

Glycooxidation:

The Menace of Diabetes and Aging

HELEN VLASSARA, M.D.¹, AND MARCIA RASHELLE PALACE, M.D.²

Abstract

Advanced glycation end products (AGE) form via the Maillard reaction *in vivo* and are also consumed from exogenous sources such as diet and smoking. They alter the structure and function of molecules and increase oxidative stress in biological systems. These consequences promote the pathogenesis of diabetic complications and changes associated with aging, including atherosclerosis, and renal, eye, and neurological disease. Both specific and nonspecific receptor mechanisms mediate these detrimental effects but also participate in the removal and degradation of AGE. AGE toxicity may be averted by promising dietary and pharmacological strategies which are currently being investigated.

Key Words: Glycooxidation, advanced glycation end product, oxidative stress, macroangiopathy, microangiopathy.

Introduction

ACUTE METABOLIC DERANGEMENTS associated with hyperglycemia are well recognized. However, only relatively recently have we become truly aware of the irreversible diabetic changes caused by elevated glucose. It is now accepted that sustained hyperglycemia causes permanent changes which promote development of progressive dysfunction, leading to diabetic complications (1). One such detrimental consequence of hyperglycemia is the formation of advanced glycation end products (AGEs) *in vivo*, via the Maillard reaction. As bioreactive molecules, AGEs have a range of chemical, cellular, and tissue effects through which they mediate not only diabetic complications, but also widespread changes associated with aging. Further-

more, the main exogenous sources of AGEs, including cigarette smoking and consumption of foods processed by heating, similarly pose major detrimental effects to living organisms.

The following review focuses on the role that advanced glycation plays in the initiation and progression of diabetic complications, the mechanisms by which AGEs exert their effects, and the *in vivo* systems that are involved in their degradation and removal. We also discuss strategies, both dietary and pharmacological, that can lessen the physiological burden of AGEs and curtail the widespread damage they impose on biological systems.

Exogenous AGE Sources

While the *in vivo* formation of AGEs detailed below clearly mediates multiple pathological processes, it is becoming apparent that exogenous sources of AGEs, such as diet and smoking, may have significant impact on disease mechanisms as well. Diet is the major source of exogenous AGE, with the highest content in cooked foods, particularly those rich in carbohydrates, proteins, and fats. As formation of AGE is enhanced by exposure to heat, AGE content, which is responsible for the browning of food as it cooks, increases with cooking temperature and duration. The exist-

¹Professor, Department of Geriatrics, Division of Experimental Diabetes and Aging, and ²Clinical Fellow, Department of Medicine, Division of Endocrinology, Mount Sinai School of Medicine, New York, NY.

Address all correspondence to Helen Vlassara, M.D., Director, Division of Experimental Diabetes and Aging, Box 1640, Mount Sinai School of Medicine, One East 100th Street, New York, NY 10029; Email: helen.vlassara@mssm.edu

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tence of AGE compounds found *in vivo*, including methylglyoxal (MG) and carboxymethyl lysine (CML), in the diet has long been recognized by food chemists. However, their pathogenic significance was not appreciated.

The study of the dietary content, bioavailability, and renal elimination of AGEs, schematically depicted in Fig. 1, has been facilitated by the availability of new AGE-specific assays. It has been confirmed that AGE absorption is approximately 10% of all AGEs ingested (2). Furthermore, only one-third of that absorbed is excreted within 48 hours in the urine of patients with normal renal function (3). AGEs that are not cleared are deposited in tissues where they remain biologically active (3), posing a serious threat to the organism, one that is clearly exaggerated in patients with renal impairment.

In vivo injury as a result of AGE accumulation is exemplified by the fact that when diabetic mice were randomized to receive either a regular diet or one which was otherwise nutritionally identical but had less AGE content, mice on the regular diet developed changes typical of diabetic nephropathy, while mice on the low AGE diet did not, even in the face of persistent hyperglycemia (Fig. 2) (4). Similarly, a low-AGE diet prevented vascular restenosis and atherosclerosis in diabetic, hyperlipidemic, ApoE-deficient mice (see below) (5).

In addition to diet, smoking remains a significant source of exogenous AGE. Glycated and oxidized derivatives form via the Maillard reaction cascade as tobacco leaves are dried in

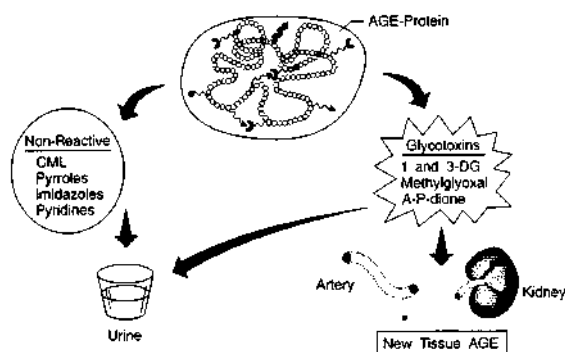


Fig. 1. Schematic representation of the fate of diet-derived AGEs. Protein or lipid glycation intermediates contained in the diet include noncrosslinking products, such as *N*- ϵ -carboxymethyllysine (CML), pyrroles, imidazoles, pyridines (left insert), and crosslink forming, reactive intermediates (glycotoxins), such as 1-,3-deoxyglycozone (3-DG), methylglyoxal, protein-linked A-P-dione (right insert). While the former are excreted in the urine, the latter may reattach onto serum or tissue components to form pathologic new AGEs.

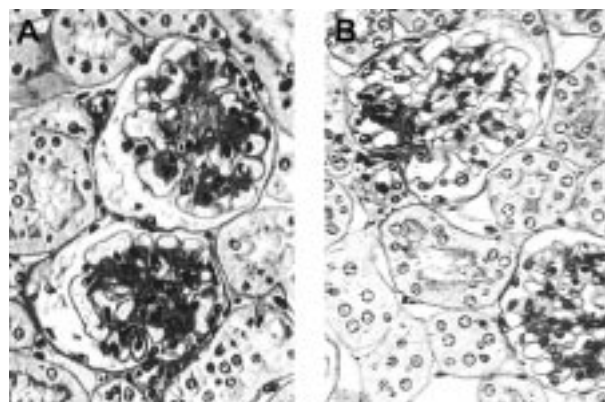


Fig. 2. Development of nephropathic glomerular changes is delayed in diabetic NOD and db/db mice fed a low-AGE diet. **Panel A:** Glomerular changes characteristic of nephropathy in db/db mice after 5 months on standard chow diet (H&E); **Panel B:** Relative sparing of glomeruli in mice fed a diet containing 5-fold lower AGE for 5 months (H&E; magnification $\times 400$).

the presence of sugars during the process of curing. Upon combustion, reactive AGE species are volatilized, inhaled and absorbed through the lungs, and become conjugated with serum proteins, including lipoproteins (6). Thus, total serum AGE and AGE-apoprotein B levels (6), as well as arterial (7, 8) and ocular lens AGE levels (8), are significantly higher in smokers than in nonsmokers, and are especially high in diabetic smokers.

Endogenous AGE Formation

Over time, even modest hyperglycemia can result in significant accumulation of AGEs on long-lived macromolecules (9–11). This is apparent on certain long-lived proteins such as those of the ocular lens. Lens crystallins are subjected to progressive modification by AGEs, which promotes lenticular browning and cumulative crosslinking (12, 13). The resultant opacification forms cataracts, a process associated with both diabetes and aging (14, 15). These changes, which can be reproduced *ex vivo* under high-glucose conditions, can be prevented by the AGE-inhibitor aminoguanidine (16). Furthermore, the collagen network of human vitreous gel contains increased levels of AGEs in diabetics (16), and vitreous AGE levels show a significant correlation with age, supporting the role of AGEs in diabetes- and age-related vitreous alterations, including progression of retinopathy and posterior vitreal detachments (16).

While glycation of long-lived molecules such as structural proteins was recognized first,

it is now apparent that AGEs also arise on short-lived molecules, including circulating plasma proteins and lipids, cytoplasmic proteins and nucleic acids (17). Furthermore, intracellular AGEs may form at a rate of up to 14 times faster in high (30 mM) glucose conditions (18) and are thus found at significantly elevated levels in diabetic patients. Such AGE modification of short-lived molecules is known to be associated with oxidation of proteins and lipids, disrupt molecular conformation, alter enzymatic activity, reduce degradative capacity, and result in abnormal recognition and clearance by receptors (19–22).

Glucose reacts with amino groups on certain phospholipids such as phosphatidylethanolamine to form AGEs (23). Glycation of lipids and lipoproteins occurs, as in the case of apoprotein B (ApoB) and low-density lipoprotein (LDL) (24). The predominant site of such modification of LDL is distal to the N-terminus of the LDL-receptor binding domain (24). Formation of AGE-LDL occurs in direct proportion to glucose concentration. In fact, AGE-ApoB levels are approximately 4-fold higher in diabetic patients than in nondiabetic patients (23, 25).

In vitro LDL oxidation occurs concomitantly with advanced glycation of the lipid component of LDL (24). During glycation, fatty acid residues can be oxidized independently of transition metals or exogenous free-radical generating systems (23). Formation of AGE-LDL and LDL oxidation are both inhibited in the presence of the AGE inhibitor aminoguanidine (23). *In vivo* clearance of injected AGE-LDL was delayed compared to that of native LDL (25) in studies of transgenic mice expressing the human LDL receptor. These data support the role of glycated Apo-B in promoting atherosclerosis, a process that will be described further, below.

AGEs as Mediators of Diabetic Complications

Macroangiopathy

Accumulation of lipids and lipoproteins in the vessel wall, resulting in fatty streaks, is a pivotal factor in the evolution of advanced atherosclerotic lesions, a process of particular concern for diabetics, who have an increased risk for vascular occlusive events (26–28). It is thought that oxidative modification of LDL (ox-LDL) *in vivo* reduces its recognition by the normal LDL receptor (29, 30), thereby decreasing

its clearance, increasing serum LDL levels (31, 32), and ultimately promoting enhanced uptake of ox-LDL by scavenger receptors on macrophages and vascular smooth muscle cells.

AGEs, which have been detected within atherosclerotic lesions in both extra- and intracellular locations (33–37), are now considered to play an important role in the initiation and acceleration of atherosclerosis even in normoglycemic patients (33), but especially in diabetics (38, 39) and more so in diabetics with renal insufficiency (34). AGE-ApoB and AGE levels in the vessel wall of carotid arteries from nondiabetic patients have been found to correlate with occlusive disease requiring endarterectomy (33). Furthermore, exposure of endothelial cells to AGEs may enhance procoagulant activity (40) and promote cell adhesion and transendothelial migration (41, 42).

As vascular endothelium expresses receptors for AGEs (43–46), accumulation of AGEs in the subendothelial space may occur as a direct result of endocytosis and transcytosis of AGE-LDL by these receptors. Intracellular accumulation of AGEs may promote phenotypic conversion of smooth muscle cells and foam cell formation within atherosclerotic plaques. This is consistent with the diffuse pattern of AGE deposition and endocytosis by endothelium, smooth muscle cells, and macrophages (34–39, 47). AGE adducts in the vessel wall can interfere with endothelium-derived nitric oxide (NO) synthase and the vasodilatory action of (NO \cdot) (48, 49), as shown in nondiabetic animals which exhibited diabetic-like disruption of vaso-relaxation after AGE-infusion (50). Hypertension, renal impairment, and erectile dysfunction seen in diabetes may be related to these alterations.

Microangiopathy

Diabetic microangiopathy is characterized by the progressive damage of endothelium and associated mural cells in microvascular beds, resulting in capillary occlusion, ischemia, and organ failure. These changes occur in the kidney, retina, and microvasculature of peripheral nerves (51, 52).

Immunohistochemical studies of kidney from normal and diabetic rats have suggested that glomerular basement membrane (BM), mesangium, podocytes, and renal tubular cells accumulate high levels of AGEs. Ultrastructural studies have indicated that AGE peptides may be reabsorbed by the renal proximal tubular

cells (53). AGE deposition can lead to glomerulosclerosis and widespread dysfunction independent of diabetes (54–56). This is evidenced by the development, in nondiabetic animals infused with AGE-albumin, of glomerular pathology resembling diabetic nephropathy, including glomerular hypertrophy, BM thickening, mesangial extracellular matrix (ECM) expansion, and albuminuria (50, 57).

In diabetic retinopathy, intraretinal blood vessels become dysfunctional in response to hyperglycemia, with progressive loss of retinal pericytes and eventually of endothelial cells, leading to capillary closure and widespread retinal ischemia. As in other vascular beds, AGEs have been localized in the retinal vessels of diabetics (58, 59). However, their exact role is not defined. Experimental studies have demonstrated that AGEs may be responsible for some retinal pathology (59) and that AGE-inhibitors, such as anti-glycated albumin (60) or aminoguanidine, can prevent the development of diabetes-associated retinal vascular lesions in rats (61) and in dogs (62).

Embryopathy

Like amino acids and lipids, nucleic acids react with reducing intracellular sugars to form Amadori and AGE-products (17). AGE formation on DNA can promote teratogenesis by causing single strand breaks in genomic DNA (17, 63, 64). Teratogenic effects may also be induced in diabetes by enhanced glycation of histone proteins, which normally function to maintain nucleosomes and thus, DNA integrity. Intracellular sugars such as glucose-6-phosphate and adenosine diphosphate (ADP)-ribose react strongly with amino groups on histones, causing crosslinking *in vitro* (65, 66) as well as *in vivo* (67).

It is well recognized that maternal diabetes is associated with congenital malformations and increased fetal mortality and morbidity (68). The incidence of perinatal infant fatalities due to congenital malformations in the offspring of women with insulin-dependent diabetes is increased 2–3-fold compared with those born to nondiabetic mothers (69). The incidence of congenital abnormalities is also increased when conception occurs in the setting of long-term hyperglycemia; this is not the case with short-term, pregnancy-induced hyperglycemia (70). This observation, as well as the marked reduction in fetal abnormalities associated with effective glucose control during preg-

nancy, supports the role of AGE-mediated teratogenicity in diabetic embryopathy.

AGE-Receptor Systems and Their Regulation

Over the past 15 years, some of the receptor systems mediating AGE-related biology have been elucidated. Several AGE-binding molecules have been described, including a specific AGE-receptor complex composed of R1, R2, and R3 (43, 44, 57, 71–75), receptor for AGE (RAGE) (45, 46), and scavenger receptors such as CD-36 (76) and SCR-II (77, 78).

Evidence indicates that AGE-R1 and -R3, components of the AGE-receptor complex, are largely responsible for AGE-recognition and high-affinity binding (43, 75). AGE-R2 is subject to AGE-induced phosphorylation (44). This suggests it plays a role in signal transduction and cell activation associated with AGE-receptor binding (41, 79).

RAGE, a multiligand member of the immunoglobulin superfamily, though less efficient in AGE endocytosis and turnover (80), is viewed increasingly as an intracellular signal-transducing or pro-inflammatory peptide. In this regard, RAGE may thus be more accurately classified in the family of oxidant-stress-inducing signaling molecules or co-factors. Consistent with the above, in animal models, brief infusion of soluble, truncated RAGE is reported to intercept diverse processes such as endothelial leakage, atherosclerosis, and inflammatory bowel disease (45, 80).

Specific and saturable binding of AGE-BSA (bovine serum albumin) to CD36-CHO (Chinese hamster ovary) cells supports the role of CD-36 as a receptor for AGE-proteins (76). As with RAGE, this scavenger receptor system, which is highly expressed on macrophages, may contribute to AGE-mediated cellular changes, particularly in the context of atherosclerosis and foam cell formation (77, 81, 82).

AGE-receptor systems may be regulated by diabetic factors such as glucose, insulin, AGEs, and reactive oxygen species (ROS) (58, 83–85). Most commonly used measures for assessing AGE-receptor modulation have centered on parameters of cell activation. While the link between AGE-receptor upregulation and cellular activation has been confirmed for a number of the presently identified receptor components, cell activation can also occur by nonreceptor pathways, or by intracellularly generated glycoxidant derivatives, leading to ROS generation and oxidant stress (72, 86).

The macrophage AGE-receptor system, which is the one most closely linked to AGE-turnover, was initially thought to include auto-regulatory switches, allowing it to respond to rising AGE levels and to reduce tissue damage (83, 84). However, the means by which this balance is maintained *in vivo* during times of excessive AGE accumulation remains largely unknown. Possibly, metabolic or genetic factors could tip this balance toward pro-inflammatory events via receptor components tied to signaling (e.g., RAGE, AGE-R2, or AGE-R3) or by receptor-independent mechanisms (43, 45, 72, 73, 81). Alternatively, it could be that delayed AGE processing and disposal, due to lack or malfunction of the endocytotic portion of the AGE-receptor, promotes cytotoxic inflammatory events, leading to organ damage, as shown experimentally in AGE-R3 deficient mice (87, 88). Findings of suppressed AGE-receptor uptake and degradation, combined with high circulating and kidney AGE levels in nonobese diabetic (NOD) mice support this hypothesis (89, 90).

Consistent with the animal data, reduced expression of AGE-R1 in human peripheral blood mononuclear cells (PBM) and in immortalized lymphoblasts from type one diabetic (T1D) patients with severe diabetic nephropathy was associated with elevated serum AGE levels and severe diabetic complications (91, 92). These data indicate that genetic modulation of the receptor system may contribute to organ damage in complication-prone patients, while other patients are spared.

For a number of the AGE-receptor-related molecules, the genomic organization and chromosomal location (45, 93–95), as well as several prevalent gene polymorphisms (96, 97), have come to light. Recently, screening for mutations was performed in 48 insulin-dependent diabetes mellitus (IDDM) patients with or without nephropathy, using single-stranded conformational polymorphism (SSCP) analysis and direct sequencing of allelic polymerase chain reaction (PCR) fragments (96). Thus far, none of the polymorphisms found have exhibited a clear connection to diabetic complications.

Elimination of AGE-Crosslinks

In vivo removal of AGE crosslink is accomplished largely through extracellular proteolysis and by scavenger cells such as tissue macrophages which ingest AGEs via AGE-specific or nonspecific receptors. Furthermore, mesenchymal cells such as vascular endothe-

lium and mesangium seem to function in AGE elimination, as suggested by the increased endocytic activity of vascular endothelium under conditions of high glucose *in vivo* and *in vitro* (98, 99).

After endocytosis, AGE macromolecules undergo intracellular degradation and are subsequently released as low-molecular-weight AGEs, known as “second-generation AGEs” (100), which include newly exposed reactive intermediates (101–105). Renal clearance subsequently limits the toxicity of these compounds. Kidney dysfunction results in failure to clear circulating AGEs, accounting in large part for the marked elevation of serum and tissue AGE levels observed in patients with renal insufficiency (101–105) and probably contributing to the acceleration of extrarenal vascular damage in patients with end-stage renal disease (ESRD).

Intracellular protective systems also limit the accumulation of reactive AGE intermediates. For example, the degradative glyoxalase enzymes metabolize the reactive dicarbonyl methylglyoxal to *S*-D-lactoylglutathione, employing reduced glutathione as a cofactor. The significance of such systems is underscored by studies in which glyoxalase-1 expression was upregulated by gene transfection in endothelial cells, resulting in significant inhibition of AGE-mediated cell abnormalities such as increased endocytosis (106). However, these systems can be attenuated under conditions of excess AGE burden, such as in diabetes, hyperlipidemia, or renal failure, or in organisms consuming an AGE-rich diet.

Recently, lysozyme (LZ), the ubiquitous host-defense protein, has been shown to exhibit high-affinity AGE-binding ($K_d=50$ nM) (107, 108). Interestingly, lysozyme contains a 17-amino-acid-long AGE-binding cysteine-bounded domain termed ABCD loop (107). *In vitro*, LZ suppressed the AGE-enhanced expression of several important modulators of kidney structure and function such as platelet-derived growth factor (PDGF)-B, $\alpha 1$ type IV collagen, and tenascin mRNA in cultured mesangial cells. LZ also normalized the AGE-suppressed MMP-9 gene expression and activity (109). *In vivo* LZ administration to NOD and *db/db* (+/+) mice normalized serum levels of AGE, increased urinary AGE clearance, and improved albuminuria in both diabetic animal models (110, 111). These data have promising implications for the development of preventive interventions to thwart AGE-associated disease processes.

Anti-AGE Strategies

As an understanding of the biology of AGEs has evolved, strategies to forestall their adverse effects have developed. Several approaches which seek to decrease accumulation of exogenous AGE, prevent AGE-formation, reduce AGE effects on cells, and break pre-existing AGE crosslink have been studied.

As diet provides a significant source of exogenous AGE, recent work has focused on determining whether it represents a modifiable risk factor for the development of AGE-induced pathology. One *in vivo* study maintained ApoE-deficient mice on chow which was either high or low in AGE content, for a total of five weeks. After one week, a femoral artery denudation injury was induced and the designated diet was maintained for another four weeks before the injured arteries were examined. Injured arteries in the animals fed the low-AGE diet showed a significant reduction in neointimal area (Fig. 3), and these animals developed less complex lesions with fewer foam cells within the neointima, associated with a 40% decrease in serum AGE levels (112).

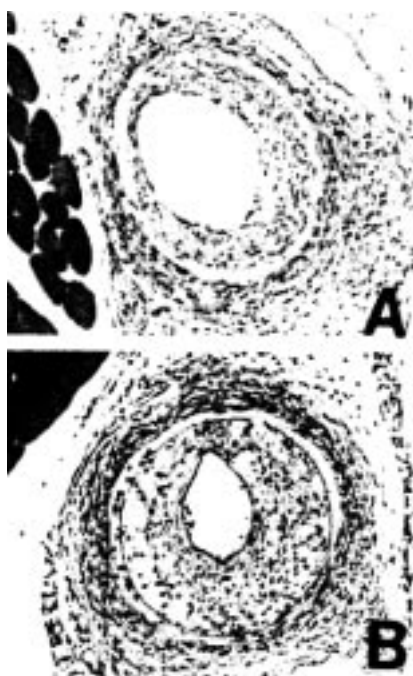


Fig. 3. Post-injury arterial restenosis is delayed in ApoE-deficient diabetic mice fed low-AGE diet. Cross-sections of injured arteries four weeks after transluminal endothelial denudation. The injury was inflicted one week after the mouse began a specific diet, which was continued until the time of sacrifice. **Panel A** represents the injured region of a mouse on the low-AGE diet while **Panel B** represents that of a mouse on the high-AGE diet. (CME staining; magnification $\times 200$).

Similarly, Apo-E-deficient streptozocin-diabetic mice which were fed a diet with AGE content tenfold lower than that of regular rodent chow had significantly suppressed aortic atherogenesis compared to animals on the regular chow, despite the persistence of elevated lipid levels. This was associated with a significant reduction in serum AGE levels (5).

A recent study of human subjects compared the effects of two diets which differed by 6-fold in their AGE content but were otherwise nutritionally equivalent. Eleven diabetic subjects with normal renal function were enrolled in the crossover study; they followed each diet for a two-week period separated by a two-week washout. After two weeks on the low-AGE diet, there was a significant decrease in fasting serum AGE levels. Furthermore, blood mononuclear cell expression of tumor necrosis factor (TNF)- α mRNA and serum levels of vascular cell adhesion molecule-1 (VCAM-1), which were significantly higher at the end of the high-AGE diet period, decreased by approximately 30–50% at the end of the low-AGE diet period (113). This reduction in levels of inflammatory mediators in humans, along with the animal data above (5, 112), underscores the potential role of a low-AGE diet in the primary prevention of atherosclerosis as well as in the prevention of restenosis after coronary angioplasty.

Other strategies in the prevention of AGE-related disease have concentrated on pharmacological interventions. The terminal amino group of the small nucleophilic hydrazine compound aminoguanidine, by virtue of its low pKa, reacts specifically with glucose-derived reactive intermediates to prevent crosslinking (114). Aminoguanidine has been shown to prevent diabetes-related vascular complications in experimental animals (23, 61, 62, 115–122). In humans, a phase I clinical trial of aminoguanidine measured advanced glycation-modified hemoglobin (AGE-Hb) in treated and untreated diabetic subjects and found that AGE-Hb, as well as LDL, was significantly reduced in the treated group (25). The fact that glycosylated hemoglobin values were not affected by aminoguanidine treatment underscores the specificity of aminoguanidine for inhibition of post-Amadori, advanced-glycation reactions. Other AGE-inhibiting drugs have been under development, including the thiazolidine derivative OPB-9195, which has been shown to prevent the progression of diabetic glomerulosclerosis in rats (123). One strategy for negating adverse AGE effects has been the employment of neutralizing

antibodies against glycated albumin. This has been shown to prevent BM thickening in diabetic db/db mice without affecting the glycemic status of the animals (60). Another approach has employed lysozyme linked to a sepharose matrix facilitating the selective depletion of AGEs from sera or dialysate in diabetic patients with kidney disease (124).

A recent therapeutic aim has been to “break” the irreversible intermolecular crosslinks in tissue and render them renally excretable (125, 126). N-phenyl-thiazolium bromide (PTB) attacks covalent carbon-carbon bonds of dicarbonyl-derived crosslinks *in vitro* (125). The AGE-breaker ALT-711 reverses AGE-mediated vascular stiffness and distensibility in diabetic rats (127). It is hoped that these and other innovations in AGE biology will empower humanity to forestall the ubiquitous disease processes associated with diabetes and with aging.

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