



## ADVANCED GLYCATION END PRODUCTS, THEIR RECEPTORS AND DIABETIC ANGIOPATHY

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**SUMMARY** - The role of chronic hyperglycemia in the development of diabetic microvascular complications and in neuropathy has been clearly established by intervention studies. However, the biochemical or cellular links between elevated blood glucose levels, and the vascular lesions remain incompletely understood. This review focuses on the consequences of hyperglycemia on the formation of advanced glycation end-products (AGEs), and on the role of AGEs and of their specific receptors (RAGE) in the functional and anatomical alterations of the vascular wall. AGEs are formed during the Maillard reaction by the binding of aldoses on free NH<sub>2</sub> groups of proteins, which, after a cascade of molecular rearrangements, result in molecules of brown color and specific fluorescence. Experimental studies have indicated that the binding of AGEs to RAGE activates cells, particularly monocytes and endothelial cells. Activated endothelial cells produce cytokines, and express adhesion molecules and tissue factor. The role of AGEs in increased oxidative stress, and in the functional alterations in vascular tone control observed in diabetes, in part related to a reduction in nitric oxide, is also discussed. The microvascular retinal, glomerular and nerve lesions induced by experimental diabetes in animals are prevented by an inhibitor of AGEs formation, aminoguanidine. The administration in diabetic animals of recombinant RAGE, which hinders AGEs-RAGE interaction, prevents hyperpermeability and vascular lesions. These data suggest a central role of AGEs and RAGE in the development of chronic complications of diabetes.

**Key-words:** diabetic angiopathy, hyperglycemia, advanced glycation end-products, receptor for advanced glycation end-products (RAGE), glycoxidation.

**RÉSUMÉ** - Produits de la glycation avancée et angiopathie diabétique.

Les mécanismes responsables des lésions micro et macrovasculaires et nerveuses observées au cours du diabète sucré ont fait l'objet de multiples hypothèses, la majorité d'entre elles essayant d'établir un lien entre l'hyperglycémie et l'angiopathie. Les travaux récents mettent l'accent sur le rôle délétère des produits de la glycation avancée (AGEs), conséquence de l'hyperglycémie. Lors de la réaction de Maillard ou glycation, les oses se lient au NH<sub>2</sub> libre des lysines puis une succession de réactions d'oxydation et de réarrangements moléculaires aboutit aux AGEs. La liaison des AGEs à leur récepteur spécifique (RAGE), entraîne une activation cellulaire en particulier endothéliale et macrophagique, qui produit des cytokines et du facteur tissulaire. Les AGEs sont ainsi à l'origine de formes réactives de l'oxygène et provoquent des modifications du contrôle du tonus vasculaire. Les lésions vasculaires observées chez les animaux diabétiques peuvent être prévenues par des inhibiteurs de la formation d'AGEs ou en empêchant l'interaction AGE-RAGE par l'injection de RAGE recombinant. L'ensemble de ces faits expérimentaux suggère le rôle central des AGE dans le déterminisme de l'angiopathie diabétique.

**Mots-clés :** angiopathie diabétique, hyperglycémie, produits de la glycation avancée, récepteurs des produits de la glycation avancée (RAGE), glyco-oxydation.

The links between chronic hyperglycemia and the development in long-term duration diabetes mellitus of micro- and macrovascular complications and of neuropathy have been evidenced by the results of intervention trials in type 1 [1], and in type 2 diabetes [2]. However, the biochemical pathway(s) between chronic hyperglycemia and functional alterations, and tissue damage remain incompletely understood. Several mechanisms have been proposed as candidates for explaining these links [3-5]. They include: 1) hyperactivity in sorbitol-aldose reductase pathway 2) increase in non-enzymatic glycation of proteins, with irreversible formation and deposit of reactive advanced glycation end-products (AGEs) 3) hyperactivity of isoform(s) of protein kinase C (PKC) and 4) increased oxidative stress.

The present review will focus on AGEs, and on their specific receptors (RAGE) in diabetes, and on their role in the development of diabetic long-term complications.

## BIOCHEMISTRY OF AGEs

Advanced glycation end products (AGEs) is a class of complex products. They are the results of a reaction between carbohydrates and free amino group of proteins. The AGEs are in fact the result of glycooxidation but as shown recently may be an end product of lipid oxidation (Fig. 1) [6, 7]. Most of the AGEs are very unstable, reactive compounds and the end products are difficult to completely analysed (Fig. 2). The brown colour of the AGEs is probably related to the name of melanoidins initially proposed by Maillard. AGEs can be formed in several conditions during fermentation, cooking or just oxidation in the atmosphere (Table I).

AGEs are toxic and can induce mutagenesis of bacteria. They are formed in excess during aging,

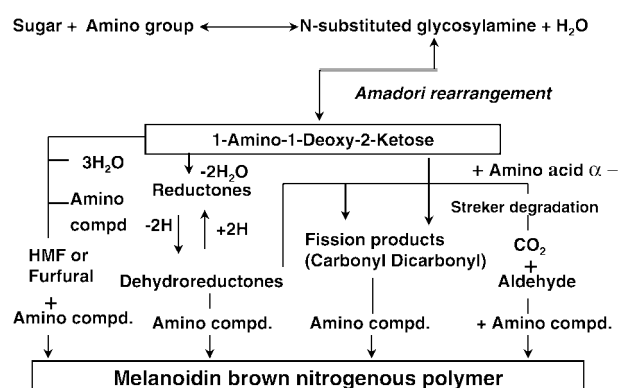


FIG. 2. Initial steps of glycation.

diabetes mellitus and renal failure [8]. The best chemically characterised AGEs compounds found in human are pentosidine [9] and carboxyl methyl lysine (CML) (Fig. 3) [10]. During the Maillard reaction, which results in AGEs formation, intermediate products are the results of dehydration, molecular rearrangement which lead to carboxyl residue as 3 deoxyglucosone which reacts with free amino groups. These reactions result in crosslinked proteins. Degradation of DNA

TABLE I. Exogenous AGEs [11-13].

Food	AGE content u/100 g	
Cereal	193,400	
Pastry	425,740	
Cake	838,400	
Duck skin	6 259,000	

Condiments	AGE u/15 ml	
Maple sirup	795	
Brown rice vinegar	2,100	
Soy sauce	8,700	

Beverage	AGE u/250 ml	Sugar g/25 cl
Soda	475	26
Orange juice	600	23
Tea	2,025	0
Coffee	2,200	0
Classic coca-cola	8,500	27
Diet coke	9,500	0

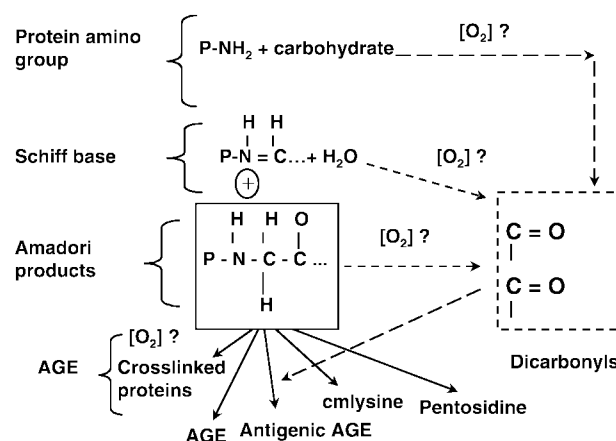


FIG. 1. Reaction of Maillard (in vitro formation of antigenic AGEs).

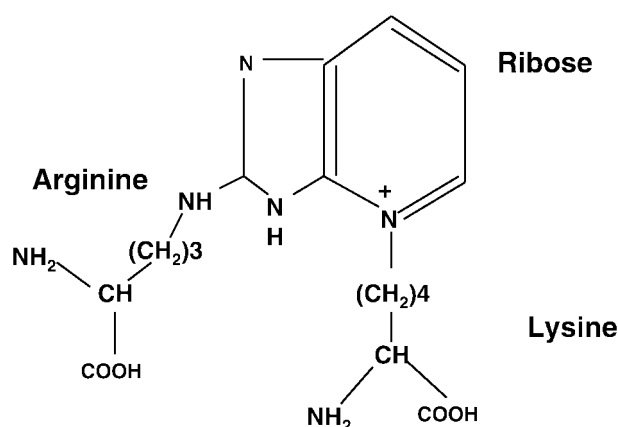


FIG. 3. Chemical structure of pentosidine.

TABLE II. AGE (imidazole) content in normal and diabetic erythrocytes.

	Normal subjects n = 10	Diabetic patients n = 33
Imidazole in red blood cells u/mg protein	4.8 ± 0.2	8.7 ± 0.2
HbA <sub>1c</sub> (%)	5 ± 0.1	8.2 ± 0.3
Fructosamine μM	285 ± 5	381 ± 12

and RNA produce free ribose, which represents a source for pentosidine formation [14]. AGEs are present on diabetic red cells [15], glycated hemoglobin being used as a clinical marker of long-term metabolic control, but other proteins and red blood cells (RBC) can be glycated (Table II). Diabetic RBC(b) bearing AGEs bind to RAGE endothelium and are responsible for the increased in vascular permeability and for endothelial cell dysfunction observed in diabetic rats [16, 17].

Arabinose and xylose present in food can also be at the origin of AGEs. Bacterias from the intestine can degrade xylanes from fruits to produce AGEs. In human, xylane is only found in the heart, and not in other tissues.

The formation of AGEs, also called the Maillard reaction, is a succession of chemical reactions which are linked in a complicated network (Fig. 1). The first step is a condensation between an amino group and a carboxyl group to form a Schiff base. This reaction is followed by a molecular rearrangement and the formation of a N-substituted glycosylamine which lead to

Amadori products (1-amino, 1-deoxy, 2-ketose). The AGEs originate from Amadori products.

Glyoxal and methyl-glyoxal can be formed by glucose auto-oxidation and by products from glycolipids which reacted which arginine or lysine resulting in the formation of:

- N-(carboxy-alkyl/lysine), N-(carboxylmethyl/lysine),
- N-(carboxyl) ethyl/lysine, Imidazole, Glyoxal lysine dimer (Gold), Methyl glyoxal lysine dimer (Mold).

## ■ ADVANCED GLYCATION END PRODUCT RECEPTORS

The first structures were identified as possible AGE receptors using radiolabelled AGE proteins. Human and murine monocytes, lymphocytes bind specifically AGEs with a dissociation coefficient between 50 and 200 nmol/L. Receptor proteins which bind AGEs, have been isolated from cell membrane and have been purified. They have different apparent molecular weights according to the cell type: 40 Kd for kidney, 36-83 Kd for macrophage cell line, 60-90 Kd for liver cells.

AGEs binding protein have been purified from endothelial cells and characterised. Two polypeptides were obtained from pulmonary endothelial cells, one was described as the receptor for AGEs (RAGE) and the second has a very high homology to lactoferrin (LFI) [18].

RAGE in a truncated form has a molecular weight of 35 Kd and belongs to the immunoglobulin superfamily. The protein possesses one extracellular region, one V and two C domains and a short cytoplasmic tail. RAGE gene is located on chromosome 6 in the MHC region (6p 21-3).

Human, rat and bovine RAGE have a high degree of homology, but slight differences in glycosylation sites and susceptibility to proteases may explain their different pharmacological parameters [19, 20].

RAGE has also some homology with molecules of the immunoglobulin superfamily (MUC, CD20). RAGE is expressed by different cell types: monocyte/macrophage, T-lymphocytes, endothelial cells, smooth muscle cells, mesangial cells, neuronal cells. RAGE expression is potentiated by hyperglycemia or TNF $\alpha$  treatment. RAGE binds different ligands such as amyloid,  $\beta$  amyloid substances or calgranulin polypeptides [21]. Carboxylmethyl lysine (CML) is the AGE which after binding to RAGE [22], is a stronger inducer of vascular cell adhesion molecule (VCAM-1) [23]. RAGE-LFI complex at the cell surface mediated AGE-albumin transcytosis and extravasation. Galectin 3 binds also carbohydrates and is present on lymphocytes, macrophages, smooth muscle cells, and fibroblasts.

## ■ MECHANISMS OF THE TOXIC EFFECTS OF AGEs IN DIABETES

### AGEs, extracellular matrix, and vessel wall components

Capillary basement membrane thickening and hypertrophy of extravascular matrix are common features of diabetic microvascular complications. The link between high plasma glucose levels and tissue damage is due, at least in part, to the formation and accumulation of AGEs in tissues [24]. AGEs accumulate in extracellular matrix proteins as a physiological process during aging [25-27]. However, this accumulation happens earlier, and with an accelerated rate in diabetes mellitus than in non-diabetic individuals. [26, 28]. Increased serum and tissue levels of AGEs, due to a reduced removal by kidney, have been evidenced in end-stage renal failure [29-31] and are more important in diabetic than in non-diabetic patients [30-31]. A highly significant correlation has been shown between the importance of the AGEs deposits and the severity of diabetic complications [32]. *In vitro* and *in vivo* studies have indicated that AGEs induce irreversible cross-links in long-living matrix structural proteins, such as type IV collagen, laminin, and fibronectin [32, 33]. AGEs are implicated in the basement membrane thickening through these alterations, *via* a reduction in susceptibility of matrix proteins to proteolytic degradation [34]. These architectural changes alter also the functional properties of the basement membrane, including permeability. Advanced glycation of proteoglycans induces a decrease in electronegative charges [35] and therefore modifies selective filtration properties of the basement membrane. Mesangial expansion is an important part of diabetic nephropathy. The role of AGEs in the overexpression of TGF- $\beta$ , which has been implicated in the pathogenesis of diabetic vasculopathy and of vascular remodeling, has been studied in a model of mesenteric vessels of streptozotocin-induced diabetic rat [36]. Vascular hypertrophy was observed, together with an increase in TGF  $\beta$ 1 and in  $\alpha$ 1 (IV) collagen gene expression. AGEs and extracellular matrix were present in abundance in diabetic, but not in control rats. Treatment of diabetic rats with the AGEs formation inhibitor aminoguanidine results in a significant reduction in pathological changes and in overexpression of TGF  $\beta$ 1 and  $\alpha$ 1 collagen genes.

### AGEs and oxidation

An important part of tissue damage and of cell death associated with chronic hyperglycemia, and diabetes is mediated by free radicals. In hyperglycemic diabetic patients, exaggerated oxidative stress is due both to an excess in free oxygen species production, secondary to increased oxidation of substrates (sugars, non-saturated fats, and glycated proteins), to increased

glucose auto-oxidation, and to a decrease in antioxidants. In animal models of diabetes, hyperproduction of free radicals is responsible for endothelial dysfunction, *via* a decrease in NO (nitric oxide) production, thus decreasing vasorelaxation of smooth muscle cells [37]. The links between oxidative stress and AGEs may explain in part the relation between hyperglycemia and both endothelial dysfunction and tissue damage. Oxidized LDL are responsible for decreased NO production, by a reduction in NO synthase [38]. AGEs quench the NO, and thus contribute to defective vasodilation observed in animal models [39]. However, some data obtained in animal models of diabetes treated with aminoguanidine do not confirm a role of AGEs in inducing abnormalities in arteriolar dilation [40]. AGEs also increase susceptibility of LDL to oxidation [41, 42]. AGEs induce apoptosis in cultured human umbilical vein endothelial cells [43]. Experimentally, we have shown that the interaction between AGEs and RAGE induces an activation of oxidative stress, and stimulates the production and release of cytokines, amplifying thus tissue damage [44].

### Consequences of engagement of the receptor RAGE

Activation of NADPH oxidase, by which AGEs-mediated generation of reactive oxygen intermediates (ROIs) and triggering of signal transduction events lead to altered gene expression in endothelial cells and macrophages *via* RAGE. The finding that enhanced expression of tissue factor in AGEs-stimulated macrophages retrieved from gp91<sup>phox</sup> null mice was suppressed compared to wild-type macrophages, strongly suggests important roles for NADPH oxidase in AGEs-mediated processes [45]. Importantly, recent studies indicating that endothelial cells express a gp91<sup>phox</sup>-containing NADPH oxidase support our hypothesis that activation of this enzyme provides source of ROIs upon AGEs engagement of RAGE in endothelial cells.

In those studies by Golligorsky *et al.*, it was shown that NADPH oxidase was a major source in the arterial wall, as its activation was associated with impaired bioavailability of endothelium-derived NO [46].

RAGE is a multiligand receptor of the immunoglobulin superfamily. In addition to AGEs, RAGE serves as a cell surface receptor for amyloid  $\beta$  peptide (A $\beta$ ), a cleavage product of the  $\beta$ -amyloid precursor protein which accumulates in Alzheimer's disease and  $\beta$  sheet fibrils [46-48]. *In vivo*, blockade of RAGE in a murine model of systemic amyloidosis suppressed amyloid-induced nuclear translocation of NF- $\kappa$ B and cellular activation [48]. RAGE is also a signal transduction receptor for EN-RAGES, and related members of the S100/calgranulin family of proinflammatory cytokines [49]. The S100/calgranulin family is comprised of closely-related polypeptides released from activated inflammatory cells, including polymorphonuclear leukocytes, peripheral blood-derived mononuclear phago-

cytes and lymphocytes [50]. Their hallmark is accumulation at sites of chronic inflammation, such as psoriatic skin disease, cystic fibrosis, inflammatory bowel disease, and rheumatoid arthritis. Ligation of RAGE by ENRAGEs mediated activation of endothelial cells, macrophages and lymphocytes. *In vivo*, blockade of RAGE suppressed inflammation in murine models of delayed-type hypersensitivity and inflammatory bowel disease. In parallel with suppression of the inflammatory phenotype, inhibition of RAGE-S100/calgranulin interaction decreased NF- $\kappa$ B activation and expression of proinflammatory cytokines in tissues, suggesting that receptor blockade changed the course of the inflammatory response. Previous studies further indicated that RAGE was likely a receptor for amphoterin, a molecule linked to neurite outgrowth in developing neurons of the central and peripheral nervous system [51]. These studies suggested that amphoterin-RAGE was linked to cellular migration and invasiveness. Consistent with this concept, the expression of amphoterin and RAGE is increased in murine and human tumors. Blockade of RAGE *in vivo* suppressed local growth and distant spread of implanted tumors, as well as the growth of tumors forming endogenously in susceptible mice. Consistent with an important role for RAGE-mediated signal transduction in these processes, blockade of RAGE/RAGE signaling on amphoterin-coated matrices suppressed activation of p44/42, p38 and SAPK/JNK kinases [52].

In settings characterized by increased accumulation and expression of RAGE and its ligands, such as diabetic atherosclerotic lesions and periodontium, chronic disorders such as rheumatoid arthritis and inflammatory bowel disease, and Alzheimer disease, enhanced inflammatory responses have been linked to ongoing cellular perturbation. One consequence of ligand-RAGE-mediated activation of MAP kinases and NF- $\kappa$ B is increased transcription and translation of vascular cell adhesion molecule (VCAM-1). At the cell surface, endothelium stimulated by a range of mediators, such as endotoxin, tumor necrosis factor  $\alpha$  (TNF  $\alpha$ ), AGEs display increased adhesion of proinflammatory mononuclear cells, at least in part, *via* VCAM-1. Recent studies have suggested that the proinflammatory effects of VCAM-1 are not limited to cellular adhesion events, as binding of ligand to VCAM-1 in endothelial cell lines and primary cultures induced activation of endothelial NADPH oxidase, a process shown to be essential for lymphocyte *migration* through the stimulated cells. These findings suggest that activation of RAGE at the cell surface may initiate a cascade of events including activation of NADPH oxidase and a range of proinflammatory mediators such as VCAM-1.

In diabetes, although oxidant stress responses are essential to eliminate pathogenic periodontal pathogens, ongoing AGE/EN-RAGE-mediated cellular activation in infected periodontium has been linked to

increased generation of proinflammatory cytokines and tissue-destructive matrix metalloproteinases, processes leading to destruction of alveolar bone [53].

## ■ AGEs, RAGE, AND COMPLICATIONS OF DIABETES

### Diabetic Retinopathy

Diabetic retinal complications result from retinal capillaries functional and morphological alterations: increased permeability to albumin and macromolecules, vascular dysfunction, loss of pericytes, and basement membrane thickening. The arguments in favor of a central role for AGEs in these alterations have been discussed above. These alterations lead to macular edema secondary to the leakage of macromolecules, and progressive capillary closures related to microthrombosis. Capillary closures are responsible for non-perfused areas (ischemic retinopathy), which induce the secretion of Vascular Endothelial Growth Factor (VEGF) and the development of neo-vessels (proliferative retinopathy). In diabetic patients, pentosidine skin concentrations have been shown to be associated with the development of proliferative retinopathy [54]. FFI, CML and total fluorescence also increase in parallel with the increasing severity of retinopathy [44]. The oxidatively formed CML is increased in diabetic rats both in neuroglial and vascular retinal components, while imidazole-type AGEs are restricted to microvessels, co-localizing with the expression of RAGE [55]. The preventive effect in animal models of the AGE-formation inhibitor, aminoguanidine, supports the role of AGEs in the development of diabetic retinopathy [56-60]. In rats with streptozotocin-induced diabetes, treatment with aminoguanidine prevents diabetic retinopathy, resulting in an 80% reduction in pericytes loss, in an absence of microaneurysms development, and of endothelial cell proliferation. The accumulation of AGEs in pre-capillary arterioles is inhibited by treatment with aminoguanidine [56]. Aminoguanidine prevents the development of retinopathy in the diabetic spontaneous hypertensive rat (SHR), and completely suppresses the deposit of PAS positive material in arterioles, and microthrombosis formation [58]. Aminoguanidine is also effective in secondary prevention of diabetic retinopathy in rats, when starting treatment 6 months after diabetes induction by streptozotocin [59]. Other studies have addressed the potential mechanisms of AGEs toxic effects on retinal components. *In vitro* toxic effects of high glucose concentrations on capillary pericytes are inhibited by aminoguanidine, suggesting a role for AGEs in this effect [60]. Evidence of this role relies on the results of studies indicating that the deleterious effects of AGEs on retinal capillary pericytes and endothelial cells are inhibited by RAGE-antibodies [61]. The role of AGEs

mediated by VEGF in vascular dysfunction related to pseudo-hypoxemic changes has been suggested by recent experiments [62]. These effects are prevented by neutralizing VEGF antibodies and markedly reduced by aminoguanidine. Moreover, an association between accumulation of CML in human diabetic retina, proliferative and non-proliferative retinopathy, and expression of VEGF has been reported [63]. The role of AGEs has been analysed in diabetic rats, treated or not with aminoguanidine. A 75% decrease in basement membrane thickening has been observed in the retinal capillaries of animals treated with aminoguanidine, compared control animals [59]. *In vitro*, an increased vascular permeability is associated with endothelial endocytosis. An increased endocytosis in retinal vascular endothelial cells is observed with high glucose concentrations, and is reduced by aminoguanidine, suggesting also that this alteration is mediated by AGEs [64]. In diabetic rats, aminoguanidine treatment exerts a preventive effect on blood-retinal barrier alterations, such as hyperpermeability, assessed by vitreous fluorophotometry [65].

### Diabetic Nephropathy

Diabetic nephropathy is characterized by abnormal deposits of matrix material in the glomerular mesangium, leading to glomerulosclerosis. Accumulation of collagen-related proteins in the glomerular extravascular matrix causes progressive capillary occlusion. An increase in serum and tissue AGEs levels in parallel with the severity of renal function impairment has been observed by Makita *et al.* [66]. Incipient nephropathy is associated with skin levels of FL, CML, pentosidine and of total fluorescence [66]. Skin AGEs levels detected by immunochemistry correlate with severity of nephropathy and increase in early stages of renal involvement [67]. A longitudinal study in type 1 diabetic patients followed during 2.5 years has indicated the predictive value of AGE serum levels for the development of the morphological changes in the kidney [68]. A direct inhibitory effect of glycated albumin has been observed on mesangial cell proliferation [69]. AGEs infusion in normal rats during 5 months results in increased AGEs renal tissue content and in alterations similar to diabetic nephropathy: increase in glomerular volume, in basement membrane thickness and in mesangial extracellular matrix [70]. These changes parallel with an increase in albumin and proteins urinary excretion. Co-treatment with aminoguanidine markedly limits structural and functional alterations. The *in vitro* data suggest that AGEs alter glomerular structure and function, leading to diabetic glomerulosclerosis. A key role in AGE metabolism has been documented for the kidney [71]. An effect of AGEs on renal gene expression has been evidenced [72]. Administration of AGE-modified albumin during 4 weeks to normal mice induces glomerular hypertrophy as well as an increase in glomeru-

lar extracellular matrix,  $\alpha 1$  (IV) collagen, laminin B1 and transforming growth factor  $\beta 1$  (TGF  $\beta 1$ ) mRNA levels. This response seems to be specific to AGEs because all these changes can be prevented by aminoguanidine co-administration. Human mesangial cells express a receptor for AGEs. The exposure of normal mouse mesangial cells to AGE albumin induces an increase in collagen IV mRNA expression and secretion. This increase in collagen IV production could be mediated, at least in part, by PDGF [73].

The role of AGEs in diabetic nephropathy development has been investigated in streptozotocin-induced diabetic rats compared to non diabetic control rats and diabetic rats co-treated with aminoguanidine [74]. After 32 weeks, diabetic rats exhibit increased fluorescence in glomeruli and renal tubes, which was prevented by aminoguanidine. Diabetic rats develop albuminuria over the 32-week period. This increase was attenuated by aminoguanidine, but not by antioxidant and by aldose reductase inhibitor. All these data emphasize the role of AGEs and of the interaction of AGE modified proteins with diabetic mesangial cells in glomerulosclerosis development. Other inhibitors of renal AGEs accumulation, as ALT-946, are also effective in preventing and retarding diabetic nephropathy in animal models [75]. However, studies with aminoguanidine (pimagedine) are no more in progress in human diabetics at the present time.

### Macroangiopathy

Macrovascular disease, and particularly coronary heart disease, is the main cause of premature death in diabetic patients. Trials comparing intensive and conventional metabolic control in type 1 [1], and in type 2 diabetes [2] have shown a reduction in the incidence of cardiovascular events in intensively treated patients. Some studies indicate the role of AGEs in the development of diabetic vascular and coronary heart disease. AGEs has been identified in coronary arteries of diabetic patients, particularly in atheromatous lesions, which suggests a role of AGEs in the accelerated development of arterial disease observed in diabetes [76]. An association between increase skin collagen fluorescence and arterial stiffness as well as an increase in systolic and diastolic blood pressure has also been reported [77]. These biomechanical alterations are associated with an increase in glycation cross-links [78]. In type 2 diabetic patients with coronary heart disease, elevated levels of AGEs and of CML have been reported [79]. In experimental diabetes, aminoguanidine has been shown to inhibit the formation the cross-links of collagen in arterial wall [80]. Decreased arterial and arteriolar elasticity and compliance related to AGEs deposits may contribute to development of systemic hypertension, which, together with arterial stiffness, may result in abnormal shear stress predisposing to endothelial injury and early atherogenesis. Finally, treatment of diabetic

mice prone to accelerated atherosclerosis by soluble extracellular domain of RAGE prevents atherosclerotic lesions, independently of glycemic and lipids control [81]. These results indicate that AGEs and their interaction with their receptor RAGE may be involved in the development of accelerated atherosclerosis in diabetes.

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