal medullary Na,K-ATPase activity and expression in diabetic rats. Cell Mol Biol. 2001; 47: 239-245.

Tsukahara H, Sekine K, Uchiyama M, Kawakami H, Hata I, Todoroki Y, Hiraoka M, Kaji M, Yorifuji T, Momoi T, Yoshihara K, Beppu M, Mayumi M: Formation of advanced glycosylation end products and oxidative stress in young patients with type 1 diabetes. Pediatr Res. 2003; 54: 419-424.

Valeri C, Pozzilli P, and Leslie D. Glucose control in diabetes. Diabetes Metab Res Rev. 2004; 20: S1-8.

Vander Jagt DL, and Hunsaker LA. Enzymology and Molecular Biology of Carbonyl Metabolism 4, H. Weiner (ed.), Plenum Press, New York, NY. USA 1993; pp.279-288.

Vasarhelyi B, Szabo T, Ver A, Tulassay T. Measurement of Na^+/K^+-ATPase activity with an automated analyzer. Clin Chem. 1997; 43: 1986-1987.

Vasdev S, Mian T, Ford CA, Longerich L, Parai S. Role of endogenous aldehydes in spontaneously hypertensive and disulfiram-induced hypertensive rats. Nutr Metab Cardiovasc Dis. 1996; 6: 130-140.

Vasdev S, Ford CA, Longerich L, Parai S, Gadag V, Wadhawan S. Aldehyde induced hypertension in rats: prevention by N-acetyl cysteine. Artery. 1998; 23(1): 10-36. Links

Vasdev S, Ford CA, Longerich L, Gadag V, and Wadhawan S. Role of aldehydes in fructose induced hypertension. MoI. Cell. Biochem. 1998; 181: 1-9.

Vasdev S, Ford CA, Parai S, Longerich L, Gadag V. Dietary alpha-lipoic acid supplementation lowers blood pressure in spontaneously hypertensive rats. J Hypertens 2000B; 18: 567-573.

Vasdev S, Longerich L, and Gill V. Prevention of fructose-induced hypertension by dietary vitamins. (Review) Clinical Biochemistry. 2004; 37: 1 – 9.

Verzijl N, DeGroot J, Thorpe SR et al. Effect of collagen turnover on the accumulation of advanced glycation endporducts. J Biol Chem. 2000; 275: 39027-39031.

Vlassara H, Fuh H, Makita Z, Krungkrai S, Cerami A, Bucala R. Exogenous advanced glycosylation end products induce complex vascular dysfunction in normal animals: a model for diabetic and aging complication. Pro Natl Acad Sci USA. 1992; 89: 12043-12047.

Vlassara H, Fuh H, Donnelly T, Cybulsky M. Acancedglycation endporducts promote adhesion molecule (VCAM-1, ICAM-1) expression and atheroma formation in normal rabbits. Mol Med. 1995; 1: 447-456.

Vlassara H, Li YM, Imani F et al. Identification of galectin-3 as a high-affinity binding protein for advanced glycation end products (AGE): a new member of the AGE-receptor family. Mol Med. 1995; 1: 634-646.

Vlassara H. and Palace MR.. Diabetes and advanced glycation endproducts. Med. 2002; 251: 87-101.

Wang H, Meng QH, Chang T and Wu L. Fructose-induced peroxynitrite production is mediated by methylglyoxal in vascular smooth muscle cells. Life Sciences. 2006; 79: 2448–2454.

Ward RA, and McLeish KR. Methylglyoxal: a stimulus to neutrophil oxygen radical production in chronic renal failure? Nephrol. Dial. Transplant. 2004; 19: 1702-1707.

Wasserfall CH, and Atkinson MA. Autoantibody markers for the diagnosis and prediction of type1 diabetes. Autoimmunity Reviews. 2006; 5: 424-428.

Wautier JL and Guillausseau PJ. Advanced glycation end products, their receptors and diabetic angiopathy. Diabetes Metab (Paris). 2001; 27: 535-542.

Well-Knecht KJ, Zyzak DV, Litchfiels JE, Thorpe SR and Baynes JW. Mechanism of autoxidative glycosylation: Identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. J. Am. Chem. Soc. 1995; 34: 3702-3709.

Well-Knecht KJ, Brinkmann E, Baynes JW. Characterization of an imidazolium salt formed from glyoxal and n-alpha-hippuryllysine: A model for maillard reaction crosslinks in proteins. J Org Chem. 1995; 60: 6246-6247.

Wendt TM, Tanji N, Guo J, et al. RAGE drives the development of glomerulosclerosis and implicates podocyte activation in the pathogenesis of diabetic nephropathy. Am J Pathol. 2003; 162: 1123-1137

Wieman TJ. Principles of management: the diabetic foot. The American Journal of Surgery. 2005; 190: 295–299

Wilker SC, Chellan P, Arnold BM, and Nagaraj RH. Chromatographic quantification of argpyrimidine, a methylglyoxal-derived product in tissue proteins: comparison with pentosidine. Anal Biochem. 2001; 290: 353-8.

Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. Circulation.1998; 97: 1837-1847.

Wu L and Juurlink BH. 2001. The impaired glutathione system and its up-regulution by sulfraphane in vascular smooth muscle cells from spontaneously hypertensive rats. J. Hypertens. 2001; 19: 1819-1825.

Wu L and Juurlink BH. Increased methylglyoxal and oxidative stress in hypertensive rat vascular smooth muscle cells. Hypertension. 2002; 39: 809-814.

Wu L. The pro-oxidant role of methylglyoxal in mesenteric artery smooth muscle cells. Can. J. Physiol. Pharmacol. 2005; 83: 6368.

Wu L. Is methylglyoxal a causative factor for hypertension development? Can. J. Physiol. Pharmacol. 2006; 84: 129-139.

Xu B, Chibber R, Ruggerio D, Kohner E, Ritter J, Ferro A. Impariment of vascular endothelial nitric oxide synthase activity by advanced glycation end products. FASEB J. 2003; 17: 1289-1291

Yamada H, Sasaki T, Niwa S, Tohru Oishi T, Murata M, Kawakamib T and Aimotob S. Intact glycation end products containing carboxymethyl-lysine and glyoxal lysine dimer obtained from synthetic collagen model peptide. Bioorganic & Medicinal Chemistry Letters. 2004; 14: 5677–5680.

Yamagishi S, Inagaki Y, Okamoto T, et al. Advanced glycation end product-induced apoptosis and overexpression of vascular endothelial growth factor and monocyte chemoattractant protein-1 in human-cultured mesangial cells. J Biol Chem. 2002; 277: 20309-20315.

Yamagishi S, Matsui T, Nakamura K. Prevention of diabetic vascular calcification by nifedipine, a dihydropyridine-based calcium channel blocker. Medical Hypotheses. 2007; 68: 1096-1098.

Yamamoto Y, Kato I, Doi T, et al. Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. J Clin Invest. 2001; 108: 261-268.

Yang CW, Vlassara H, Peten EP, He CJ, Striker GE, Striker LJ. Advanced glycation end products up-regulate gene expression found in diabetic glomerular disease. Proc Natl Acad Sci. 1994; 91: 9436-9440.

Yao D, Taguchi T, Matsumura T, Pestell T, Edelstein D, Giardino ., Suske G, Ahmed N, Thornalley PJ, Sarthy VP, et al. Cell. 2006; 124,

Yki-Jarvinen H, Kauppila M, Kujansuu E, et al. Comparison of insulin regimens in patients with non-insulin-dependent diabetes mellitus. N Engl J Med. 1992; 327:1426.

Zeng J, Davies MJ. Evidence for the formation of adducts and S-(carboxymethyl)cysteine on reaction of α-dicarbonyl compounds with thiol groups on amino acids, peptides, and proteins. Chem Res Toxicol. 2005; 18: 1232-1241.

Appendix 1

October 2003

Faculty of Medicine, Schools of Nursing and Pharmacy of Memorial University of Newfoundland; Health Care Corporation, St. John's; Newfoundland Cancer Treatment and Research Foundation

Consent to Take Part in Health Research

TITLE: New diagnostic markers for children and young people with Type 1 diabetes

INVESTIGATOR(S): Drs. E Randell, L. Newhook, S. Vasdev, and V. Gadag

SPONSOR:

Your child/ward has been asked to take part in a research study. It is up to you to decide whether he/she can be in the study or not. Before you decide, you need to understand what the study is for, what risks your child/ward might take and what benefits he/she might receive. This consent form explains the study.

The researchers will:

- **discuss the study with you**
- **answer your questions**
- **keep confidential any information which could identify you personally**
- **be available during the study to deal with problems and answer questions**

If you decide for your child/ward not to take part or to leave the study this will not affect his/her usual health care.

1. Introduction/Background:

A1C is a lab test measured in patients with diabetes because it is related to the average blood sugar level. A1C is also related to the risk of developing diabetes related problems called complications. These complications include: vision problems, kidney problems, nerve damage, and high blood pressure. There is no test, including A1C, that can predict early on when and if these problems will occur in a patient with diabetes. A number of recently discovered chemicals in blood may be useful in identifying patients who will develop complications. If these chemicals are more useful than A1C they may allow doctors to better treat their patients. If patients can be better treated this could mean that many patients with diabetes can live longer without complications.

Initials: _____

2. **Purpose of study:**

We wish to study some newly discovered chemicals that are in blood to see how they are related to the risk of developing diabetes complications.

3. **Description of the study procedures and tests:**

If you agree for your child/ward to take part in this study, the researchers will measure some of these recently discovered chemicals in the blood sample that has been sent to the lab for A1C. A researcher will also record information from your child's/ward's chart. This information will include: age, sex, how long he/she has had diabetes, what type of insulin he/she is receiving, A1C results, and if he/she has experienced any complications related to diabetes.

4. **Length of time:**

Your participation in this study will involve the time required to read this form and consent to have your child participate. If you consent to have your child/ward participate the researchers will make arrangements with the Lab for the additional tests to be performed on his/her A1C sample.

5. **Possible risks and discomforts:**

There are no risks directly related to participating in this study. Your child/ward will give a blood sample for the A1C test. Some of this sample will be used to do the additional lab tests. No additional blood will be collected from your child/ward for this study.

6. **Benefits:**

It is not known whether this study will benefit your child/ward.

7. **Liability statement:**

Signing this form gives us your consent to have your child/ward be in this study. It tells us that you understand the information about the research study. When you sign this form, you do not give up your legal rights. Researchers or agencies involved in this research study still have their legal and professional responsibilities.

8. **Questions:**

Initials: _____

If you have any questions about taking part in this study, you can meet with the investigator who is in charge of the study at this institution. That person is:

Dr. Edward Randell (777-6375)

Or you can talk to someone who is not involved with the study at all, but can advise you on your rights as a participant in a research study. This person can be reached through:

Office of the Human Investigation Committee (HIC) at 709-777-6974
Email: hic@mun.ca

Initials: _____

Signature Page

Study title: New diagnostic markers for children and young people with type 1 diabetes

Name of principal investigator: Dr. Edward Randell

To be filled out and signed by the parent of the participant:

	Please check as appropriate:	
I have read the consent	Yes { }	No { }
I have had the opportunity to ask questions/to discuss this study.	Yes { }	No { }
I have received satisfactory answers to all of my questions.	Yes { }	No { }
I have received enough information about the study.	Yes { }	No { }
I have spoken to Dr. Newhook and she has answered my questions	Yes { }	No { }
I understand that I am free to withdraw from the study	Yes { }	No { }

- at any time
- without having to give a reason
- without affecting my future care

I understand that it is my choice to have my child/ward be in the study and that he/she may not benefit . Yes { } No { }

I agree that the study doctor or investigator may read the parts of my child's/ward's Hospital records which are relevant to the study. Yes { } No { }

I agree to have my child take part in this study. Yes { } No { }

_____ _____

Signature of participant Date

_____ _____

Signature of witness Date

<u>To be signed by the investigator:</u>

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the participant fully understands what is involved in being in the study, any potential risks of the study and that he or she has freely chosen to be in the study.

_____ _____

Signature of investigator Date

Initials: _____

Telephone number: _____

Assent of minor participant (if appropriate):

_____ _____

Signature of minor participant Date

_____ _____

Relationship to participant named above Age

Initials: _____

Appendix 2

Examination of advanced-glycation end products (AGEs) in children with Type 1 diabetes

Chart Audit Form

Date:_____.

Recruitment Date:_____.

Study #:_____

Name:_____

MCP#:_____

Age:_____

Sex:_____

Number of years with the diagnosis of

diabetes:_____.

Summary of past A1C values

Date (D/M/Y)	**A1C (%)**
1)	
2)	
3)	
4)	
5)	
6)	

Check if the subject has any of the following complications:

- ☐ Hypertension
- ☐ Nephropathy
- ☐ Retinopathy
- ☐ Neuropathy

Type of Insulin management:_____

_____

_____

Signature of Data Collector

Appendix 3

In this research, synthesis for the other three methylglyoxal-derived AGE residues, including argpyrimidine, MOLD and CEL, were also successfully performed. The procedure is described as follows:

1. Synthesis of argpyrimidine

Argpyrimidine was synthesized as described by Shipanova et al (1997). Briefly, 311 mg of t-BOC Arginine (1 mmol) was dissolved in 10 ml of 200 mM sodium phosphate buffer (pH 7.4), followed by adding 0.175 ml of 1 mmol MG solution and 4 mg of DETPA. The mixture was incubated at 55°C for 4 days. This was followed by slowly adding 0.85 ml of concentrated HCl and allowing the mixture to stand at room temperature for 2 hours to hydrolyze the t-BOC group. The HCl was then evaporated under vacuum and the solution was lyophilized and purified using a C18 reverse phase column as previously described (Shipanova et al., 1997). The recovered argpyrimidine product was further purified using LC-MS/MS by a procedure similar to that used for MG-H1. The peak corresponding to argpyrimidine by MRM 255.2>192.2 moniting was collected and lyophilized. The final argpyrimidine product was stored at –70°C for AGEs measurement.

2. Synthesis of MOLD

MOLD was prepared as previously described by Nagaraj, et al. (1996). Briefly 500 mg of N^{α}-*t*-BOC lysine and 200 mg of MG was dissolved in 5.0 ml of 0.2 M sodium phosphate buffer (pH 7.4), and incubated at 37 °C for 16 h. The resulting solution was separated by cation exchange chromatography on an AG-50W-X4 (Bio-Rad) (2.5 × 7-cm) column equilibrated with 0.02 M sodium acetate buffer (pH 5.0) at a flow rate of 20 ml/h. The column was then washed with 250 ml of buffer and eluted in a gradient of 0 to 1 M NaCl in 100 ml followed by 100 ml of buffer containing 1 M NaCl. Fractions (3 mL) were then collected to which 200 µl of 2 N HCl was added and allowed to stand overnight to hydrolyze t-BOC group. Fractions were then pooled and lyophilized. MOLD was further purified by HPLC on a Synmetry Prep C_{18} column in a linear gradient of 0.1% trifluoroacetic acid (TFA) in water, to 50% acetonitrile in water containing 0.1% TFA; 0-100% over 35 min at a flow rate of 2.0 ml/min. Fractions containing MOLD were further purified by HPLC on a Gemini C18 semi-prep column and separation and identification of MOLD were made by MRM of the 341.3>212.2 transition. The collection was then vacuum dried and the final products of MOLD was stored at –70°C.

3. Synthesis of CEL

Synthesis of CEL was carried out based on the method described by Teerlink et al. (2004) which involves protecting the α-amino group of lysine by converting to its copper complex. To do this, 2.2g of lysine was dissolved in 5 ml of water and heated to 100°C. Solid $CuCO_3$ was added slowly to produce a saturated copper solution. The copper-lysine complex was then treated with 2.3g (1.4 ml) of 2-bromopropionic acid in

2.5 ml of 2 N NaOH for 3 days at room temperature under sterile conditions. The mixture was finally adjusted to pH 2 with HCl and copper ion was removed by treating with hydrogen sulfide gas. The precipitated copper sulfide was removed by filtration. The remaining solution was evaporated under vacuum on rotary evaporator and further purified by preparative HPLC using a reverse phase prep C_{18} column as previously described (Teerlink et al., 2004). CEL was then further purified on a Gemini C18 semi-prep column (250x10mm; 5 μm; Phenomenex, USA) with monitoring by MS/MS by MRM of transition 219>130 and the peak was collected as CEL. The collection product was evaporated to dryness in a 2 ml plastic microfuge tube and the dried product was stored at –70°C for use as a standard to measure plasma AGEs. (See Figure 3.9 for chromatogram of these compounds)