Retinopathy is the most common microvascular complication of diabetes, and it remains a major cause of visual impairment worldwide. Epidemiology studies indicate that, at 20 years after diagnosis, most patients with type 1 diabetes will experience retinopathy, whereas ~80% of insulin-dependent patients with type 2 diabetes and ~50% of patients with non-insulin-dependent type 2 diabetes will have retinopathy. Microvascular lesions, such as microaneurysms, blood barrier dysfunction, and capillary dropout, are key features of diabetic retinopathy. However, it should be appreciated that the sole purpose of the retinal circulation is to support the metabolic demands of the inner retinal neurons and glia and that these cells are also damaged appreciably during diabetes. Such neuronal and glial dysfunction occurs in union with blood flow abnormalities and often before the appearance of overt microvascular damage. In diabetic patients and short-term animal models of diabetes, reversible retinal psychophysical and electrophysiological alterations are evident. Such early-stage alterations may be signposts for the irreversible retinal microvascular, neuronal, and glial damage observed after longer term diabetes.

The DCCT (Diabetes Control and Complications Trial) and UKPDS (UK Prospective Diabetes Study) population studies in type 1 and type 2 diabetic patients, respectively, have established the relationship between hyperglycemia and retinopathy. These seminal studies, and many others, point toward hyperglycemia as being critical in the pathogenesis, although it often occurs in unison with dyslipidemia and hypertension. This epidemiology provides the foundation for ongoing research, seeking to identify the cellular and molecular mechanisms that underpin diabetic retinopathy. The formation of advanced glycation end products (AGEs) and the activation of receptors for AGEs are the focus of this article, although it should be appreciated that hyperglycemia can simultaneously provoke a range of other pathogenic mechanisms in retinal cells in vitro and in vivo. Such pathways to diabetic retinopathy should not necessarily be viewed as independent phenomena. Brownlee et al. have proposed a unifying concept whereby hyperglycemia increases superoxide production (via the mitochondrial electron transport chain) which in turn initiates accelerated AGE formation and also exacerbates interrelated pathogenic responses. This hypothesis has been reinforced in the field of retinopathy, in which three biochemical abnormalities involving AGE formation, flux through the hexosamine pathway and diacylglycerol-mediated activation of PKC-β can be attenuated with benfotiamine. This vitamin B1 thiamine derivative stimulates transketolase activity and shunts excess triose phosphates toward the reductive pentose phosphate pathway, which is impaired in high-glucose diabetes. By converging on three harmful pathways, benfotiamine prevents high-glucose-induced dysfunction in retinal microvascular cells, whereas treatment of diabetic animals protects against the key lesions of retinopathy.

**Biochemistry of AGE Formation**

**How AGEs Are Formed**

Nonenzymatic-glycation reactions between reducing sugars and the free amino groups on proteins, lipids, and DNA are an inevitable consequence of aldehyde reactivity. As a consequence, many proteins in vivo carry some burden of chemically attached carbohydrate. An understanding of this chemistry was established in 1912 by the food chemist, Louis Camille Maillard, who reported formation of brown products upon heating mixtures of amino acids and sugars. The Maillard, or browning, reaction begins with the formation of a Schiff base between glucose and ε-amino groups that slowly rearranges to relatively stable Amadori adducts, an example of which is hemoglobin A1c (HbA1c). Both the Schiff base and the Amadori product can undergo further oxidation and dehydration with concentrations ultimately dependent on both forward and reverse reactions. The forward reactions give rise to additional irreversible protein-bound compounds collectively termed advanced glycation end products (AGEs). During diabetes, the rate of formation of AGEs exceeds that predicted by first-order kinetics. Thus, over time, even modest hyperglycemic excursions can result in significant adduct accumulation on long-lived macromolecules.

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AGE adducts are highly stable at physiological pH, and their rate of accumulation in tissues depends on factors such as availability of metal ions, redox balances, and longevity of the modified protein. Although AGEs may lead to pigmentation or fluorescence, they can also inflict considerable damage on proteins through cross-linking, changing tertiary structure, conferring resistance to digestion, altering enzymatic activity, or impairing receptor recognition. AGE adducts form from many different precursors that contribute to the heterogeneity of these chemical structures. Numerous AGE adducts have been identified in vivo, including: N^\text{\textdagger}-(carboxymethyl) lysine (CML), crossline, pentosidine, fururyluranyl imidazole (FFI), hydroimidazolone, argpyrimidine, glyoxal lysine dimer (GOLD), and methylglyoxal lysine dimer (MOLD).^{15}

**AGEs Form from a Range of Precursors**

Glucose is often viewed as the principal AGE precursor; however, it is considerably less reactive than α-oxoaldehydes such as glyoxal (GO), methylglyoxal (MGO), and 3-deoxyglucosone (3-DG), which arise from glycolytic metabolism and can form AGEs very rapidly.\(^19,20\) For example, GO reacts with arginine residues to form carboxymethyl-arginine (CMA),\(^21\) whereas MGO can give rise to the AGEs N^\text{\textdagger}-(carboxylthoxy) lysine (CEL) and arginine-hydroimidazolone.\(^19,22\) The concentrations of these reactive carbonyls rise in high-glucose-exposed cells and occur at elevated levels in diabetic serum, and they institute the major source of AGEs in vivo.\(^22-23\) Indeed, in diseases of carbonyl stress, such as diabetic nephropathy, the AGEs can reach exceptionally high levels.\(^24\)

**AGEs Are Also Derived from Food**

AGEs and ALEs are abundant in food. Indeed a typical Western diet contains high levels of fat and sugar and, being highly thermally processed, can lead to high levels of harmful adducts.\(^25\) Consumption of a conventional Western diet typically leads to a daily intake of \(\sim 25\) to \(75\) mg AGE/ALEs (advanced lipoxidation end products),\(^25\) and a proportion of these adducts pass the gut epithelium and can appear as plasma AGEs.\(^26\) Food-derived AGEs may be linked to vascular dysfunction, especially in situations in which there is renal dysfunction and poor clearance of plasma AGEs.\(^27,28\) The potential involvement of food-derived AGEs and ALEs in diabetic retinopathy has not been studied.

**Detoxification Systems Can Limit AGE Formation In Vivo**

Cells have evolved systems that provide endogenous protection against dicarbonyls, and several detoxifying enzymes have been identified. For example, a glutathione-dependent glyoxalase complex (formed from glyoxalase I [GLO1] and glyoxalase II [GLO2] components) acts as a detoxification system for GO and MGO, which are converted to \(\text{o-lactate}.\(^29\)\) Endothelial cells transfected to overexpress GLO1 accumulate less MGO-derived AGEs\(^30\) and are protected against high-glucose-induced responses.\(^31\) The GLO1 detoxification system has been shown to be critical for retinal pericyte survival, but this may be insufficient during diabetes, since these cells undergo apoptosis as a direct result of MGO-derived AGE formation.\(^32\) The importance of this enzyme system is supported by findings in studies in which *Caenorhabditis elegans* was engineered to overexpress GLO1. These worms contain fewer AGEs and show a significantly increased lifespan when compared with wild-type counterparts.\(^33,34\) Clearly, there is a potential for harnessing the detoxifying property of enzymes such as GLO1, to protect against diabetic retinopathy.

**ALEs May Play an Important Role in Retinal Disease**

Dyslipidemia is often overlooked as a pathogenic force in diabetic retinopathy.\(^35\) In the context of this review, lipid peroxidation reactions can also form a class of Maillard products, the ALEs, which are linked to diabetes and dyslipidemia.\(^36\) ALEs form through the lipoperoxidative production of reactive aldehyde species. Among the best characterized are \(N^\text{\textdagger}-(2-propenal)\)lysine and dihydroxyaldehyde-type adducts (malondialdehyde-derived), hemiacetal and pyrrole adducts (4-hydroxy-2-nonenal, and 4-hydroxyhexenal-derived), and \(N^\text{\textdagger}-(3-formyl-3,4-dehydropiperidino)\)lysine, or FDP-lysine; acrolein-derived.\(^37\) Recent work has also shown that hemoglobin levels of the ACR-derived ALE, FDP-lysine, are associated with the severity of retinopathy in patients with type 1 or type 2 diabetes.\(^38\) Clearly, ALEs represent an important source of protein modification, especially in lipid-rich, highly oxidative environments, such as that in the retina. The understanding of the role of ALEs in diabetic retinopathy lags far behind that which is known about AGEs, and these adducts therefore warrant further study.

**Pathogenic Role of AGES in Diabetic Retinopathy**

**AGEs and Clinical Correlation with Diabetic Retinopathy**

AGEs affect cells from three main perspectives: as adducts occurring on modified serum proteins, as endogenous adducts formed as a consequence of glucose metabolism, or as extracellular matrix-immobilized modifications on long-lived structural proteins (Fig. 1). All these AGEs can be analyzed in serum, cells, and tissues using analytical and/or immunocytochemical approaches. Clinically, most quantification in patient tissues and serum has been based on ELISA of a range of AGE-antibodies, but this method has often produced confounding outcomes. With this proviso, patient-based studies have revealed that the levels of AGEs in serum correlate with the clinical progression of diabetic retinopathy.\(^39\) Although many reports have measured a range of ill-defined AGE moieties, others have used adduct-specific antibodies or chemical analysis for CML, pentosidine, or hydroimidazolone\(^40-42\) and also have found association with diabetic retinopathy. It should be acknowledged that some reports have demonstrated no correlation between AGE levels and retinopathy in diabetic patients,\(^43\) although the apparent disparity may be related to variations in patient populations, the presence of nephropathy, and/or the nonuniformity of assays for AGE quantification.

**AGEs as Robust Biomarkers for Disease Risk**

AGE-modified proteins are readily cleared from the bloodstream (except during renal dysfunction), and thus quantification of these adducts in serum may not always provide robust biomarkers for disease. By contrast, AGE modification of extracellular matrix isolated from skin biopsies often provides more meaningful data,\(^44\) illustrated by the DCCT skin collagen ancillary study group.\(^45\) They demonstrated that cross-linked AGEs on long-lived skin proteins are significantly associated with the progression of diabetic retinopathy. More than 200 patients from the original DCCT were followed up for a further 10 years under the auspices of the Epidemiology of Diabetes Interventions and Complications (EDIC) Trial.\(^46\) The results revealed that levels of diabetic retinopathy were significantly lower in the group initially maintained under tight glycemic control and that these benefits extended far beyond the period of intensive insulin therapy.\(^46\) The patients under conventional
control for the first 10 years maintained a hyperglycemic or metabolic memory and retained a strong association with retinopathy progression. CML-modified skin collagen predicted the progression of retinopathy (and nephropathy), even after initiation of intensive insulin therapy.\(^{46}\) Furthermore, the predictive effect of hemoglobin A1c (HbA1c) vanished after adjustment for AGES, suggesting that accumulation of these adducts on long-lived protein is an excellent marker for retinopathy risk and could offer a molecular-based explanation for the metabolic memory phenomenon.\(^{47}\)

**AGES in the Diabetic Retina**

AGES have been extensively quantified in various ocular tissues and are often elevated during ageing and in diabetic subjects when compared to nondiabetic control subjects.\(^{47}\) This includes vitreous collagen,\(^{48}\) where the AGE levels correlate with diabetic retinopathy.\(^{49}\) In the diabetic retina, AGES and/or late Amadori products have been localized to vascular cells, neurons, and glia.\(^{50-55}\) This would be expected to have pathogenic implications for the individual cells and retinal function. Although differential accumulation of AGES exists in the retina over the course of life, diabetes significantly enhances the occurrence of these adducts in the vascular and neural tissue components.\(^{54}\)

It has been demonstrated that MGO-derived hydroimidazolone is increased 279% in 24-week diabetic rat retina,\(^{56}\) and this finding emphasizes the fact that MGO could be the major source of AGES in this tissue. In terms of cell localization for AGES, many adducts occur at high levels in the Müller macroglia, and these increase as diabetes progresses.\(^{57}\) This fact is significant, because Müller glia have a unique role in the architecture and physiology of the retina and show considerable dysfunction during the hyperglycemia and hypoxia experienced by diabetic retina.\(^{58,59}\) This dysfunction is manifested by increased expression of glial fibrillary acidic protein (GFAP),\(^{60}\) NO production,\(^{61,62}\) and concomitant synthesis of glutamate (as a function of disruption of the glutamate transporter\(^^{63}\)) which may contribute to excitotoxicity in retinal neurons.\(^{64}\) Significantly, Winkler et al.\(^{65}\) have demonstrated that Müller cells exposed to high glucose conditions in vitro produce excess lactate, indicative of increased glycolytic flux,\(^{66}\) and this effect leads to greater production of MGO. Hypoxia is also known to enhance glycolytic metabolism through increased HIF-1α-dependent expression of glycolytic enzymes in the conversion of cells to a predominantly glycolytic metabolic state in the absence of oxygen (the Pasteur effect). Hypoxia, by increasing glycolysis and MGO synthesis, could lead to significant AGE formation in the diabetic retina.

**AGES Evoke Retinal Oxidative Stress and Retinal Cell Death**

Oxidative stress results from the disequilibrium between pro- and antioxidants in biological systems,\(^{66}\) and this pathway is intimately linked to the formation of AGES.\(^{13,67,68}\) Numerous studies have reported that oxidative stress is increased in diabetic patients and that it plays an important role in the pathogenesis of diabetic complications, including retinopathy.\(^{69,70}\) Previous work has demonstrated that the concentration of superoxide is elevated in the retina of diabetic rats and in retinal cells incubated in high-glucose media.\(^{61,71,72}\)

Of importance, it has been shown that inhibition of superoxide with antioxidants\(^{73,74}\) or overproduction of mitochondrial superoxide dismutase (SOD)\(^{75}\) can protect against capillary degeneration during diabetic retinopathy in experimental diabetes, although how this influences AGE accumulation in the retina has not been studied. Retinal capillary degeneration remains a hallmark of retinopathy in diabetic animal models and patients\(^{76}\) and these vessels seem to be important targets for both AGE- and superoxide-induced toxicity.\(^{77}\) For example, AGES induce toxic effects on retinal pericytes by causing oxidative stress and subsequent apoptosis.\(^{78}\) In addition, some
Inflammation, RAGE, and Diabetic Retinopathy

The involvement of inflammatory processes in the initiation of neurovascular lesions during diabetic retinopathy has received recent attention. Global mRNA expression profiling has highlighted altered expression of proinflammatory cytokines and interrelated pathways, not only in the retinal vessels, but also in the neuroglia. There is undoubtedly a complex milieu of dysregulated proinflammatory factors apparent in diabetic retina, but there is strong evidence of involvement of major mediators of inflammation such as IL-1α, IL-1β, and IL-6 and TNFα that may be linked to microglial activation and infiltrating monocytes that are increased in diabetic retina, both in humans and in animal models.

RAGE as a Component of the Innate Immune Response

The receptor for AGEs (RAGE) is the most established AGE-binding protein and acts as a signaling receptor for at least two distinct AGEs: CML and hydroimidazolone adducts. RAGE is now known to be a key component of the innate immune response and binds to multiple ligands including S100B, high-mobility group box (HMG)-1, amyloid-β, and Mac-1 (CD11b/CD18).

RAGE is constitutively expressed in a range of tissues, such as brain, kidney, liver, heart, and the vasculature, and in various cell types, including neurons, endothelium, smooth muscle, epithelium, and inflammatory cells. RAGE and downstream proinflammatory signaling on ligand-binding are associated with several disease states such as diabetic complications, Alzheimer's disease, cancer, and viral infections. A receptor for AGEs (sRAGE), which is composed of the extracellular domains but lacks the transmembrane and cytosolic sequences, sRAGE may act as a dominant negative isoform and block RAGE signaling by function as an extracellular "decoy receptor" to inhibit RAGE ligand-binding.

RAGE dimerization occurs on ligand binding and, in association with a binding peptide diaphanous (Dia)-1, intracellular signaling is initiated. This process can result in phosphorylation of various protein kinases involving MAPKs, Rac/Cdc42, and JAK/STATs and subsequently activate the NF-κB pathway. This signal transduction links RAGE to several inflammation-related cell responses, such as apoptosis, mobility, migration, and proinflammatory gene expression.

Thus, RAGE has been the focus for several small-molecule drugs or neutralizing antibodies that can regulate ligand binding or downstream signal transduction and thereby prevent disease.

RAGE Involvement in Diabetic Retinopathy

In the retina, RAGE expression has been predominantly localized to glia in the inner retina, and this receptor appears to be upregulated in diabetic conditions. AGE-RAGE ligands have also been demonstrated in the retina, and they often occur at higher levels during diabetes. Other RAGE ligands including S100/calgranulins and HMGB1 are evident in the retinal vessels, and such receptors may also be present in the neuroglia. This finding opens a further new paradigm for possible RAGE-mediated involvement in retinal neuropathic abnormali-
ties during diabetes, and recent studies have indicated a role for the receptor in Müller cell dysfunction.\textsuperscript{125} It has been reported that hyperglycemia increases RAGE, S100A8, S100A12, and HMGB1 expression in human aortic endothelial cells, a response that can be normalized by a superoxide dismutase mimetic.\textsuperscript{126} We have recently shown that this response occurs in the diabetic retina whereby hyperglycemia in vivo and HG exposure to Müller cells in vitro induce the expression of RAGE and its ligands, leading to cytokine signaling. This signaling, in turn, leads to RAGE signaling and proinflammatory cytokine expression, a response that further implicates involvement of this receptor in retinal inflammatory disease.\textsuperscript{127}

### RAGE Blockade as a Potential Therapeutic Strategy for Diabetic Retinopathy

sRAGE can prevent Müller cell dysfunction\textsuperscript{125} during diabetes and retinal capillary leukostasis in AGE-infused normal mice.\textsuperscript{106} The clinical potential for reducing RAGE signaling in the diabetic retina is further underscored by development of new RAGE-regulating agents. One such agent is TTP488, which is an orally delivered small molecule, for which phase II studies have been completed in patients with Alzheimer’s disease. Use of these agents and other similar strategies is exciting, because they directly address RAGE-ligand binding and show great potential for diabetic complications. Evaluation of the role of RAGE inhibition in diabetic retinopathy is ongoing in several laboratories.

Some commonly used diabetes drugs, such as thiazolidinediones (e.g., rosiglitazone),\textsuperscript{128} or calcium channel blockers, such as nifedipine,\textsuperscript{129} have been shown to reduce RAGE expression in endothelial cells and could serve to limit the proinflammatory effects of AGEs. In addition, angiotensin II receptor blockers (ARBs) can reduce RAGE expression, and this effect should be factored into the benefit that these agents have in preventing diabetic retinopathy.\textsuperscript{130} In addition to the downregulation of RAGE and associated reduction in oxidative stress, ARBs can act as selective regulators of PPAR\textgamma, which suggests some cross-talk between the AGE–RAGE axis and PPAR\textgamma modulation.\textsuperscript{131} Thus, a complex interplay between enhanced levels of AGEs, suppressed antioxidant status and upregulation of the RAGE axis may together play a pivotal role in the progression of diabetic retinopathy.

### Conclusion

Long-term management of retinopathy in the ever-expanding diabetic patient population involves precise regulation of glycemic, vasotensive, and lipidemic profiles, most effectively in combination with drugs that ameliorate an array of biochemical and metabolic abnormalities. Early-stage experimental work suggests that AGE formation and activation of AGE-receptors represent important, interconnected pathogenic mechanisms in diabetic retinopathy, and inhibition of these pathways presents a valid avenue for therapeutic exploitation. As our knowledge of Maillard chemistry and its biological impact improves, alongside elucidation of AGE-receptor signaling in various retinal cell-types, there may be exciting new opportunities for therapeutic management of diabetic retinopathy at all stages of the disease.

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