

## Minireview

### **Glycotoxines, Carbonyl Stress and Relevance to Diabetes and its Complications**

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Short title: Glycotoxins, carbonyl stress and diabetes

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## Summary

A large body of evidence has implicated reactive carbonyl compounds as glycotoxic mediators of carbonyl stress. This review is focused on the pathophysiologic effects of  $\alpha$ -oxoaldehydes in diabetes and related complications, summarizing the state-of-the-art on the endogenously produced carbonyls methylglyoxal, glyoxal and 3-deoxyglucosone, formed as glycolytic intermediates during metabolic conversion of glucose, *via* Maillard reaction by degradation of glycated proteins, and during lipid peroxidation process. Their role in the advanced glycation process and detrimental effects on vascular tissue are discussed.

## Key words:

Diabetes, Carbonyl stress, Methylglyoxal, Glyoxal, 3-Deoxyglucosone and Advanced glycation end-product.

## Introduction

Glycation process leading to advanced glycation end-product (AGE) formation has been identified as a biologic phenomenon in the pathogenesis of diabetes and its complications (Brownlee 2005, Ahmed and Thornalley 2007, Huebschmann *et al.* 2006). AGE-proteins are formed by post-translational modification when blood glucose reacts with free amino groups of amino acids, peptides, phospholipids and nucleic acids. This chemical reaction between an amino group and carbonyl group of reducing sugar is not enzymatically catalyzed. It is a covalent interaction, generally termed nonenzymatic glycosylation\*. French chemist L.C. Maillard conducted pioneer work on sugar-amino acid condensation (Maillard 1912) and this type of reaction has been termed after him in chemical nomenclature. In the course of Maillard reaction, the first formed Schiff base adduct is converted to a more stable Amadori rearrangement product. These Amadori products then undergo further rearrangement to form irreversibly bound AGEs, in a cascade of chemical reactions. The intermediate stage of Maillard reaction is characterized by the formation of numerous secondary products. The sugar moiety of an early glycation product can undergo chemical reactions, consequently producing low molecular weight carbonyls such as methylglyoxal, glyoxal and 3-deoxyglucosone. These reactive carbonyls again react with free amino groups, leading to cross-linking and formation of AGEs in the late stage of Maillard reaction (Finot, 1982).

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\*The term glycation is arbitrarily adopted to distinguish this type of chemical reactions from many other N- or O-glycosylations.

AGEs represent a potpourri of very heterogeneous chemical structures, and a number of them have been detected in body proteins (Thorpe and Baynes 2003).

*In vivo*, AGE-modified proteins have been found to be involved in the development of vascular lesions, thus potentially contributing to micro- and macrovascular pathology. Glycated hemoglobin (HbA1c) determination has been accepted as a standard measure of glycemia control and risk marker of long-term complications in diabetes (DCCT 1993, Genuth *et al.* 2002). A significant increase in HbA1c level is a consequence of hyperglycemia. However, glucose *per se* appears to be by far less potent in the formation of AGE-proteins than glycotoxins, the intermediary metabolites of  $\alpha,\beta$ -dicarbonyl structure. This review summarizes current evidence on the physiologically produced carbonyl compounds methylglyoxal, glyoxal and 3-deoxyglucosone, their role in the advanced glycation process, and detrimental effects on vascular tissue.

#### *$\alpha$ -Oxoaldehydes as AGE precursors*

Endogenously formed  $\alpha$ -oxoaldehydes are potent glyating agents acting as key factors in the phenomenon called carbonyl stress (Thorpe *et al.* 2000). In fact, carbonyl stress results from impaired balance between the generation of carbonyl intermediates and efficiency of scavenger pathway. Dicarbonyls, in general, are formed as (i) glycolytic intermediates in metabolic conversion of glucose, (ii) *via* Maillard reaction by degradation of glycated proteins, and (iii) during lipid peroxidation (Fig. 1). Whereas glucose is involved in glycation reactions exclusively in its aliphatic form, accounting for only 0.001% of total glucose concentration,  $\alpha$ -oxoaldehydes are characterized by extremely high chemical activity. They are up to 20,000-fold as reactive as glucose, thus being able to form AGE-structures even at very low concentrations. Physiologically,  $\alpha$ -oxoaldehydes react directly with protein terminal amino acid residues. The potency of  $\alpha$ -oxoaldehydes in glycation under physiologic conditions was demonstrated by *in vitro* incubation of 1  $\mu\text{mol/L}$  ( $^{14}\text{C}$ )methylglyoxal in human plasma (Thornalley 2005). All the methylglyoxal was irreversibly bound to plasma proteins after 24 h. It has been postulated that the biogenesis of these intermediary metabolites is not exclusively dependent on persistent hyperglycemia but may also be influenced by glycemia fluctuation. It appears that reactive  $\alpha$ -oxoaldehydes can discretely promote the pathology of diabetic complications, even in the presence of good glycemia control, as indicated by HbA1c ( $\leq 8\%$ ). Evaluation of physiologic  $\alpha$ -oxoaldehydes has not yet been fully investigated, mostly due to the complex methods of detection. However, their role has been postulated in the

carbonyl stress reactions, which activate a series of inflammatory responses leading to accelerated vascular damage in diabetes and end-stage renal disease.

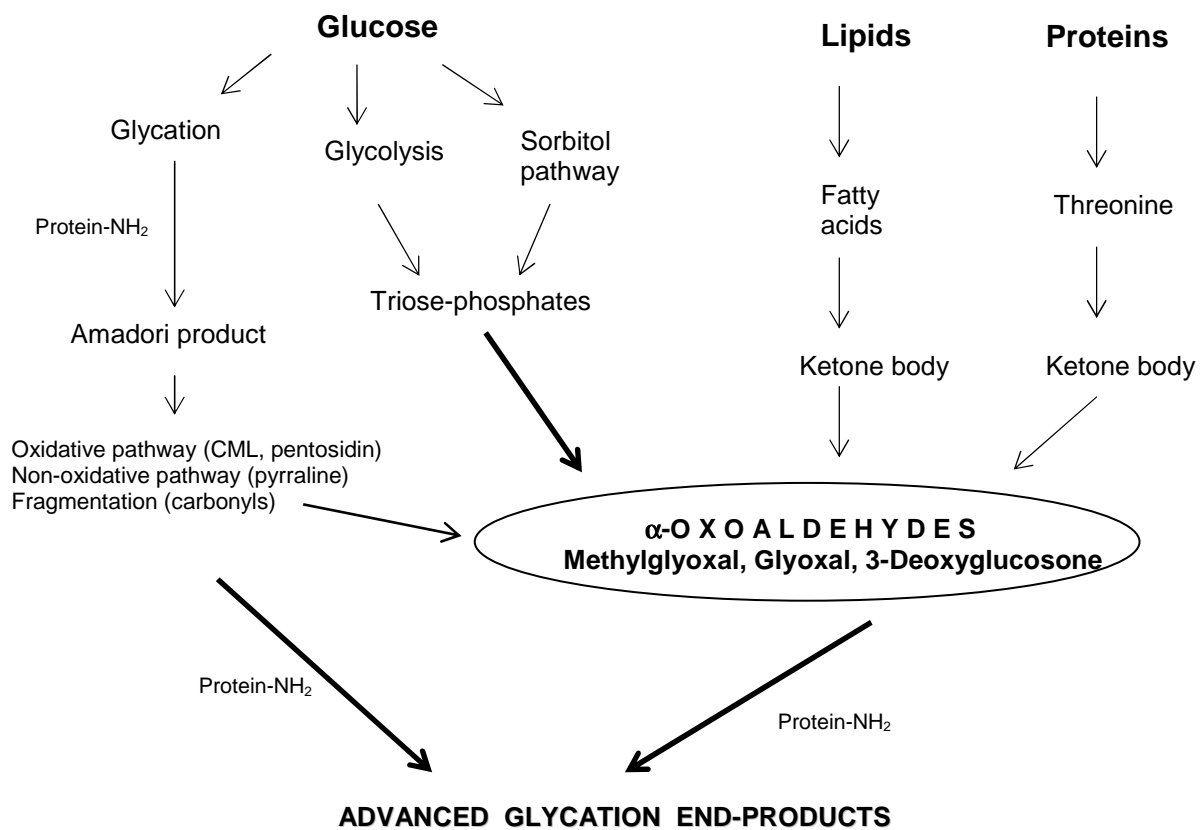


Figure 1. Reaction scheme for reactive carbonyl compounds biogenesis.

## Methylglyoxal

### *Biogenesis*

Methylglyoxal (MG) is a distinctive representative of  $\alpha$ -oxoaldehyde compounds, generated intracellularly in all mammalian cells, through both enzymatic and non-enzymatic pathways. The majority of MG production occurs from the triose phosphate intermediates in the glycolytic pathway, which includes fragmentation and elimination of phosphate from glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Richard 1993). It is also formed in the ketone body metabolism and in the catabolism of threonine. MG is very

reactive, but its interactions with body proteins and nucleic acids are controlled by glyoxalase (Thornalley 1998). The glyoxalase system is present in the cytosol of all mammalian cells. It consists of two enzymes, glyoxalase I and glyoxalase II, and a catalytic amount of reduced glutathione (GSH). Physiologic  $\alpha$ -oxoaldehydes, MG and glyoxal are metabolized by glyoxalase pathway. The metabolism of MG leads to the production of the inert end product D-lactate (Thornalley 2003). The concentrations of  $\alpha$ -oxoaldehydes in human tissues and body fluids are usually low. For example, the concentrations of glyoxal and MG in human blood samples were 211 and 80 pmol/g, respectively (Thornalley 1998). In terms of physiopathology, such low concentrations of  $\alpha$ -oxoaldehydes are protective because of the carbonyl high reactivity with nucleic acids and body proteins. The glyoxalase system *in situ* suppresses irreversible modifications of DNA, RNA and amino acid residues by the action of  $\alpha$ -oxoaldehydes. The accumulation of  $\alpha$ -oxoaldehyde-derivative nucleic acids is associated with mutagenesis and may promote cell apoptosis. In physiologic conditions, MG can form MG-derivative AGE structures accumulated on humoral and tissue proteins. For example, MG readily binds and modifies arginine, lysine and cysteine residues in protein, and intracellularly reacts with nucleic acids, participating in the formation of a variety of AGEs (Lo *et al.* 1994). MG-induced AGEs on mitochondrial proteins lead to a decline of mitochondrial function and have been hypothesized to act as a contributing factor in the phenomenon of so-called “metabolic memory” (Ihnat *et al.* 2007).

Metabolic disorders such as diabetes can induce an increase in the MG formation. Increased MG production arises mainly from hyperglycemia (Brownlee 2001) in cells accumulating high level of glucose. This might be additionally exacerbated by low glyceraldehyde-3-phosphate dehydrogenase activity (Beisswenger *et al.* 2003). In fact, the rate-controlling enzymes of the glycolytic pathway modulate up and downstream of MG. Intracellularly produced MG crosses cell membranes, probably by passive diffusion, and humoral MG is mainly cell-derived. Additionally, minor amounts of MG might arise from ketone body metabolism, degradation of threonine, and lipid peroxidation. The sustained increase in the MG level may also be the consequence of decreased clearance by the glyoxalase system. Optimal activity of the glyoxalase system is dependent on the adequate level of reduced glutathione (GSH). In diabetes, both hyperglycemia and oxidative stress are associated with GSH depletion. This may entail impaired detoxification of MG and consequently its increased production in diabetic state.

The possible sources of MG include the catabolism of threonine *via* aminoacetone (Lyles and Chalmers 1992). The catabolism of threonine normally produces glycine and acetyl-CoA, however, in a low CoA state such as diabetic metabolic crisis, where much of the CoA is in the form of acetyl-CoA, threonine is catabolized to aminoacetone (Tressel *et al.* 1986). Enzymatic oxidation of ketone bodies (acetoacetate and acetone) can also generate MG. Its production from acetoacetate is mediated by myeloperoxidase, whereas P450 2E1 cytochrome catalyzes acetone conversion into MG (Kalapos 2003). In healthy people, it has been shown that the popular and widely used Atkins diet, characterized by low carbohydrate intake, leads to a significant increase in MG level (Beisswenger *et al.* 2005). The highly significant relationship between MG and acetol levels, demonstrated by the same authors, suggests that MG is produced directly from acetol by oxidative mechanisms. Although ketone bodies are likely to be an important source of MG, it is also possible that some MG is derived from increased triose phosphate resulting from the increased production of glycerol caused by accelerated triglyceride breakdown, or from lipoxidation products (Baynes and Thorpe 2000). The sustained increase in the MG level may also be the consequence of decreased clearance by detoxification pathways. Several enzymes are involved in detoxification of MG and make a network of four recognized catabolism pathways, as follows: the glyoxalase system, aldose reductase, betaine aldehyde dehydrogenase, and 2-oxoaldehyde dehydrogenase (Nemet *et al.* 2006).

Importantly, diet is an underappreciated source of distinct AGE derivatives (Huebschmann *et al.* 2006). In humans with or without diabetes, a meal with high AGE content was shown to lead to its elevation in plasma. Some 10% of AGEs ingested are absorbed into the body's circulation, and over 65% of those absorbed are retained (Koschinsky *et al.* 1997). The influence of diet on serum MG level was also demonstrated in renal failure patients (patients with diabetes were excluded). It was shown that a high dietary AGE intake increased serum MG (26%) in contrast to a diet with low AGE intake, which caused a decrease in serum MG (35%) (Uribarri *et al.* 2003).

MG has been postulated to play a role in the development of hypertension (Chang 2006). Studies using animal model and cell cultures showed a significant increase in blood pressure to coincide with elevated MG level in plasma and aortic tissues (Wang *et al.* 2004, 2005). However, functional links between MG biogenesis and hypertension, in part mediated by ROS and AGEs, have only been documented in rat model but not yet in humans with these conditions.

### *Significance in diabetes*

It is considered that monitoring of MG biogenesis in diabetic patients might help assess the risk of progression of diabetic complications. In physiologic conditions, MG exists in two forms, i.e. as a free dicarbonyl compound (unbound form) and bound to protein residues, known as MG-derivative AGEs. MG in the free form is very unstable, has short biological survival, and sophisticated techniques are required for its quantitative evaluation. Our results of free MG measurement have shown excessive production in diabetic state, detected in both plasma and whole blood samples (Nemet *et al.* 2005). We found MG overproduction to correlate tightly with glycemia fluctuation. Glycemic lability is one of the known risk factors in the progression of diabetic complications (Beisswenger *et al.* 2001). In another recent study, we observed MG biogenesis during the course of diabetic ketoacidosis and recorded an extremely high MG production during the first 24 h of metabolic crisis (Turk *et al.* 2006). MG as a side-product of glycolysis consequently arises from an increased flux during hyperglycemia. However, the treatment for ketoacidosis and glycemia control failed to produce significant MG reduction toward control values of diabetic population in general. This observation suggests involvement of other MG sources, probably ketone body metabolism. It is postulated that ketone bodies might be alternative MG precursors not only in ketoacidosis but also in poor diabetes control. The involvement of ketone body metabolism may explain why type 1 diabetic patients suffer the highest level of MG (McLellan *et al.* 1994). In addition, the above study of MG production during ketoacidosis supports the hypothesis that other toxic metabolites that are actively involved in post-translational protein modifications are probably generated in acute metabolic crisis. Therefore, excessive production of glycotoxic intermediates such as  $\alpha$ -oxoaldehydes or other  $\alpha,\beta$ -dicarbonyls may be a link connecting an acute metabolic event with accelerated tissue damage, a feature characteristic of long-term complications of diabetes.

As emphasized above, due to the extremely high chemical reactivity, the physiologically produced MG participates in the formation of MG-derivative AGE structures. Imidazolone adducts are formed in reaction with arginine residues, whereas carboxyethyllysine (CEL) and MG-lysine dimer (MOLD) are formed in reaction with lysine residues. A recent study by Ahmed *et al.* (2005) has demonstrated that hydroimidazolones are the most important biomarkers of advanced glycation process. This study has offered evidence that the majority of AGE in plasma proteins of diabetic and non-diabetic subjects are hydroimidazolones. In diabetic patients, the level is significantly higher, and the largest quantitative increase in protein glycation adducts of plasma protein occurs as structural isomers of MG and 3-DG

derivative hydroimidazolones. In hemoglobin, MG and 3-DG derivative hydroimidazolones are, again, the major glycation adducts in quantitative sense. The authors report that diabetes type 1 under moderate metabolic control is associated with a profound increase of plasma level and urinary excretion of protein glycation and oxidation free adducts, particularly MG-derivative hydroimidazolone. MG-derivative adducts are excreted in the urine. As reported by Ahmed *et al.* (2005), urinary excretion of a variety of AGE-compounds increased up to 15-fold in diabetic patients. Considering renal function and MG-adduct level, we found a significant inverse correlation between plasma MG-derivative AGEs and creatinine clearance (Nemet *et al.* 2005). Elevation of plasma MG was also observed in non-diabetic patients with mild to moderate renal failure and in patients on hemodialysis (Beisswenger *et al.* 2005). These results suggest that renal function might affect the clearance of plasma MG-derivative AGE and consequently influence its concentration in the blood stream.

#### *Relevance to diabetic complications*

The knowledge of advanced glycation end products has considerably expanded over years, and a large body of evidence has documented their implication in diabetes related complications (Brownlee 2005, Huebschmann *et al.* 2006, Singh *et al.* 2001, Monnier *et al.* 2005, Turk *et al.* 2001). The recently recognized relationship between  $\alpha$ -oxoaldehydes and biologically important macromolecules highlights the intermediate step of advanced glycation cascade (Beisswenger *et al.* 2003, Thornalley 2005). The involvement of MG in modification of tissue proteins has been demonstrated in diabetes and uremic patients by means of immunohistochemistry and immunochemistry (Bourajjaj *et al.* 2003). However, structural and functional modification of host-protein is a common feature of all AGEs irrespective of their generating precursors. Through their effects on the functional properties of extracellular matrix, intracellular signal transduction and protein function, AGEs may contribute to the pathogenesis of diabetic retinopathy (Pokupec *et al.* 2003), neuropathy (Misur *et al.* 2004), renal failure (Makita *et al.* 1994, Rabbani *et al.* 2007, Turk N *et al.* 2004) and macrovascular disease (Turk *et al.* 2003) (for detailed reviews of glycation and complications of diabetes see Turk 1997, 2001).

A mechanism by which AGE-modified proteins may exert their effect is binding to AGE-specific cell-surface receptors (RAGE) identified on a variety of cells including endothelial and smooth muscle cells, and by internalization and degradation *via* monocyte/macrophage AGE-receptors. Increased RAGE expression has been found on endothelial cells, vascular smooth muscle cells and cardiac myocytes of diabetic patients (Schmidt *et al.* 1999). It has



been reported that ligation of AGE with RAGE causes activation of intracellular signaling, gene expression, and production of proinflammatory cytokines and free radicals, thus playing an important role in the development and progression of diabetic micro- and macroangiopathy (Kim *et al.* 2005). A central observation in human diabetes has been reported by Cipollone *et al.* (2003). The authors found that up-regulated RAGE expression in diabetic atherosclerotic plaques retrieved from human subjects co-localized with a range of pro-oxidant and inflammatory markers. These authors also showed that the increased expression of RAGE in the plaques correlated linearly with the glycated hemoglobin level, suggesting that RAGE expression is correlated with the degree of ligand accumulation.

Considerable progress has been made in the understanding of the AGE/RAGE action in the past few years. Unifying outcomes of RAGE engaged with its ligand family in inflammatory and vascular cells is up-regulation of the cascade of proinflammatory cytokines, growth factors, adhesion molecules and matrix metalloproteinases, with multiple consequences in tissues and organs (Ramasamy *et al.* 2008). Recent studies have incriminated AGEs and their receptor in the pathogenesis of diabetic nervous system complications (Lukic *et al.* 2008). Increasing evidence indicates that RAGE with its ligands can initiate and sustain significant cellular perturbations in the inner and outer retina, as a relevant pathway leading to the pathogenesis of diabetic retinopathy (Wang *et al.* 2008). When made diabetic, RAGE-overexpressing transgenic mice exhibited exacerbation of the indices of nephropathy, while RAGE-deficient animals showed amelioration of diabetic nephropathy (Yamamoto *et al.* 2007). Genetic variation in the gene coding for RAGE may alter gene expression and function of the receptor and affect diabetic vascular disease development and outcome. Investigation of the association between RAGE polymorphisms and parameters of glucose homeostasis indicated that gene polymorphisms might contribute to the development of insulin resistance and diabetes (Gaens *et al.* 2008). Therefore, therapeutic interruption of AGE and/or AGE-RAGE interactions seems to be a logical approach in reducing the sequels of diabetic complications.

### **3-Deoxyglucosone**

#### *Biogenesis*

3-Deoxyglucosone (3-DG) is a small molecular weight aliphatic aldehyde of potential physiologic significance, which has been demonstrated to participate in adduct formation with proteins. The biogenesis of 3-DG in humans occurs *via* Maillard reaction in its intermediate stage, and also from fructose-3-phosphate through the polyol pathway. Physiologically produced 3-DG was detected in the lens and hearts of diabetic rats and in an increased level in erythrocytes of diabetic hemodialysis patients (Lal *et al.* 1995 and 1997, Tsukushi *et al.* 1999). 3-DG reacts rapidly with protein amino residues forming 3-DG-related AGEs such as carboxymethyllysine (CML), 3-DG-imidazolone, pyrraline, and pentosidine as a minor product (Niwa *et al.* 1999). 3-DG-imidazolone and pyrraline are considered to be markers of the presence of 3-DG derivative AGEs *in vivo*. There is evidence that 3-DG-imidazolone is the major product in 3-DG derivative AGEs (Ahmed *et al.* 2005, Jono *et al.* 2004). 3-DG itself shows certain biologic activities such as the induction of apoptosis, suppression of cell proliferation and induction of heparin-binding of epidermal growth factor-like growth factor, which acts as a potent mitogen in aortic smooth muscle cells (Niwa *et al.* 1999). Moreover, 3-DG, likewise MG, inactivates glutathione reductase that enhances the potential for oxidative damage, and also inhibits the activities of hepatic enzymes responsible for glucose metabolism.

#### *Clinical relevance*

In humans, 3-DG is involved in aging and plays an important role in the end-stage renal disease and pathogenesis of diabetic complications. Its plasma level was found to be increased in uremic patients as well as in diabetic patients (Hamada *et al.* 1997, Niwa *et al.* 1995). Investigations based on plasma measurement of 3-DG and its urinary metabolite 3-deoxyfructose have suggested a relationship between impaired detoxification of this carbonyl compound and early nephropathy development (Wells-Knecht *et al.* 1994). 3-DG involvement in modification of tissue proteins has been demonstrated by immunohistochemistry and immunochemistry in diabetic and uremic patients. Niwa *et al.* (1997) demonstrated 3-DG-imidazolone to accumulate in nodular lesions and to expand mesangial matrix of the glomeruli and renal arteries in advanced stages of diabetic nephropathy as well as in atherosclerotic lesions of the aorta. Pyrraline has been detected in sclerosed glomeruli, arteries affected with arteriosclerosis and senile plaques of patients with Alzheimer's disease. Higher pyrraline content was demonstrated in cataractous lenses as

compared with age-matched controls (Nagaraj and Sady 1996). 3-DG derivative AGE structures were localized not only in the extracellular matrix but also in intracellular lesions. 3-DG-imidazolone and carboxymethyllysine might be recognized by AGE receptor expressed on macrophages and smooth muscle cells, and taken up into intracellular lesions, inducing expression of cytokines and growth factors that contribute to the pathology of diabetic vascular disease.

### **Other dicarbonyls**

The second step of Maillard reaction is characterized by the formation of numerous secondary products. The sugar moiety of an early glycation product can undergo enolization, followed by dehydration, oxidation and/or fragmentation reactions, consequently producing dicarbonyls and other low molecular weight compounds (Finot 1982). Most of the products formed are by far more reactive than the precursor molecules. As reported by Thornalley (2005), from this cascade of reactive intermediates, 11 dicarbonyl compounds are currently being investigated for their role in glycation in food chemistry or for their physiologic relevance.

Glyoxal is definitely an interesting issue in the context of carbonyl stress and its physiologic significance in diabetes. In addition to Maillard reaction, glyoxal is a side product of lipid peroxidation. It is important as a glycation agent in physiologic systems and a precursor of AGEs. A specific glyoxal-derived AGE is a lysine-lysine crosslinking structure named glyoxal-lysine dimer or GOLD. Investigators have reported an age-related increase of GOLD in human lens proteins as well as in diabetic state (Sady *et al.* 2000, Chellan and Nagaraj 1999, Frye *et al.* 1998, Shamsi and Nagaraj 1999). The rapid reaction of glyoxal with proteins may account for the difficulty in its measurement in biologic samples (Nakajima *et al.* 2007). However, publications report significant increases in plasma glyoxal concentrations in uremic (Niwa *et al.* 1999) and diabetic patients (Lapolla *et al.* 2003, Han *et al.* 2007).

### **Conclusion**

Protein glycation leading to AGE formation is enhanced in diabetes by increases in blood glucose *per se*, and collaterally, by the production of small molecular weight carbonyl compounds. The increased generation of glycotoxins occurs consequentially to both alterations in cellular metabolism and inefficiency of the related scavenger mechanisms. From the large body of literature reports presented, it is evident that physiologically  $\alpha$ -dicarbonyl

compounds accelerate the kinetics of AGE formation, thus potentiating their detrimental effects in vascular pathophysiology.

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