Importance of Advanced Glycation End Products in Diabetes-Associated Cardiovascular and Renal Disease

Mark E. Cooper

Although the features of diabetic cardiomyopathy, atherosclerosis, and nephropathy have been clinically characterized, the pathogenesis and the mechanisms underlying the abnormalities in the diabetic heart and kidney are not fully understood. During the past several years, in an attempt to discover interventions for diabetes-related complications, researchers have refocused their attention from the hemodynamic aspects of the disease to the biochemical interactions of glucose and proteins. Diabetes is a disorder of chronic hyperglycemia, and glucose participates in diabetic complications such as atherosclerosis, cardiac dysfunction, and nephropathy. Chronic hyperglycemia accelerates the reaction between glucose and proteins and leads to the formation of advanced glycation end products (AGE), which form irreversible cross-links with many macromolecules such as collagen. In diabetes, these AGE accumulate in tissues at an accelerated rate. The development of the novel compound dimethyl-3-phenacylthiazolium chloride (alagebrium chloride), which chemically breaks AGE cross-links, led to several preclinical animal studies that showed an attenuation or reversal of disease processes of the heart and kidney. In diabetes, AGE not only structurally stiffen structural collagen backbones but also act as agonists to AGE receptors (RAGE) on various cell types, which stimulate the release of profibrotic growth factors, promote collagen deposition, increase inflammation, and ultimately lead to tissue fibrosis. In the heart, large vessels, and kidney, these reactions produce diastolic dysfunction, atherosclerosis, and renal fibrosis. Administration of the cross-link breaker alagebrium chloride in these diabetic animals attenuates these pathologic phenomena, restoring functionality to the heart, vasculature, and kidney. Am J Hypertens 2004;17:31S–38S © 2004 American Journal of Hypertension, Ltd.

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many age- and diabetes-related disorders through non–receptor-mediated as well as receptor-mediated pathways, which activate growth factors, induce a number of processes, and initiate inflammatory reactions. This putative role of AGE in the pathogenesis of cardiovascular and renal disease has prompted a widespread search for effective AGE inhibitors.

In this review, we describe the basic mechanism of AGE formation, look at some of the interventions that have been recently developed to reduce AGE-related injury, and present several preclinical studies that have been carried out with these interventions. These studies have confirmed the role of AGE in the development of myocardial, vascular, and renal dysfunction in animal models of diabetes and provide a rationale for testing these new therapies in clinical trials.

**Glycation and AGE**

**Glycation**

The basic glycation pathways are shown in Figure 1. With a constant excess supply of glucose in diabetes, long-lived proteins such as collagen become glycated (Fig. 1, upper portion). Over months and years, these early glycated end products, such as Schiff bases and Amadori products (eg, glycosylated hemoglobin [HbA1c] and fructosamine) form irreversible cross-links between protein moieties in the connective tissues of skin, heart, kidney, eye, and other body structures. These advanced glycation end products include n-carboxymethyllysine (CML), known to be involved in diabetic nephropathy; pentosidine, which is seen in patients with diabetes and chronic inflammatory disorders; and the imidazolium salt cross-link, glyoxal-lysine dimer (GOLD), present in the serum of diabetic patients. The formation of AGE cross-links promotes fibrosis and decreases connective tissue flexibility. Specifically, AGE cross-links are increasingly considered partly responsible for atherosclerosis, nephropathy, cataracts, neuropathy, retinopathy, and myocardial dysfunction, as seen in diabetes.

**Interventions to Reduce AGE-Related Injury**

Interventions to reduce AGE-mediated injury aim to chemically break existing AGE-formed cross-links as well...
as to prevent the formation of new cross-links. The compound alagebrium chloride (dimethyl-3-phenacylthiazolidium chloride [alagebrium, Alteon Inc., Parsippany, NJ]) breaks AGE cross-links without disrupting the natural glycosylation sites or peptide bonds that are maintained within collagen. Another approach to inhibit AGE accumulation is to attenuate AGE formation. A number of AGE-formation inhibitors have been studied, the prototype being AG. Both AG and alagebrium chloride have been studied in animal models of experimental diabetes. These animal models developed accelerated cardiac and renal dysfunction partly as a result of AGE accumulation, which makes them useful tools for the study of AGE. Here, we report several studies in animal models of diabetes that examined the effects of alagebrium chloride and AG on reducing AGE and concomitant myocardial, renal, and atherosclerotic changes.

**Mechanisms of Diabetic Cardiomyopathy**

**Diabetes-Induced Myocardial Structural Changes in Diabetic Rats**

Candido et al. investigated the molecular mechanisms underlying diabetic cardiomyopathy as part of a study that assessed the effects of alagebrium chloride on diabetes-associated myocardial disease in diabetic rats. In this study, diabetes was induced by streptozotocin (STZ) (Boehringer-Mannheim, Mannheim, Germany) in Sprague-Dawley rats. After 16 weeks, diabetic and control rats (no STZ) were randomized to receive the cross-link breaker, alagebrium chloride at 10 mg/kg body weight daily by oral gavage or no treatment for a subsequent 16 weeks. This delayed intervention protocol was used because the clinical use of a therapy such as alagebrium chloride would often be initiated in diabetic subjects with established disease. Thus, four groups of animals were studied: control (n = 11), control + alagebrium chloride (n = 10), diabetic (STZ rats, n = 12), and diabetic + alagebrium chloride (n = 10). At 32 weeks, 16 weeks after initiating treatment, hearts from the rats that were killed were removed for determination of collagen content and cross-linking, AGE accumulation, gene and protein expression of growth factors, collagens, and various AGE receptors.

A significant reduction in left ventricular (LV) hypertrophy was observed in diabetic rats treated with alagebrium chloride. Specifically, compared with control rat hearts, diabetic rat hearts showed significant hypertrophy, and this correlated with the presence of cross-linked collagen in the left ventricle. At week 32, compared with control rats with and without alagebrium chloride, untreated diabetic rat hearts showed a significantly increased LV mass and an upregulation of brain natriuretic peptide (BNP) gene expression (approximately twice that of the control group), a marker of cardiac dysfunction. In rats treated with alagebrium chloride, these measures were significantly decreased to nondiabetic control levels at 32 weeks.

Increased collagen expression and cross-linking also appeared to be a prominent feature in the diabetic hearts of these animals. Candido et al. found that gene expression of LV type III collagen, a fibrillar collagen, was significantly increased in diabetic rat hearts. This was prevented in part by the cross-link breaker alagebrium chloride (Fig. 2, top). Immunohistochemical staining of the LV for collagen III showed an increase in positive brown staining for collagen III in diabetic LV sections (Fig. 2C) compared with control animals without and with alagebrium chloride (Figs. 2A, 2B). This increase in collagen III immunostaining was significantly reduced in alagebrium chloride–treated animals (Fig. 2D). Furthermore, collagen removed from the diabetic rat hearts was significantly less soluble in pepsin and acid, indicating increased cross-linking, which was reduced in diabetic rats treated with alagebrium chloride. These results indicate that the reduction in LV size and improvement in function with alagebrium chloride may be related to the reduction in collagen III expression and AGE cross-links. These findings are consistent with recent research in diabetic dogs, which showed that alagebrium chloride restored LV ejection fraction and reduced aortic stiffness and LV mass, with no reduction in blood glucose level—changes that were associated with a reversal of collagen upregulation.

To assess further the characteristics of glycated collagen, Candido et al. examined the myocardial collagen AGE accumulation by fluorescence and immunostaining. These investigators found that AGE accumulation was significantly increased in diabetic rat hearts compared with control rat hearts. Treatment of diabetic rats with alagebrium chloride completely prevented the increases in LV AGE fluorescence and immunostaining. Specifically, immunohistochemical staining with antibodies to CML—an AGE that is increased in diabetes—showed a significant increase in untreated diabetic hearts and a reduction in diabetic hearts treated with alagebrium chloride.

Many cellular responses elicited by AGE have been reported to have deleterious effects on organ structure and function. In particular, AGE act as ligands to various AGE receptors (RAGE, AGE-R1, AGE-R2, AGE-R3) that are present on macrophages, epithelial cells, mesangial cells, and endothelial cells. This interaction triggers a number of effects, including an inflammatory response, angiogenesis, increased expression of proinflammatory cytokines transforming growth factor–β (TGF-β) and connective tissue growth factor (CTGF), and induction of extracellular matrix protein production. Preclinical evidence shows that RAGE expression is increased in blood vessels and kidneys of diabetic animals and that AGE-R3 expression is increased in diabetic rat kidneys. In the study by Candido et al., expression of RAGE and AGE-R3 was upregulated in diabetic rat hearts, and this was attenuated by alagebrium chloride. Furthermore, CTGF gene expression was increased twofold in diabetic rat hearts compared...
with control rat hearts. This was confirmed by in situ hybridization studies of the LV from untreated diabetic rats, which revealed increased cardiac CTGF mRNA levels in the LV from diabetic hearts (Figs. 3C and 3G). These increases were not evident in diabetic rats treated with alagebrium chloride (Figs. 3D and 3H). The CTGF gene expression was not clearly evident in the hearts of untreated or alagebrium chloride–treated control rats (Figs. 3A, 3B, 3E, and 3F). Immunohistochemical staining for CTGF in LV sections confirmed an increased expression of this prosclerotic growth factor, which was reduced in diabetic rat hearts by the cross-link breaker alagebrium chloride.

The evidence from this study suggests that AGE play a central role in many of the alterations seen in the diabetic heart, and that cleavage of preformed AGE cross-links with alagebrium chloride attenuates collagen III expression, as well as growth factors and AGE receptors. These changes occurred in association with a reduction in cardiac hypertrophy. The results from this study and other studies have provided a better understanding of AGE-related damage to the diabetic heart. Specifically, the AGE that accumu-
mulate in diabetes interact with cellular receptors to activate growth factors that promote collagen accumulation, which ultimately leads to cardiac dysfunction. In addition, AGE cross-links increase cardiac stiffness. By reducing AGE, the cross-link breaker alagebrium chloride not only reduces cross-linked collagen levels but also attenuates many of the molecular and cellular pathways, possibly via RAGE-dependent pathways, ultimately resulting in improved cardiac function.

Atherosclerosis in Diabetic apoE KO Mice

To delineate further the effects of AGE on cardiovascular disease, researchers have studied atherosclerosis in diabetic mice. Atherosclerotic disease remains the major cause of morbidity and mortality in diabetes, yet the precise mechanisms by which diabetes promotes macrovascular disease have not been fully elucidated.

To examine the molecular mechanisms and structural changes underlying diabetes-associated atherosclerosis, Candido et al\textsuperscript{15} studied atherosclerosis-prone apoE knock-out (KO) mice with STZ-induced diabetes, a well-established model of diabetes-associated atherosclerosis. A total of 60 apoE KO mice were rendered diabetic by six daily intraperitoneal injections of STZ at a dose of 55 mg/kg in citrate buffer. Control mice ($n=30$) received citrate buffer alone. Compared with nondiabetic apoE KO (control) mice, those treated with STZ showed a fourfold increase in plaque area of the entire aorta within 20 weeks of becoming diabetic (Figs. 4A and 4B). The atherosclerotic plaques were seen primarily in the aortic arch, thoracic aorta, and abdominal aortic regions. In nondiabetic control mice, histologic examination revealed that most lesions were fatty streaks (Fig. 4D) and complex fibrous plaques were seen only occasionally at the aortic arch. However, in diabetic mice, the individual lesions were predominantly complex fibrous plaques, which were present in all segments of the aorta. Specifically, compared with control mice, the prevalent pathologic characteristics of the lesions in diabetic mice were an asymmetrically thickened intima composed of a fibrous cap with smooth muscle cells, foam-filled macrophages, and a lipid-rich necrotic core with cholesterol clefts within the extracellular matrix, as shown in Figure 4E. Compared with control mice, apoE KO diabetic mice showed an increase in macrophage/monocyte infiltration, $\alpha$-smooth-muscle actin ($\alpha$-SMA) levels, collagen content, and CTGF gene expression in these atherosclerotic lesions.

Based on evidence exploring alagebrium chloride in diabetic rats, and studies that used other approaches to inhibit AGE-dependent phenomena in diabetic mice,\textsuperscript{16,17}
it was postulated that treatment to prevent AGE cross-links would be also effective in retarding atherosclerosis in these apoE KO mice.

To study this, Forbes et al.18 randomly treated STZ-induced diabetic apoE KO mice with either AG (n = 20) administered in the drinking water (1 g/L) or alagebrium chloride (n = 20) administered by gavage (10 mg/kg/day) and compared them with control and diabetic apoE KO mice (no treatment) over 20 weeks. These two chemically distinct compounds were commenced during the first week of STZ-induced diabetes.

These two disparate approaches to prevent aortic AGE accumulation—either with the inhibitor of AGE formation (AG) or with the AGE cross-link breaker (alagebrium chloride)—were associated with attenuation of plaque area in diabetic apoE KO mice, a model of diabetes-associated atherosclerosis. A sixfold increase in plaque area in diabetic mice was attenuated by 30% with alagebrium chloride and by 40% with AG, implying that AGE play a role in the pathogenesis of atherosclerosis in this model. This was confirmed by plasma measurements that showed a 50% to 100% increase in AGE peptides in diabetic apoE KO mice, which was completely prevented by each of the treatments. Within the atherosclerotic plaques of untreated diabetic apoE KO mice, there was approximately a fourfold increase in the immunostaining area for the AGE, n-CML, which was decreased to control levels by alagebrium chloride and significantly reduced by AG.

Furthermore, as had been observed in the hearts of diabetic rats, AGE appeared to upregulate the expression of the AGE receptor RAGE, and this expression was significantly reduced by both alagebrium chloride and AG. Diabetic mice showed large areas of RAGE immunostaining within the atherosclerotic plaques as well as in adjacent areas. This increase in plaque RAGE staining was significantly reduced by the administration of alagebrium chloride.

Also, collagen levels were significantly increased in atherosclerotic plaques. Although the percentage of total collagen within the plaque was increased tenfold in the atherosclerotic plaques, type III and IV collagens were significantly increased. Treatment with alagebrium chloride attenuated the overall increase in plaque collagen content by 50%, but displayed differential effects on the three types of collagen studied. The reduction with alagebrium chloride treatment was most profound for collagen IV, which was normalized to nondiabetic levels, and with collagen III, which was attenuated by 50%. However, as was also seen in the diabetic rat LV,9 the increase in type I collagen in atherosclerotic plaques was not altered by either treatment in apoE KO diabetic mice.

As we had also seen in the diabetic rat model, diabetic apoE KO mice showed upregulation of CTGF gene and protein expression. Specifically, in situ hybridization and immunohistochemical staining for CTGF showed large increases in this growth factor in atherosclerotic plaques, which was attenuated by alagebrium chloride (Fig. 5).

The CTGF protein (Figs. 5A to 5C) and mRNA (Figs. 5E to 5G) were localized within aortic plaques in the diabetic mice. Both treatments attenuated these increases in CTGF gene and protein expression to levels seen in control apoE KO mice. A similar pattern was observed for TGF-β1 expression, which was increased in diabetic apoE KO mouse aortas compared with control mice and was decreased in the treated groups. The α-SMA protein expression, as assessed by immunohistochemistry, was increased in aortas from untreated diabetic mice compared with control mice. This parameter was reduced by each of the treatments, although it was not normalized to control levels.

From these studies, we can surmise that AGE play a complex and important role in diabetes-associated atherosclerosis. Alagebrium chloride and AG significantly reduced both atherosclerotic plaque formation and complexity and abrogated the expression of putative molecular mediators of this process, such as growth factors. These findings extend our understanding of the molecular and cellular mechanisms responsible for atherosclerotic lesion formation in diabetic vessels. As discussed later, many of the AGE-induced biochemical alterations seen in the cardiovascular system also play a central role in the development of diabetic nephropathy.
Mechanisms of Diabetic Nephropathy

Many studies have identified important changes in the components of the AGE pathway, including AGE receptors in the diabetic kidney. For example, short-term (6 weeks) diabetes induced in apoE KO mice increased renal expression of AGE receptors (eg, RAGE), with an associated inflammatory cell infiltration into the glomerulus. In addition, the role of AGE in diabetic kidney disease is emphasized in studies of various interventions to reduce renal AGE accumulation. The substance AG has been shown to be an effective agent in preventing nephropathy in the diabetic rat model.

Recently, Forbes et al studied the role of AGE in renal fibrosis and the effectiveness of alagebrium chloride at 10 mg/kg body weight daily by oral gavage as an early and delayed intervention in the STZ-induced diabetic rat model. In this study, STZ diabetic rats were randomized into three treatment groups: 1) no treatment; 2) treatment with the AGE cross-link breaker alagebrium chloride from week 16 to week 32 (early); and 3) treatment with alagebrium chloride, from week 24 to week 32 (late). Both the glomerulosclerotic index and tubulointerstitial area, two markers of renal structural injury, were significantly increased in diabetic rats, with both measures significantly reduced to control levels by early but not later alagebrium chloride treatment. In addition, total renal collagen, nitrotyrosine, and protein expression of collagen IV showed improvement only with early alagebrium chloride treatment. Renal TGF-β1 gene and protein expression were increased in diabetic rats. Although both early and late intervention with alagebrium chloride reduced gene expression, protein expression was reduced only by the early intervention strategy. The increased expression of this important profibrotic growth factor was also associated with an increased accumulation of collagen, specifically the basement membrane collagen type IV, which is also markedly attenuated by alagebrium chloride. This is illustrated in Fig. 6, which shows renal immunostaining for TGF-β1 and collagen IV in control (Figs. 6A and 6E), diabetic (Fig. 6B and 6F), alagebrium chloride early (Fig. 6C and 6G), and alagebrium chloride late (Fig. 6D and 6H) groups. These changes in TGF-β1 and type IV collagen with treatment were also seen with respect to CTGF gene expression in this study.

Similarly, in another model of accelerated kidney injury, Lassila et al showed in diabetic apoE KO mice that CTGF is also elevated in diabetic nephropathy (as had been observed in the heart and aorta of diabetic rats and mice). The expression of CTGF and TGF-β1 was markedly upregulated in the diabetic glomerulus, which was prevented by both AG and alagebrium chloride. In situ hybridization of CTGF in this animal model showed a marked reduction in CTGF mRNA levels with both agents, particularly alagebrium chloride. Similar to that observed in the study by Forbes et al, diabetes-associated glomerular and tubulointerstitial injury was associated with increased expression of collagen type IV and TGF-β1, increased α-SMA immunostaining, and macrophage infiltration, as well as increased serum and renal AGE. Treatment with alagebrium chloride and AG attenuated renal AGE accumulation, and was associated with less albuminuria, renal structural injury, macrophage infiltration, and TGF-β1 and collagen expression.

Summary and Conclusion

Throughout a person’s life, reducing sugars such as glucose react nonenzymatically and reversibly with free amino groups in proteins to form small amounts of stable Amadori products. As a person ages, the further spontaneous irreversible modification of proteins by glucose results in the formation of a series of AGE, a heterogeneous family of biologically and chemically reactive compounds with cross-linking properties. In diabetes, this...
continuous process of protein modification is amplified as a result of chronic hyperglycemia.

The AGE bind to specific receptors such as RAGE on a range of cell types including macrophages, epithelial cells, and endothelial cells, triggering specific cellular responses in the vasculature. Such responses include increased release of cytokines (eg, tumor necrosis factor–α [TNF-α] and interleukin-1 [IL-1]), and increased production of growth factors (eg, TGF-β, CTGF, and vascular endothelial growth factor [VEGF]), which ultimately lead to angiogenesis and increased extracellular matrix accumulation. As we have reviewed here, in animal models of diabetes, a number of pathologic manifestations of diabetes complications have been observed, including inflammation, collagen deposition, and increased expression of growth factors, all of which manifest clinically as diastolic dysfunction, atherosclerosis, and nephropathy.

Potential treatment strategies for these AGE-derived complications include prevention of AGE formation and breaking of pre-existing AGE cross-links. The therapeutic potential of the AGE inhibitor AG has been extensively investigated in animal models and in phase 3 clinical trials. Furthermore, alagebrium chloride, a highly potent AGE cross-link breaker, has the ability to reverse already-formed AGE cross-links. By breaking existing cross-links, alagebrium chloride significantly reduces sclerosis and inflammation, partly by downregulating critical mediators, such as the growth factors CTGF and TGF-β1. In a number of preclinical studies with alagebrium chloride, we have seen a significant reduction in collagen deposition, ultimately leading to less cardiac dysfunction, reduced atherosclerosis, and attenuation of renal disease.

Thus, an intervention such as the AGE cross-link breaker alagebrium chloride may ultimately be an excellent treatment for diabetic complications because it addresses multiple manifestations of vascular disease including atherosclerosis, nephropathy, and cardiac disease.

References