Glycation Products and the Pathogenesis of Diabetic Complications

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Glucose irreversibly modifies long-lived macromolecules by forming AGEs as a function of glucose concentration and time. AGEs cause qualitative and quantitative changes in extracellular matrix components such as type IV collagen, laminin, and vitronectin. These AGE-induced changes can affect cell adhesion, growth, and matrix accumulation. AGE-modified proteins also alter cell function by interacting with specific receptors on macrophages and endothelial cells, inducing changes that promote matrix overproduction, focal thrombosis, and vasoconstriction. DNA and nuclear proteins also may be targets for AGE damage. The persistence of accumulated AGEs during periods of normal glucose homeostasis may explain the phenomenon of hyperglycemic memory. Pharmacological inhibition of in vivo AGE formation by aminoguanidine prevents or ameliorates diabetic retinopathy, nephropathy, and neuropathy in animal models. These data suggest that aminoguanidine and other AGE inhibitors have a potential therapeutic role in the treatment of diabetic patients.

COMPLICATIONS CAN DEVELOP AND PROGRESS DURING POSTHYPERGLYCEMIC EUGLYCEMIA

— Chronic hyperglycemia appears to be the central initiating factor responsible for the development of all diabetes-specific complications. The extent and rate of progression of diabetic microvascular disease correlate strongly with both duration and magnitude of hyperglycemia, although factors such as genetic determinants of tissue response to hyperglycemic injury and hypertension clearly influence the clinical course (1–3). The epidemiological relationship between hyperglycemia and non–diabetes-specific macrovascular disease is complicated by the many other factors that influence atherogenesis in nondiabetic individuals. Nevertheless, in the Framingham Heart Study, a strong independent association was found between hyperglycemia and macrovascular disease in older women (4), and in the Gothenberg study, hyperglycemia was found to be the most important coronary risk factor in elderly men (4a). Acute hyperglycemia has been associated with various reversible biochemical abnormalities, including changes in polyol pathway activity, an increased rate of de novo diacylglycerol synthesis and protein kinase C activation, decreased cellular uptake of *myo*-inositol, which leads to reduced Na/K-APase activity, and impaired endothelium-dependent arterial relaxation (5). These reversible abnormalities and others yet to be discovered all may play a role in the pathogenesis of complications, but they cannot account for a major characteristic of diabetic complications—hyperglycemic memory.

Hyperglycemic memory refers to the persistence or progression of hyperglycemia-induced microvascular alterations during subsequent periods of normal glucose homeostasis. The most striking example of this phenomenon is the development of severe retinopathy in histologically normal eyes of diabetic dogs that occurred entirely during a 2.5-yr period of normalized blood glucose that followed 2.5 yr of hyperglycemia (6). Similarly, when euglycemia is restored by islet transplant after 16 wk of diabetes, retinopathy still develops over the following months in rats (H.P. Hammes, unpublished observations). Hyperglycemia-induced increases in selected matrix gene transcription also persist for weeks after restoration of normoglycemia in vivo, and a less pronounced but qualitatively similar prolongation of hyperglycemia-induced increases in selected matrix gene transcription occurs in cultured endothelial cells (7). Together, these observations imply that hyperglycemia induces prolonged and sometimes irreversible changes in long-lived molecules that persist and cause continued pathological function in the absence of continued hyperglycemia.

HYPERGLYCEMIA PERMANENTLY ALTERS TISSUE MACROMOLECULES THROUGH ACCELERATED AGE FORMATION

— In chemical terms, pathogenic changes induced by antecedent hyperglycemia imply that glucose or glucose-derived metabolites irreversibly modify long-lived extracellular and/or

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AGE, ADVANCED GLYCATION END PRODUCT; HSPG, HEPARAN SULFATE PROTEOGLYCAN; LDL, LOW-DENSITY LIPOPROTEIN; PDGF, PLATELET-DERIVED GROWTH FACTOR; G6P, GLUCOSE-6-PHOSPHATE.

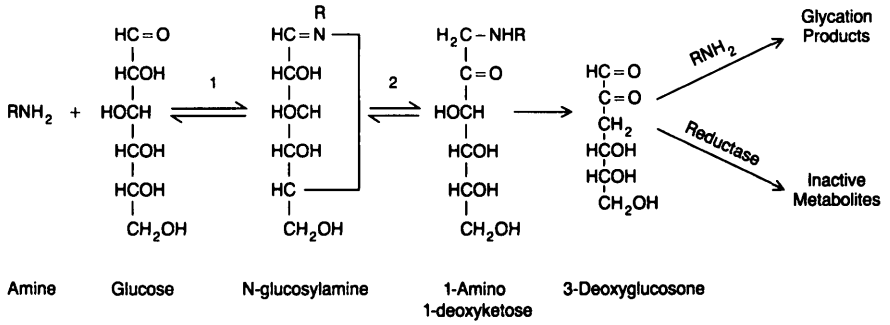


Figure 1—*Formation of AGEs from glucose. Reversible early products can give rise to irreversible advanced products through generation of highly reactive carbonyl compounds such as 3-deoxy-D-glucosone. AGE formation in vivo may be retarded by the action of reductase enzymes.*

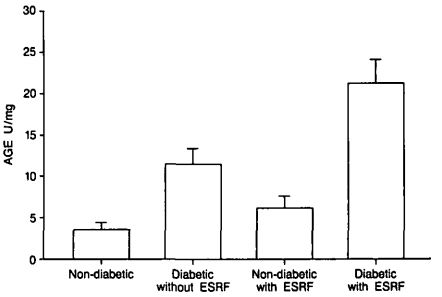


Figure 2—*Level of AGEs in arterial wall collagen determined by a radioreceptor assay (34).*

intracellular macromolecules. The most well-characterized and best understood example of such irreversible modification by sugars is the formation and accumulation of AGEs (8). The formation of irreversible AGEs begins with the nonenzymatic formation of reversible early glycation products (Fig. 1). These products form from the condensation of a sugar aldehyde or ketone with a free amino group via nucleophilic addition, resulting first in the rapid formation of a Schiff base. With glucose and epsilon-amino groups of Lys residues, equilibrium is reached in a matter of hours at a steady-state level that is proportional to ambient glucose concentration. Through acid-base catalysis, these Schiff base adducts then undergo rearrangement to the more stable 1-amino-1-deoxy-D-ketose (Amadori) product. With glucose, equilibrium is reached over several weeks. Thus, even on very long-lived proteins, the total amount of Amadori product is only proportional to the integrated glucose concentration of the preceding 4 wk. After the relatively brief time necessary to attain equilibrium, measured levels of Amadori products reach a constant steady-state value that does not increase as a function of time beyond that point. The Amadori product also is degraded into various highly reactive carbonyl compounds, such as 3-deoxy-D-glucosone and sugar fragmentation products that react again with free amino groups to form various intermediate and advanced glycation products (9–14).

AGEs on extracellular macromolecules are exclusively glucose-derived. Inside cells, AGEs most likely form from various other more highly reactive glycating sugars as well. The formation of covalently modified Hb as a function of the time-integrated extracellular glucose concentration is the best studied example of nonenzymatic glycation inside cells. In vivo glycation of the enzyme alcohol dehydrogenase has subsequently been demonstrated (15). However, glucose has the slowest rate of Schiff base formation of any sugar found in cells, because the rate of Schiff base formation is directly proportional to the percentage of sugar in the open chain form (16). Thus the rate for D-fructose is 7.5 times faster than that for glucose (0.002% open chain), and the rate for D-ribose (0.05% open chain) is 16.6 times faster. Most strikingly, the glycolytic intermediate glyceraldehyde-3-phosphate (100% open chain) forms >200 times more glycated protein than do equimolar amounts of either glucose or fructose (17). For this reason, the elevated levels of this compound that occur during hyperglycemia (17–20) would modify intracellular amino groups at a rapid rate compared with glucose.

AGEs arise from a complex series of dehydrations, rearrangements, and reactions that are poorly understood (21,22). The rate of AGE formation has a nonlinear dependence on sugar concentration. The rate of formation is approximately second order with respect to the concentration of glycated amino groups, suggesting that even modest elevations of glucose significantly increase AGE accumulation (23). Some evidence suggests that AGE formation may be increased markedly by various oxidative processes (24,25).

The chemical structures of several AGEs have been elucidated (14,25–27), but these compounds do not appear to comprise the major fraction of AGEs found in vivo. Studies with antibodies to AGEs suggest that immunologically similar structures form from the incubation of different sugars with different proteins. None of the known AGEs compete for binding to the AGE antibodies (28–31).

AGE formation in vivo may be retarded by the action of reductase enzymes (Fig. 1) that reduce compounds such as 3-deoxy-D-glucosone to less reactive ones (e.g., 3-deoxy-D-fructose). Indirect evidence supporting this concept comes from measurements indicating that several milligrams of 3-deoxy-D-glucosone are formed in the body each day and detoxified by reduction to 3-deoxy-D-fructose (32). Recently, a candidate reductase from liver has been characterized, cloned, and sequenced

(N. Taniguchi, unpublished observations).

Because AGEs are irreversibly attached to macromolecules, the level of AGEs does not decline when hyperglycemia is corrected. Instead, these products continue to accumulate at varying rates over the lifetime of the diabetic tissue component. This relationship with time has been demonstrated in tissues from normal, nondiabetic individuals by using both AGE-specific fluorescence and AGE-specific antibodies (28,33). In diabetic arterial wall collagen (Fig. 2), the level of accumulated AGEs is elevated threefold (34).

AGES CAUSE QUALITATIVE AND QUANTITATIVE CHANGES IN EXTRACELLULAR MATRIX

— AGE formation alters the functional properties of several important matrix components. AGE formation on type IV collagen decreases binding of the noncollagenous NC1 domain to the helix-rich domain, inhibiting lateral association of these molecules into a normal networklike structure. AGE formation on laminin causes decreased polymer self-assembly, decreased binding to type IV collagen, and decreased binding of HSPG (35,36). Decreased binding of anionic HSPG is the primary mechanism responsible for the absence of this component in long-term diabetic basement membrane (37), and this defect appears to be attributable to AGE formation on the HSPG-binding protein vitronectin (38). The absence of HSPG is thought to stimulate a compensatory overproduction of other matrix components, possibly through altered partitioning of growth-regulatory factors (such as basic fibroblast growth factor and plasminogen activator inhibitor-1) between matrix-bound proteoglycans and cells (39,40).

AGE formation on matrix components also may contribute to the evolution of diabetic complications by altering the normal interactions of specific matrix ligands with integrinlike receptors on target cells in affected tissues.

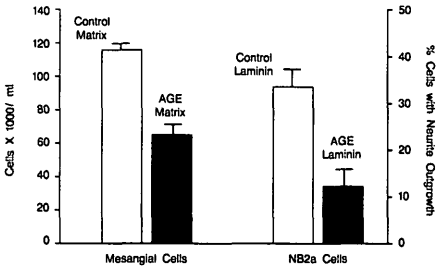


Figure 3—*Inhibitory effect of AGE-modified mesangial matrix (left) and AGE-modified laminin (right) on mesangial cell proliferation and neurite outgrowth, respectively (42,44).*

Modification of the cell-binding domains of type IV collagen causes decreased endothelial cell adhesion, for example (41), whereas modification of the neurite-promoting sequence of laminin inhibits neurite outgrowth by 55–65% (Fig. 3) (42–44). AGE modification of mesangial cell matrix also decreases cell proliferation by 50% (Fig. 3), while increasing fibronectin production, perhaps by altering responsiveness to cytokines such as TGF-β (44–46). These effects, in conjunction with those mediated by specific cell-surface receptors for AGEs themselves (described below), explain in part the increased production of basement membrane material in chronic diabetes.

The progressive occlusion of diabetic vessels involves more than the expansion of extracellular matrix components, however. Extraluminal accumulation of plasma proteins also makes a significant contribution. In vitro and in vivo experiments indicate that short-lived plasma proteins, such as LDL and immunoglobulin G, are chemically bound by reactive AGE precursors on matrix proteins (47–53). More extensive oxidative modification of bound LDL by vascular cells could accelerate the atherosclerotic process in hyperglycemic patients. Once immobilized, plasma proteins cross-linked by AGEs then serve as substrate for additional AGE formation. Decreased degradation of diabetic basement membrane components, as well as increased production, contributes to basement membrane accumulation (54), and this most likely reflects the reduced susceptibility of AGE proteins to protease digestion.

Collagen was the first matrix protein used to demonstrate unequivocally that glucose-derived AGEs form covalent, heat-stable, intermolecular bonds (55,56). Cross-links derived from AGEs were found throughout the collagen molecule, in marked contrast to normal cross-links generated by the enzyme lysyl oxidase, which occur only on two peptides at the NH_2- and COOH-terminal ends of the molecule. The degree of AGE-derived cross-link formation was unchanged after selective enzymatic removal of lysyl oxidase-generated cross-links. Glomerular basement membrane glycated in vitro is similarly more resistant to digestion by pepsin, papain, trypsin, and endogenous glomerular proteases than is normal basement membrane (57).

Matrix accumulation of AGEs may accelerate diabetic vascular occlusion further by blunting the effect of vasodilatory and antiproliferative factors. The endothelium-derived relaxing factor and antiproliferative factor nitric oxide is quenched by AGEs in a dose-dependent fashion. In diabetic animals, defects in the vasodilatory response to nitric oxide correlate with the level of accumulated AGEs and are prevented by inhibition of AGE formation (58). In cell culture, AGEs block the cytostatic effect of nitric oxide on aortic smooth muscle cells and mesangial cells (58A.)

AGE PROTEINS ALTER CELLULAR FUNCTION BY INTERACTING WITH SPECIFIC RECEPTORS

— AGE formation on extracellular matrix components appears to affect target tissue function adversely by altering two types of receptor-mediated interactions with cells. One type of altered interaction (described above) involves the family of cell receptors that binds matrix peptide ligands. The other type of altered inter-

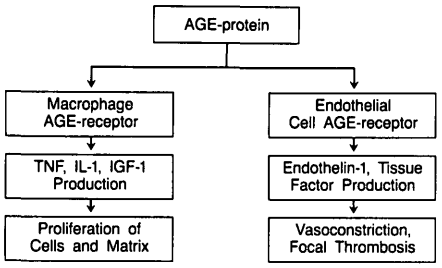


Figure 4—*Schematic of the mechanisms by which AGE-protein binding to specific receptors on macrophages and endothelial cells may cause pathological changes in diabetic vessels.*

action of AGE matrix with cells involves specific cell receptors for a common structural element in AGEs.

This high-affinity receptor was first identified on monocytes and macrophages (59), which are critical cells in the development of atherosclerotic plaques. There are 1.5×10^5 macrophage receptors for AGE-modified proteins per cell, with a binding affinity of 1.75×10^7 M^{-1}. This receptor has a unique biological significance because it is the first receptor that recognizes a posttranslational protein modification known to occur extensively in vivo. When macrophages interact with AGE-modified proteins (Fig. 4), they secrete tumor necrosis factor-α, interleukin-1, and insulinlike growth factor-I in concentrations that have been shown to stimulate glomerular synthesis of type IV collagen and proliferation of endothelial, mesangial, and smooth muscle cells (60,61).

A 90,000-M_r AGE-binding protein has been purified from the murine cell line RAW 264.7 (62), and more recently, two novel and distinct AGE-binding proteins were isolated from rat liver. Both the 60,000- and 90,000-M_r proteins are present on rat monocyte/macrophages, and antisera to either protein blocks AGE binding to macrophages (63). AGE receptors have been identified on glomerular mesangial cells by using antisera against these two proteins, and interaction with AGE proteins increases PDGF-mediated mesangial cell production of type IV collagen, laminin, and HSPG (45,64).

Endothelial cells also express AGE-specific receptors. Ligand binding to this receptor on macrovascular endothelial cells induces two additive procoagulatory changes in the endothelial surface (65). First, tissue factor activity increases (Fig. 4), which activates coagulation factors IX and X through factor VIIa binding. At the same time, a rapid reduction in thrombomodulin activity occurs, which prevents activation of the anticoagulant protein kinase C pathway. In addition to these procoagulatory changes, AGE protein binding to the endothelial cell AGE receptor also induces increased production of the potent vasoconstrictor peptide endothelin-1 (Fig. 4) (66). This effect involves the AP-1 DNA-binding site for transcriptional regulatory proteins, and perhaps activation of the transcription factor NF-κB also (P.P. Nawroth, presented at the Deutschen Diabetes-Gesellschaft, Hannover, Germany, May 28–30, 1992). The consequences of these AGE-induced changes in endothelial function would be excessive vasoconstriction and focal thrombosis (Fig. 4).

Recently, endothelial cell AGE-binding proteins have been isolated and characterized (67). Both a 35,000- and 80,000-M_r protein were purified to homogeneity. The NH_2-terminal sequence of the 80,000-M_r protein was identical to lactoferrin, whereas the 35,000-M_r protein was novel. Antibodies to either protein blocked binding of AGEs, and immunoelectron microscopy suggested that the two proteins were closely associated on the cell surface. An apparent full-length 1.5 kb cDNA for the 35,000-M_r protein has been cloned, which codes for a protein with a 322 amino acid extracellular domain, a 19–amino acid transmembrane domain, and a 43–amino acid carboxyterminal domain. This endothelial AGE-binding protein appears to be a new member of the superfamily of immunoglobulin-related proteins (68).

DNA AND NUCLEAR PROTEINS MAY BE TARGETS FOR AGE DAMAGE— The sustained increase in matrix component mRNA observed in tissues and cells transiently exposed to high glucose concentrations (7,69) may be a consequence of AGE-induced alterations in DNA and nuclear proteins, as well as of AGE-induced alterations in extracellular matrix. Amino groups of both DNA nucleotides and histones react with G6P in vitro. The spectral and fluorescent properties of AGEs formed on DNA are similar to those of AGEs on proteins (70,71). By using class I and class II apurinic endonuclease digestion, both modified nucleotide bases and apurinic/apyrimidinic sites have been detected in DNA damaged by AGEs (72).

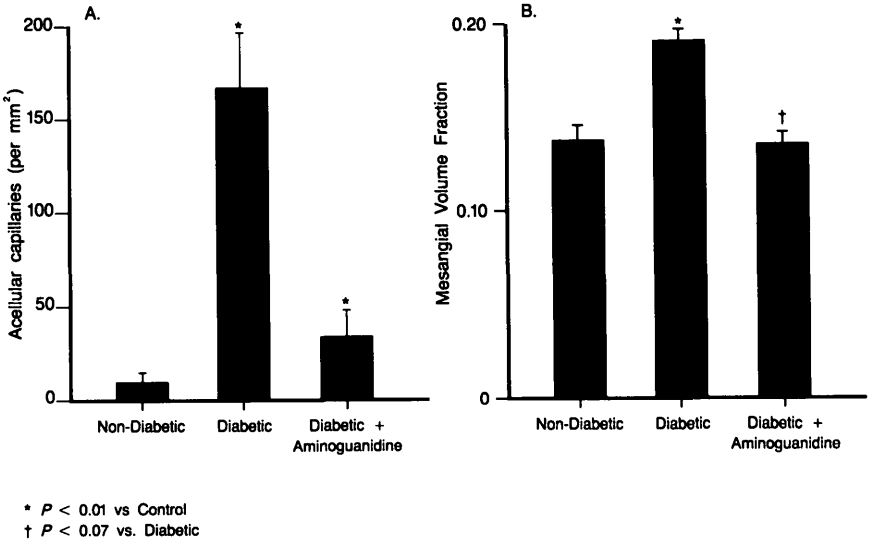
AGE modification of DNA is associated with mutations and altered gene expression in prokaryotic model systems. Glycation of the plasmid pBR322 before transfection into *E. coli* inactivates the plasmid's tetracycline-resistance gene (73). The mutations induced in this gene appear to arise during attempted enzymatic repair of DNA segments that have been modified by AGEs, because glycated pBR322 functions normally in mutant bacteria that lack the repair enzyme uvrABC excision nuclease. This ATP-dependent nuclease hydrolyzes pBR322 DNA modified by AGEs in vitro (E. Mullokandov, W. Franklin, M.B., unpublished observations). Glycation of plasmid DNA after transfection also causes mutations and gene inactivation (74). When pAM006 is transfected into *E. coli* mutants that accumulate G6P, inactivation of the plasmid's β-galactosidase gene is proportional to the concentration of glycating sugar.

A similar phenomenon may occur in eukaryotic cells. When human endothelial cells are cultured in 30 mM glucose, increases in single-strand breaks and in DNA repair synthesis occur (75). Increased single-strand breaks in DNA

Aminoguanidine

$$H_2N-NH-C(=NH^+_2)-NH_2$$

Figure 5—*Structure of aminoguanidine, an inhibitor of AGE formation.*



* $P < 0.01$ vs Control
† $P < 0.07$ vs. Diabetic

Figure 6—*Effect of aminoguanidine, an inhibitor of AGE formation, on pathognomonic structural abnormalities of long-term diabetes in retina (A) and glomerulus (B) (78,79).*

also occur in lymphocytes from chronically hyperglycemic diabetic patients.

PHARMACOLOGICAL INHIBITION OF AGE FORMATION PREVENTS DIABETIC COMPLICATIONS IN ANIMAL MODELS— To test the hypothesis that AGE formation and its consequences play an important role in the pathogenesis of diabetic complications in vivo, pharmacological agents that inhibit AGE formation were sought (55). The prototype inhibitor selected was the nucleophilic hydrazine aminoguanidine (Fig. 5). This compound effectively inhibits AGE formation in vitro, preventing AGE-induced cross-links in collagen, structural alterations in laminin, and AGE matrix-induced defects in mesangial cell proliferation (36,44,55). Aminoguanidine inhibits AGE formation primarily by reacting with Amadori-derived products such as 3-deoxy-D-glucosone in solution, rather than forming adducts with peptide-bound early glycation products (76). Importantly, aminoguanidine does not interfere with the formation of normal, lysyl oxidase-derived collagen cross-links, as determined both indirectly (55) and by direct quantitation of the lysyl oxidase-dependent cross-link products hydroxylysinonorleucine and dihydroxylysinonorleucine (77).

In vivo, the effect of aminoguanidine on diabetic complications has been examined in retina, renal glomerulus, and peripheral nerve, using animal models (Table 1). In the diabetic retina, aminoguanidine treatment for 26 wk prevented a 2.6-fold accumulation of AGEs at sites of PAS-positive protein accumulation, the branching sites of precapillary arterioles (78). The effect of this AGE inhibition was evaluated after 75 wk of diabetes. Untreated diabetic animals developed the characteristic pathological features of background diabetic retinopathy in humans, saccular capillary microaneurysms, and an 18.6-fold increase in the number of acellular capillaries. In contrast, aminoguanidine-treated diabetic animals had no microaneurysms and an 80% reduction in the number of acellular capillaries (Fig. 6A). Abnormal endothelial cell proliferation was prevented completely, and pericyte dropout was reduced significantly.

Aminoguanidine treatment of diabetic animals for 32 wk likewise prevented accumulation of AGEs in the renal glomerulus (79). Untreated diabetic animals developed the characteristic structural and functional features of human diabetic nephropathy, increased fractional mesangial volume, and albuminuria. Increased fractional mesangial volume is the morphological parameter that correlates most closely with extent

Table 1—*Effects of aminoguanidine treatment on diabetic complications*

Retina	Kidney	Nerve
↓ Intercapillary protein deposits	↓ Urinary albumin excretion	↓ Axonal atrophy
↓ Microaneurysms	↓ Basement membrane thickening	↑ Motor nerve conduction velocity
↓ Acellular capillaries	↓ Mesangial expansion	↑ Sensory nerve conduction velocity

of clinical disease in patients (80,81), and this abnormality was completely prevented in diabetic animals by aminoguanidine treatment (Figure 6*B*). Concomitantly, a near normalization of the associated 10-fold increase in urinary albumin excretion occurred in the treated diabetic group. Aminoguanidine also ameliorated albuminuria in hypertensive diabetic rats without affecting blood pressure (82). In another animal model of diabetic nephropathy, aminoguanidine treatment for 9 mo prevented glomerular basement membrane thickening (83).

Functional and structural abnormalities of the diabetic peripheral nerve also are improved by aminoguanidine treatment. In one study, a 57% reduction in diabetic peripheral nerve blood flow was normalized by 8 wk of aminoguanidine treatment (84). Nerve action-potential amplitude fell progressively with duration of diabetes, reaching 63% of controls at 24 wk (84). In contrast, action-potential amplitude at 24 wk in diabetic animals receiving 50 mg/kg aminoguanidine was 97% of controls. Similarly, both sensory and motor nerve conduction velocities became progressively slower with duration of diabetes in untreated animals, whereas both were nearly normal at 24 wk in aminoguanidine-treated diabetic animals. In another study of shorter duration, treatment with a lower dose of aminoguanidine ameliorated axonal atrophy and normalized the frequency of fibers undergoing myelin wrinkling and paranodal demyelination (85).

The prevention of diabetic retinopathy, nephropathy, and neuropathy by pharmacological inhibition of AGE formation in animal models suggests that aminoguanidine and other AGE inhibitors have a potential therapeutic role in the treatment of diabetic patients. Phase II/III clinical studies have been designed to assess the effects of aminoguanidine on various end points in different stages of diabetic nephropathy. Studies such as these will define the ultimate place of AGE inhibitors in the treatment of diabetic complications.

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