Protein Glycation
A Firm Link to Endothelial Cell Dysfunction

Jean-Luc Wautier, Ann Marie Schmidt

Abstract—The advanced glycation end products (AGEs) are a heterogeneous class of molecules, including the following main subgroups: bis(lysyl)imidazolium cross-links, hydroimidazolones, 3-deoxyglucosone derivatives, and monolysyl adducts. AGEs are increased in diabetes, renal failure, and aging. Microvascular lesions correlate with the accumulation of AGEs, as demonstrated in diabetic retinopathy or renal glomerulosclerosis. On endothelial cells, ligation of receptor for AGE (RAGE) by AGEs induces the expression of cell adhesion molecules, tissue factor, cytokines such as interleukin-6, and monocyte chemoattractant protein-1. A chief means by which AGEs via RAGE exert their effects is by generation of reactive oxygen species, at least in part via stimulation of NADPH oxidase. Diabetes-associated vascular dysfunction in vivo can be prevented by blockade of RAGE. Thus, agents that limit AGE formation, increase the catabolism of these species, or antagonize their binding to RAGE may provide new targets for vascular protection in diabetes. (Circ Res. 2004;95:233-238.)

Key Words: glycated proteins ■ (carboxymethyl)lysine ■ 3-deoxyglucosone ■ endothelial cells ■ vascular permeability ■ oxidant stress

Nonenzymatic protein glycation (glucosylation or glycosylation) by glucose is a complex cascade of reactions yielding a heterogeneous class of compounds, collectively termed advanced glycation end products (AGEs). Nonenzymatic glycation of proteins described by Louis-Camille Maillard1 have been implicated in the pathogenesis of diabetes, renal failure, and aging.2 In vitro–prepared AGE modifications of proteins have been shown to be toxic, immunogenic, and capable of triggering cellular injury.

Maillard Reaction
The Maillard reaction begins with the reaction of the carbonyl (aldehyde or ketone) of the reducing sugar to form a reversible Schiff base with the amino group of the biomolecule. The Schiff base can undergo an intramolecular rearrangement to form the Amadori products;3 this can undergo a series of further rearrangements, dehydration, and condensation to form irreversible end products, which may be fluorescent and yellow–brown in color; some can form stable intermolecular and intramolecular cross-links (Figure 1).4 In vivo, the amount of AGEs on a protein has been found to be dependent on the inherent reactivity of specific amino groups, as determined by their microenvironment, the glucose concentration, and the half-life of the protein. Realization of the importance of Maillard-like reactions in vivo began in the mid-1970s with the study of hemoglobin A1c (HbA1c),5 a naturally occurring minor human hemoglobin that is elevated in diabetic patients. HbA1c is known to be a post translational...
adduct of glucose with the N-terminal valine amino group of the \( \beta \) chain of hemoglobin, in which the glucose was thought to be attached via a nonenzymatically formed Schiff base structure. In fact, the carbohydrate in HbA1c is attached as a 1-deoxy-1-fructosyl residue to the N-terminal valine nitrogen derived from an initially formed Schiff base via an Amadori rearrangement. HbA1c is used for assessing extended metabolic control in diabetic patients and provides an average integrated blood glucose concentration for approximately a 28-day period.

Glycation adducts are formed by the reaction of proteins with glucose-reactive \( \alpha \)-oxoaldehydes such as glyoxal, methylglyoxal, and 3-deoxyglucosone, and other saccharide derivatives.\(^6\) The initial Schiff base adducts formed from glucose and lysine and N-terminal amino-acid residues rearrange to form fructosamine. Fructosamine degradation and the direct reaction of \( \alpha \)-oxoaldehydes with protein form many AGEs. Oxidative reactions may be increased by oxidative stress arising from mitochondrial dysfunction and activation of NADPH oxidase.\(^7\)\(^-\)\(^8\) Some AGEs are cross-linked, for example, the bis(lysyl)imidazolium salts may denature proteins and confer resistance to proteolysis. When AGEs are formed at critical sites in enzymes or proteins, they may be associated with enzyme inactivation.\(^9\)

Cross-linked AGEs, GOLD [glyoxal-derived lysine dimer, 1,3-di(\( \alpha \)-lysino imidazolium salt], MOLD [methylglyoxal-derived lysine dimer, 1,3-di(\( \alpha \)-lysino)-4-(methyl-imidazolium salt], DOLD [3-deoxyglucosone-derived lysine dimer, 1,3-di(\( \alpha \)-lysino)-4 (2,3,4-trihydroxybutyl)imidazolium salt], and pentosidine may alter protein structure and function (Table).\(^3\)\(^-\)\(^10\)

**Cellular and Extracellular AGE-Modified Proteins**

The concentrations of AGEs in protein of human blood cells and plasma in vivo and human kidney mesangial cells can be determined by liquid chromatography mass spectrometry.\(^10\) The concentration of AGEs is higher in cellular protein than in plasma protein. \( N' \)-carboxymethyl-lysine plasma protein is \( \approx 21 \mu \text{mol/mol of lysine} \), whereas cellular \( N' \)-carboxymethyl-lysine concentration reaches 68 to 233 mmol/mol of lysine. MOLD was found in plasma (0.8 \( \mu \text{mol/mol of lysine} \)) and in red blood cells (5.3 \( \mu \text{mol/mol of lysine} \)).

AGE content may increase rapidly; a 13.8-fold increase in AGE levels within 1 week has been demonstrated in endothelial cells. Hyperglycemia-induced increases in endothelial cell macromolecular endocytosis can be prevented by inhibition of methylglyoxal-derived intracellular AGEs.\(^6\) AGEs arise from both metal-catalyzed auto-oxidation of glucose with the dicarbonyl glyoxal and arabinose as intermediates, and from decomposition of Amadori products to the dicarbonyl 3-deoxyglucosone. The dicarbonyl methylglyoxal produced by nonenzymatic fragmentation of triose phosphate also forms AGE in vitro.

**AGE Products**

- Bis(lysyl)imidazolium cross-links
  - GOLD (glyoxal-derived lysine dimer)
  - MOLD (methylglyoxal-derived lysine dimer)
  - DOLD (3-deoxyglucosone-derived lysine dimer)

- Hydroimidazolones
  - G-H (glyoxal-derived hydroimidazolone)
  - MG-H (methylglyoxal hydroimidazolone)
  - 3DG-H (3-deoxyglucosone hydroimidazolone)

- Monolysyl adducts
  - \( N' \)-carboxymethyl-lysine (CML)
  - \( N' \)-carboxyethyl-lysine (CEL)
  - Pyrraline

According to Ahmed and Thornalley.\(^3\)
Methylglyoxal Is Efficiently Metabolized by the Glyoxalase System

Incubation of GM7373 endothelial cells that stably express human glyoxalase-I in high glucose concentration (30 μmoI/L) did not result in increased AGE formation whereas the AGE content in the nontransfected cells was increased 13.6-fold, demonstrating that enzymatic defense against glycation may represent an approach to limit the deleterious consequences of AGES. Oxidative stress is inextricably linked to glycation because the depletion of GSH and NADPH also decreases the in situ activities of glyoxalase-I and NADPH-dependent aldehyde reductases.

Endothelial Cell Dysfunction

Endothelial cell dysfunction, a broad term that implies reduced production of nitric oxide (NO) and an imbalance in the relative contribution of endothelium relaxing and contracting factors and oxidants, is emerging as a key component in the pathophysiology of diverse cardiovascular abnormalities associated with atherosclerosis, hypertension, diabetes, renal failure, and aging. In addition to its vasodilatory effect, NO also protects against vascular injury, inflammation, and leukocyte adhesion. When endothelial cells undergo inflammatory activation, the increased expression of selectins, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 may promote the adherence of monocytes. Adherent monocytes transmigrate into the arterial wall, passing between the endothelial cells. Endothelial cells, rather than providing protection against thrombosis, then become prothrombotic and may produce tissue factor (Figure 2).

Two major microvascular complications of diabetes are retinopathy and nephropathy. In both settings, several lines of evidence suggest a deleterious effect of AGES, either by direct action on endothelial cells or through a mechanism involving another cell type or the matrix components, present in the vicinity of the endothelium.

Retinopathy

The first studies of ocular complications linked to glycation established the deleterious effects of AGE formation in pig crystallin in the pathogenesis of diabetic cataract. One of the earliest changes observed in retinal microvessels is the selective loss of intramural pericytes, a process that may be linked to the effects of AGES. AGES may induce apoptosis and trigger oxidative stress of bovine pericytes. In reaction to hypoxemia-induced microvascular damage, the retinal epithelial and endothelial cells increase production of vascular endothelial growth factor and promote neoangiogenesis, a process prevented by antibodies to RAGE. Pyridoxamine, an inhibitor of AGE formation and lipoxidation end products, protects against diabetes-induced retinal vascular lesions. The thiamine monophosphate derivative, benfotiamine, inhibits hyperglycemia-dependent pathways and NF-κB activation, and prevents experimental diabetic retinopathy. Beraprost sodium, a prostaglandin I2 analog, has been reported to protect retinal pericytes from AGE-induced cytotoxicity through its anti-oxidative properties and was previously shown to decrease vascular hyperpermeability in diabetic rats.

Nephropathy

Glomerulosclerosis observed in diabetic animals is associated with AGE deposition in mesangium and hyalinated and/or sclerotic lesions. Levels of N-carboxymethyl-lysine adducts are increased in soluble proteins and insoluble collagen in patients with diabetes and renal impairment. Overexpression of RAGE accelerates glomerulosclerosis development in mice. Pharmacological blockade of RAGE in db/db mice, or genetic deletion of RAGE in mice with streptozotocin-induced hyperglycemia, results in decreased albuminuria and mesangial expansion/glomerulosclerosis.

In renal failure, peritoneal dialysis treatment causes chemical peritonitis because of the limited biocompatibility of peritoneal dialysis fluids that contain high glucose concentrations (up to 45 g/L) and, thus, glucose-derived products that are precursors of AGES. Because RAGE is expressed on endothelial and mesothelial cells, the receptor may bind AGES present in patients or formed during peritoneal dialysis. The binding of AGES to RAGE produces a local inflammatory reaction, likely as a consequence of vascular cell adhesion molecule-1 overexpression, leukocyte adhesion, and cytokine release.

Endothelial Cell Activation

A key consequence of ligand engagement of RAGE is activation of multiple signaling pathways, including p21ras, erk 1/2 (p44/p42), MAP kinases, p38 and SAPK/JNK MAP kinases, rho GTPases, phosphoinosit-3 kinase and the JAK/STAT pathway, and downstream effectors such as NF-κB and CREB.

Engagement of RAGE by AGE triggers the generation of reactive oxygen species. The incubation of human endothelial cells with specific AGE (carboxymethyl lysine-modified adducts) prompted intracellular generation of hydrogen peroxide, a process suppressed by diphenyliodonium but not by inhibitors of nitric oxide. Consistent with an important role for NADPH oxidase in AGE–RAGE–triggered events, macrophages derived from wild-type mice expressed enhanced levels of tissue factor on stimulation with AGE. In contrast, macrophages derived from mice deficient in gp91 phox failed...
to display enhanced tissue factor on incubation with AGEs (Figure 3).  
Forearm resistance vessel responses to endothelium-dependent and independent agonists are abnormal in individuals with type 1 diabetes. The individuals with the highest HbA1c levels showed the most marked impairment in the response to acetylcholine.  
Increased vascular permeability is characteristic of early diabetic vasculopathy. Postconfluent cultured endothelial cells incubated with red blood cells harvested from diabetic patients revealed increased diffusional transit (permeability of macromolecular tracers 125I albumin and 3H inulin) compared with endothelial cells incubated with red blood cells from nondiabetic subjects. The diminution of endothelial cell barrier function was completely inhibited by anti-RAGE antibodies. Furthermore, in diabetic rats infused with soluble RAGE (60 μg/mL), hyperpermeability was blocked in intestine and skin and suppressed by 90% in the kidney. Inhibition of AGEs and diabetic hyperpermeability by antioxidants both in vitro and in vivo suggested a central role of AGE–RAGE–induced oxidant stress in the development of hyperpermeability. These studies strongly suggested that reactive oxygen species formation is a likely means by which oxidative stress can reversibly increase vascular permeability by rapid changes in endothelial cell shape via calcium-mediated pathways. Reactive oxygen species can also increase permeability by quenching NO. Elevated serum levels of AGEs are correlated with the presence of microvascular complications (retinopathy, nephropathy); both of these conditions are associated with increased vascular permeability.

Endothelial Cell Growth, Angiogenesis, and Accelerated Aging: The Impact of Glycation

Angiogenesis and microvascular function are impaired in animal models of diabetes and collateral vessel development is inhibited in diabetic patients. Several factors have been proposed to contribute to abnormal neoangiogenesis in diabetes. For example, glycation of fibroblast growth factor 2 (FGF2) lowered its chemotactic effect toward endothelial cells, and when injected in normoglycemic mice, glycated FGF2 displayed a weaker angiogenic effect compared with nonglycated FGF2. Glycation of bFGF with the intracellular sugars, fructose and glucose-6-phosphate, reduced its high-affinity heparin-binding capacity and its mitogenic activity. These results suggested that reduced angiogenesis observed in diabetes may be a consequence of growth factor glycation. Direct inhibition of endothelial cell growth can be induced by high concentrations of ribose, which results in the generation of protein cross-links and eventually apoptosis.

Endothelial cells cultured on matrices containing glycated collagen display a defect in branching angiogenesis, in part by the early induction of PAI-1, which is accompanied by a time-dependent reduction in cell proliferation. Glycated collagen induces premature endothelial cell senescence as indicated by the appearance of senescence-associated β-galactosidase, increased cell size, and rate of apoptosis. This glycated collagen-induced senescence is associated with a decreased synthesis of NO. Premature senescence was also demonstrated in the aorta of Zucker diabetic rats, suggesting that premature senescence may contribute to diabetic vasculopathy.

RAGE overexpression is associated with an enhanced inflammation and expression of cyclooxygenase-2 and PGE synthase-1 in human diabetic atherosclerotic plaques. AGE formation has been shown to reduce proteolysis of glycated proteins and therefore may affect angiogenic pathways. In diabetic and normal mice, hind limb ischemia was induced by right femoral artery ligation. After 28 days, the ischemic/nonischemic leg angiographic ratio was decreased by 1.4-fold in diabetic animals when compared with control animals. When diabetic mice were treated with aminoguanidine, the angiographic score was similar to the level observed in control animals. AGE blood levels were increased in the diabetic group 12.3-fold compared with control mice, and aminoguanidine treatment decreased the AGE plasma level by 4.2-fold compared with untreated diabetic mice. Blockade of AGE formation normalized impaired ischemia-induced angiogenesis in diabetic mice. This effect is probably mediated by restoration of matrix degradation processes that are disturbed as a result of AGE accumulation.

Endothelial cells from different locations in the vascular tree respond differently to various stimuli and receptors are expressed to different degrees. Whether other receptors in addition to RAGE transduce the effects of AGEs in the vasculature remains to be fully determined. AGE–R3 galectin 3 is proposed as a scavenger receptor, because mice genetically devoid of AGE–R3 develop a rapid glomerulosclerosis compared with wild-type animals.

CD34+ cells from nondiabetic human subjects displayed no benefit when infused into nondiabetic mice with hind limb ischemia; these same cells exerted a profound increase in restoration of blood flow when infused into diabetic mice. Consistent with the premise that progenitor cells may be impaired in diabetes, cultured CD34+ blood cells retrieved from type 1 diabetic subjects produced fewer endothelial cells.
per milliliter of blood compared with cells retrieved from non-diabetic subjects.

Conclusion
Blockade of AGE and cross-link formation may be achieved by administration of compounds such as aminoguanidine, pyridoxamine or OPB9195 $\left[\text{2-(2-isopropylidenhydrodrazono-4-oxothiazolidin-5-ylacetanilide}\right]$. A second method for inhibiting AGE effects is to target RAGE. Agents mimicking the protective effects of soluble RAGE are under development. Cross-link breakers that contain thiazolium structure can break a carbonyl compounds. ALT-711 (4,5-dimethyl-3-phenacyltiazolium chloride) has been the most widely studied agent in this class and has been shown to enhance collagen solubility and to reduce expression of RAGE and AGE-R3 mRNA in diabetic rats. This molecule is now in advanced phase II clinical trials to clarify its potential therapeutic use.

The negative impact of hyperglycaemia on endothelial function and pathological changes is supported by a large series of published studies. High-glucose flux through glucose transporters on endothelial cells overwhelms the mitochondrial electron transport system. Excess mitochondrial substrate flux results in the generation of reactive oxygen species. In conclusion, although reduction of AGEs is possibly an achievable goal in the clinic, such an outcome may not be compatible with life. It is highly likely that age gracefully, a delicate balance between tolerable versus deleterious levels of AGEs in the body must be struck.

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References


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