REVIEW

Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation

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Received on December 22, 2004; revised on March 7, 2005; accepted on March 7, 2005

The products of nonenzymatic glycation and oxidation of proteins and lipids, the advanced glycation end products (AGEs), accumulate in a wide variety of environments. AGEs may be generated rapidly or over long times stimulated by a range of distinct triggering mechanisms, thereby accounting for their roles in multiple settings and disease states. A critical property of AGEs is their ability to activate receptor for advanced glycation end products (RAGE), a signal transduction receptor of the immunoglobulin superfamily. It is our hypothesis that due to such interaction, AGEs impart a potent impact in tissues, stimulating processes linked to inflammation and its consequences. We hypothesize that AGEs cause perturbation in a diverse group of diseases, such as diabetes, inflammation, neurodegeneration, and aging. Thus, we propose that targeting this pathway may represent a logical step in the prevention/treatment of the sequelae of these disorders.

Key words: age/glucose/injury/oxidative stress/receptors

Introduction

Advanced glycation end products (AGEs), the products of nonenzymatic glycation and oxidation of proteins and lipids, accumulate in diverse biological settings, such as diabetes, inflammation, renal failure, and aging. AGEs have multiple potential effects on the vessels and tissues. Indeed, in this context, many “receptors” for AGE have been identified, such as lactoferrin, scavenger receptors types I and II, oligosaccharyl transferase-48 (OST-48), 80K-H phosphoprotein, galectin-3, and CD36 (El Khoury et al., 1994; Vlassara et al., 1995; Li et al., 1996; Ohgami et al., 2002). This review will focus on the role of AGEs and their interaction with receptor for advanced glycation end products (RAGE), a central signal transduction receptor for these species. RAGE function is inextricably linked to its capacity to activate an array of signal transduction cascades, strongly suggesting that RAGE transduces the effects of AGEs by its capacity to engage such signaling cascades, rather than simply mediating AGE removal/detoxification.

Although RAGE was first described as receptor for AGEs (Neeper et al., 1992; Schmidt et al., 1992), an emerging view is that RAGE is a multiligand receptor of the immunoglobulin superfamily. RAGE has been identified as receptor for amyloid-beta peptide (Aβ) and β-sheet fibrils (Yan et al., 1996, 2000); S100/calgranulins (Hofmann et al., 1999); amphoterin (Horı et al., 1995; Taguchi et al., 2000); and, most recently, Mac-1 (Chavakis et al., 2003). It is our hypothesis that the engagement of RAGE by AGEs in a variety of settings triggers rapid generation of reactive oxygen species (ROS) and the up-regulation of inflammatory pathways, mechanisms dependent on RAGE signal transduction. Once set in motion, this ligand/RAGE axis markedly perturbs cellular properties and sets the stage for the sequelae of AGE generation/accumulation, such as diabetic complications, amplification of inflammation and tissue injury/breakdown, and the myriad consequences of natural aging. Here, we will discuss classical settings that promote the generation of AGEs. We present evidence that key roles for RAGE in translating the signature of AGEs in the organism may represent a targeted strategy to suppress the long-term maladaptive consequences of AGEing.

AGEs: diabetes, RAGE, and complications

A large body of evidence suggests that AGE formation and accumulation are enhanced in diabetes (Brownlee, 1992). AGEs are a heterogeneous class of compounds that are composed of both fluorescent and nonfluorescent species. Glycation adducts of proteins are formed when proteins react with glucose-reactive alpha-oxoaldehydes such as glyoxal, methylglyoxal, and 3-deoxyglucosone (3-DG) (Brownlee, 1996). The initial Schiff base adducts formed from glucose and lysine and N-terminal amino acid residues may rearrange to form the key intermediate, fructosamine. Fructosamine degradation and the direct reaction of alpha-oxoaldehydes with proteins may form many AGEs. Of the various types of AGEs that may be generated, it has been shown that both cross-linked and noncross-linked AGEs may be generated. In vivo, a diverse array of AGE products has been detected and characterized such as bis(lysyl)imidazolium...
cross-links, hydroimidazolones, and monolysyl adducts (Wautier and Schmidt, 2004).

Experimental evidence has been presented that both carboxymethyl lysine (CML) adducts of proteins/lipids, as well as AGEs derived through the generation of hydroimidazolone, species that accumulate in diabetes, are specific ligands for RAGE (Thornalley, 1998; Kislinger et al., 1999). Indeed, evidence indicates that CML-AGEs are highly prevalent in diabetes, as well as in aging and renal failure (Reddy et al., 1995; Ikeda et al., 1996; Schleicher et al., 1997; Tauer et al., 2001).

AGE and RAGE: insights from cell culture into diabetic complications

In our first studies, we identified RAGE as receptor for AGE based on the ability of $^{125}$I-AGE albumin to bind RAGE in plastic wells, or on RAGE-expressing endothelial cells (EC), with $K_D \sim 50 \text{nM}$ (Schmidt et al., 1992). From these first observations, we tested the premise that AGE-cellular RAGE interaction would modify critical properties that promoted complications. In EC, AGE-RAGE interaction modulates the expression of adhesion molecules and the expression of proinflammatory/prothrombotic molecules such as vascular cell adhesion molecule-1 (VCAM-1); in fibroblasts, AGE-RAGE interaction modulates the production of collagen; in smooth muscle cells (SMC), AGE-RAGE interaction modulates the migration, proliferation, and expression of matrix modifying molecules; in mononuclear phagocytes (MP), AGE-RAGE interaction modulates chemotaxis and haptotaxis and the expression of proinflammatory/prothrombotic molecules; and in lymphocytes, AGE-RAGE interaction stimulates the proliferation and generation of interleukin-2 (IL-2) (Schmidt et al., 1993, 1995; Miyata et al., 1996; Owen et al., 1998; Sakaguchi et al., 2003). A large body of evidence suggests that one consequence of AGE–RAGE interaction is the generation of ROS, at least in part via the activation of NADPH oxidase (Yan et al., 1994; Lander et al., 1997; Wautier et al., 2001), although other sources of ROS dependent upon AGE have not been ruled out.

Studies have suggested that a key target of ROS in the cell is the activation of the transcription factor, nuclear factor (NF)-kB; indeed, AGE–RAGE interaction activates NF-kB (Yan et al., 1994). NF-kB is a critical factor transducing a variety of inflammatory and pro- or anti-apoptotic signals in the cell, depending on the time course, site, and chronicity of stimulus. Importantly, one consequence of RAGE-dependent activation of NF-kB is the up-regulation of RAGE itself. A molecular mechanism underlying such an effect has been elucidated by the observation that the gene encoding RAGE contains functional binding elements for NF-kB, as well as other factors (Li and Schmidt, 1997).

The heterogeneity of AGEs and the specific circumstances in which they are generated may lead to varied degrees of modification. This must be considered when interpreting experimental findings. For example, Valencia and colleagues reported that bovine albumin incubated in up to 500 mM glucose, fructose, or ribose failed to bind RAGE and up-regulate VCAM-1 or tumor necrosis factor (TNF)-alpha on human microvascular EC or peripheral blood mononuclear cells, respectively, if contaminating endotoxin was thoroughly removed (Valencia et al., 2004). In contrast, other non-AGE signaling ligands of RAGE did up-regulate these factors. Such findings support the concept that AGEs are indeed heterogeneous, and, most importantly, that the specific “characteristic(s)” of these modified species that allow them to bind RAGE are yet to be fully elucidated.

These studies in plastic wells and cell culture indicated that it was important to determine if RAGE was present in human tissue, and, if so, if its expression was modulated in diabetes.

AGE and RAGE: a definite presence in human diabetes

A central question addressed by our studies was to what extent was RAGE up-regulated in human diabetic tissues? In two key settings, atherosclerosis and nephropathy, RAGE appears to be in the right place and time to contribute, at least in part, to diabetic perturbation. In the macrovessels, Cipollone and colleagues showed that enhanced RAGE expression in human diabetic atherosclerotic plaques colocalized with COX-2, type 1/type 2 microsomal prostaglandin E$_2$, and matrix metalloproteinases, particularly in macrophages at the vulnerable regions of the atherosclerotic plaques (Cipollone et al., 2003). Supportive of the indelible mark of AGEs in mediating regulation of RAGE in the vessel wall, these authors demonstrated that the increased levels of RAGE in the plaques, as determined by western blot, correlated with the level of glycosylated hemoglobin. Glycosylated hemoglobin species, although not themselves AGEs or ligands for RAGE, are common precursors to AGE generation. In another setting, Tanji and colleagues showed that RAGE expression was increased in human diabetic kidney (Tanji et al., 2000). In those studies, it was demonstrated for the first time that the principal site of RAGE expression in the diabetic kidney was the podocyte (glomerular epithelial cell), with virtually no expression of RAGE evident in mesangium or in the tubules.

Taken together, such evidence, although strictly an association at this time, provides support for the view that RAGE is present in human diabetic tissues targeted for dysfunction in chronic hyperglycemia. Furthermore, such studies do not fully establish whether such expression of the receptor was contributory or solely a consequence of vascular perturbation, triggered, perhaps, by RAGE-independent pathways. To begin to address these concepts, we turned to animal models. Only by testing in vivo settings might we be able to address the key question: is up-regulation of RAGE in diabetic tissues reflect a cause or effect phenomenon?

Mice are not human—but it is a good start in the heart and great vessels

Although large animal models such as pigs and nonhuman primates may be useful tools to model human-like atherosclerosis, the inability to (readily) genetically modify such creatures renders these models difficult to rigorously test the roles of key players in atherogenesis. There is little doubt that in both type 1 and type 2 diabetes, a chief cause of morbidity and mortality is accelerated atherosclerosis, causing heart attacks and strokes (Kannel and Mceee, 1979; UK Prospective Diabetes Study (UKPDS) Group, 1998).
Our group first reported on the impact of hyperglycemia on vascular perturbation in apolipoprotein (apo) E null mice. In these animals, hypercholesterolemia and atherosclerosis spontaneously develop on normal rodent chow (Plump et al., 1992; Zhang et al., 1992). The induction of diabetes with streptozotocin (stz) resulted in a relative insulin deficiency type diabetes, mimicking, at least in part, type 1 disease. In addition to elevated levels of blood glucose, levels of cholesterol also rose in this model. Our findings demonstrated that in parallel with increased tissue and plasma AGE levels, significant acceleration of atherosclerotic lesion area and complexity at the aortic sinus was noted after 6 weeks of established hyperglycemia (Park et al., 1998). To test the role of the AGE–RAGE axis, we treated mice with once-daily administration of murine soluble RAGE (sRAGE) immediately upon the documentation of diabetes. sRAGE is the extracellular ligand-binding domain of RAGE that binds ligands and blocks their interaction with, and activation of, cell surface receptors. Produced in a baculovirus expression system and purified to homogeneity, sterile, lipopolysaccharide (LPS)-free, sRAGE may be administered to mice once daily by intraperitoneal injection, based on the pharmacokinetics of its plasma and tissue distribution. Mice receiving sRAGE displayed a dose-dependent decrease in atherosclerosis area/complexity compared with vehicle-treated animals (Park et al., 1998). The blockade of RAGE failed to affect levels of lipids or glucose, thereby suggesting that RAGE acted downstream of these key risk factors. Thus, our observations reinforced the premise that RAGE was an amplification factor in the vessel wall affected by atherosclerosis in diabetes.

Despite their promising nature, the potential for elucidating clinical translation of these findings was limited by the experimental design. Specifically, in those experiments, RAGE blockade commenced immediately upon the induction of hyperglycemia. To test the extent to which RAGE blockade might attenuate established and complex atherosclerosis, we rendered 6-week-old apo E null mice diabetic with stz. By 8 weeks of age, mice displayed uniform and consistent hyperglycemia. At 14 weeks of age, mice were begun treatment with either murine sRAGE, 100 μg/day, or vehicle, murine serum albumin (MSA). Mice were killed at 20 weeks. By this age, sRAGE-treated mice displayed striking stabilization of lesion area and the complexity at the aortic root compared to diabetic mice treated with MSA (Bucciarelli et al., 2002). Compared to diabetic mice at 14 weeks, no significant increase in either lesion area or complexity was noted in RAGE-blocked mice at 20 weeks. In sRAGE-treated mice, numbers of MP and SMC in atherosclerotic plaques were reduced at 20 weeks and more closely resembled numbers of infiltrating cells at 14 weeks of diabetes. Most strikingly, vascular inflammation and oxidant stress were also attenuated in the aortae of sRAGE-treated mice, as indicated by decreased cox-2 and nitrotyrosine epitopes, JE-MCP-1, and tissue factor antigens; MMP9 antigen/activity; and phosphorylated p38 MAP kinase. Certainly, oxidative/nitrosative stress is linked to AGE generation. Although AGES generate ROS, in part via RAGE, as well as RAGE-independent means (Yim et al., 2001), there is evidence that ROS themselves may fuel further generation of these AGE species. The pioneering work of Brownlee’s laboratory has shown conclusively that glucose-induced enhanced generation of mitochondrial superoxide is a key factor in AGE generation, particularly in EC (Nishikawa et al., 2000). We speculate that RAGE is “in the middle” of this complex interplay between the biochemical generation of these adducts, and the resultant cell signaling leading to the generation of adducts.

More complex webs woven by AGES: the polyol pathway

In addition to a vicious cycle of AGE generation, ROS formation, and RAGE activation, another central pathway in hyperglycemia, the polyol pathway, may be modulated by diabetes. There is experimental evidence that in cultured human microvascular EC, AGES may induce a key enzyme of the polyol pathway, aldose reductase (AR) (Nakamura et al., 2000). By the polyol pathway, glucose is reduced to sorbitol by AR; fructose generated by this pathway is converted into fructose-3-phosphate by the action of 3-phosphokinase (3-PK). One product of this reaction is 3-DG, a major precursor in the generation of an array of AGES, in particular, CML adducts and others (Niwa, 1999; Hasuik et al., 2002). Levels of plasma 3-DG increase with increased AR levels in erythrocytes in the presence of renal failure (Hasuik et al., 2002). Consistent with a key role for AR in AGE generation, the administration of the AR inhibitor, epalrestat to diabetic subjects reduced levels of CML adducts and their precursors in erythrocytes and decreased plasma levels of thiobarbituric acid reactive substances (TBARs), a measure of oxidant stress (Hamada et al., 2000). In other studies, epalrestat reduced plasma levels of AGES (CML adducts) in patients with type 2 diabetes (Nakamura et al., 2003). Consistent with the premise that elevated 3-DG contributes significantly to diabetic complications, it has been shown that human subjects with higher levels of 3-DG experienced more severe complications of the disorder (Kusunoki et al., 2003).

Furthermore, the tangled web of the polyol pathway and its products provides a further direct stimulus to the generation of AGES; it has been shown that fructose may induce protein oxidation and AGE formation, as well (Takagi et al., 1995; Schalkwijk et al., 2004). From these experiments, it is perhaps not surprising that polyol pathway inhibition alone may not suffice as treatment for the complications of diabetes. These findings suggest the possibility that combined therapies, such as AR inhibition coupled with AGE and/or AGE-RAGE antagonism, might synergize to block primary stimuli for AGE generation, but, as well, the refueling pump driven by factors such as RAGE and ROS.

AR: beyond the micro- and great vessels—to the heart of the matter

Work from our laboratories has elucidated the role of AR in myocardial injury. In the isolated perfused heart, AR inhibition protects hearts from ischemic injury (Ramasamy et al., 1997, 1998; Trueblood and Ramasamy, 1998; Hwang et al., 2002). The apparent interdependence of AGE-RAGE and AR pathways in the generation of AGES and ROS led us to test the concept that RAGE mediates at least in part, cardiac dysfunction in the diabetic heart. Recent preliminary studies from our laboratory have shown that CML-AGES...
are increased in the diabetic mouse and rat heart after 3 months of hyperglycemia; in parallel, RAGE expression was enhanced particularly in EC and infiltrating MP (Buccarelli et al., 2000). When diabetic rats or mice were treated daily with sRAGE, the expression of inducible nitric oxide synthase (iNOS) and levels of NO and cGMP were decreased in sRAGE-treated diabetic hearts. Preliminary studies in the isolated perfused diabetic heart subjected to ischemia/reperfusion showed that lactate dehydrogenase (LDH) release was reduced in rodents treated with RAGE blockade (Lee et al., 2003).

These findings underscore the premise that AR- and RAGE-dependent pathways likely synergize in the diabetic heart to drive exaggerated inflammation and metabolic dysfunction. The relevance of these findings to the treatment of human diabetic heart disease has yet to be tested. However, in the context of AR inhibition alone, it has recently been shown that the administration of the specific inhibitor of AR (zopolrestat) for 1 year in human diabetic subjects resulted in increased resting left ventricle ejection fraction, cardiac output, LV stroke volume, and exercise ejection fraction (Johnson et al., 2004).

**AGE-RAGE and diabetic complications: beyond the heart of the matter**

It is well established that other organ systems are strikingly susceptible to injury and dysfunction in chronic hyperglycemia. As discussed above, human diabetic kidneys display increased AGEs and increased expression of RAGE. Although strictly an association, studies in animal models suggest that blockade of the receptor is beneficial in murine diabetes and nephropathy.

Specifically, we have shown that RAGE blockade provides benefit in diabetes-associated nephropathy in db/db mice (Wendt et al., 2003). In addition, the administration of stz triggered albuminuria and mesangial expansion in wild-type mice; these processes were significantly suppressed in diabetic homozygous RAGE null mice. Consistent with findings in human diabetes, in the mouse kidney in diabetes, podocytes were the principal RAGE-expressing cells. Intensive studies are underway to elucidate the precise link between AGE, RAGE, and podocytes in diabetic and, perhaps, other nephropathies, especially those characterized by heightened oxidative stress.

In other studies, the role of RAGE blockade in the neuropathy of diabetes has been tested. Based on the hypothesis that the activation of NF-kB was a key and critical RAGE-dependent contributing factor to the development of diabetic nephropathy, diabetes was induced with stz in transgenic mice in which the expression of β-globin transgene was under control of an NF-kB-dependent promoter. In the sciatic nerve, diabetes was associated with ~20-fold increase in the induction of β-globin transcripts (which was reduced strikingly with intense insulin therapy to reduce hyperglycemia, and, thus, AGE generation). This was inhibited fully by pretreatment of the diabetic mice with sRAGE (Bierhaus et al., 2004). To further dissect the role of RAGE, we rendered wild-type mice and homozygous RAGE null mice diabetic with stz. After 6 months, mice were killed. Although sciatic nerve demonstrated increased expression of IL-6 and activated NF-kB in the peripheral nerve of wild-type mice, there was no up-regulation of either IL-6 or NF-kB activation in RAGE null mice. The hot plate test was performed to test for thermonociception after 3 months of stz diabetes. Treatment with sRAGE for 3 weeks completely restored pain perception and corrected the latency time. In RAGE null mice rendered diabetic with stz, compared to wild-type mice with diabetes, a significant protection against impaired thermonociception was observed. However, the protection against pain in the diabetic RAGE null mice was not associated with changes in PGP9.5 cutaneous nerve fibers in the footpad. Thus, these findings distinguished morphological versus functional roles of RAGE in the footpad. The mechanisms by which the deletion of RAGE protects against loss of pain perception in chronic diabetes are under active investigation.

In addition to these classical microvascular complications, blockade of RAGE has also been shown to attenuate complications in distinct AGE-enriched environments of diabetes. In the periodontium, blockade of RAGE diminishes *Porphyromonas gingivalis*-triggered alveolar bone loss; in peripheral wounds, blockade of RAGE limits the intense inflammatory response, thereby accelerating wound closure and facilitating angiogenesis (Lalla et al., 2000; Goova et al., 2001). Taken together, these studies elucidated the important observation that even in an environment in which bacterial infection is common, RAGE is involved in the inflammatory response that impedes repair. In other studies, experiments using sRAGE and homozygous RAGE null mice demonstrated that in a model of lethal peritonitis, RAGE null mice displayed increased survival versus wild-type littermates (Liliensiek et al., 2004).

These considerations underscore the implications of our finding that RAGE is a multiligand receptor. Key insights into roles for RAGE even in euglycemic inflammation were elucidated by the identification of non-AGE ligands of RAGE.

**AGEs, oxidant stress, and inflammation: round and round we go**

A plethora of evidence suggests that AGEs are involved in a vicious cycle of inflammation, generation of ROS, amplified production of AGEs, more inflammation, and so on. In addition to the ligation of RAGE, AGEs may be linked to increased generation of ROS by multiple mechanisms, such as by decreasing activities of superoxide dismutase (SOD) and catalase, diminishing glutathione stores, and activation of Protein Kinase C (Yan and Harding, 1997; Obrosova, 2002; Jiang et al., 2004). The direct link of inflammation to AGE formation was suggested by studies in which the activation of myeloperoxidase (MPO) pathways was shown to directly generate CML-AGEs (Anderson et al., 1999). Further evidence for the vicious cycle of oxidative stress begetting AGE and back again was suggested by studies in which malondialdehyde (a lipoperoxidation product) caused secondary oxidative damage to proteins (Traverso et al., 2004).

In the context of RAGE, non-AGE ligands for RAGE may accumulate in oxidative/inflammatory settings as well.
We hypothesize that although AGEs may be rapidly generated by glucose, ROS, or other acute inflammatory stimuli, a key consequence of this interaction is the migration of inflammatory cells into the initial nidus of stress. What are the alternative ligands of RAGE and what is their biologic context?

S100/calgranulins: extracellular newly-identified RAGEs of RAGE

S100/calgranulins are a family of at least 20 polypeptide members. The principal and prototypic function of these cells involve key intracellular mechanisms linked to calcium binding. The EF-hand, calcium-binding domains of these species place them within this family of molecules. S100/calgranulins may be expressed by a wide array of cell types; in the specific context of inflammation and RAGE, these species may be produced in polymorphonuclear leukocytes, dendritic cells (DC), MP, and lymphocytes (Zimmer et al., 1995; Schafer and Heinzmann, 1996; Donato, 2001). When first identified as signal transduction ligands for RAGE, essentially by the finding that S100A12 (calgranulin C or extracellular newly-identified RAGE binding protein, ENRAGE) bound RAGE in a saturable and dose-dependent manner on cultured EC, SMC, MP, and lymphocytes (Hofmann et al., 1999), a puzzling concept was that S100/calgranulins largely existed as intracellular proteins. Distinct mechanisms were identified by which these molecules may be released by inflammatory cells (Rammes et al., 1997; Frosch et al., 2000), thereby allowing them to engage the cell surface receptor RAGE in an autocrine and paracrine manner. Indeed, other S100s such as S100B are linked to nervous system stress, and others, such as S100P, are linked to cancer. In this context, RAGE-dependent ligation of S100P increases the proliferation and survival of cancer cells in vitro (Arunugam et al., 2004).

The direct evidence linking RAGE to the euglycemic inflammatory response was deduced from the following studies. First, in euglycemic mice, blockade of RAGE suppressed delayed type hypersensitivity in mice sensitized challenged with methylated bovine serum albumin, and decrease colonic inflammation in IL-10 deficient mice (Hofmann et al., 1999). Later studies indicated that RAGE blockade suppressed experimental autoimmune encephalomyelitis in mice exposed to encephalitogenic T cells and myelin basic protein and suppressed joint inflammation and destruction in DBA/1 mice sensitized/challenged with bovine type II collagen (Hofmann et al., 2002; Yan et al., 2003). Important roles for S100/calgranulins in diabetic inflammatory stress are underscored by the observation that S100/calgranulins are increased in diabetic macrovessels and renal cortex in murine models, thus suggesting that these molecules may contribute to the acceleration of perturbation in diabetes (Kislinger et al., 2001; Wendt et al., 2003).

Amphoterin: from the nucleus to the extracellular space

Amphoterin, also known as high mobility group box 1 (HMGB1), has important functions within the cell as a DNA binding protein. In addition, it is well described that amphoterin may also exist extracellularly and on the surface of cells, especially cells actively involved in migration (Rauvala and Pihlaskari, 1987). Our studies have explored this molecule’s interaction with RAGE in the context of neurite outgrowth and tumors (Hori et al., 1995; Taguchi et al., 2000). The work of Kevin Tracey’s group first implicated amphoterin directly in mechanisms linked to the inflammatory response. Like S100/calgranulins, amphoterin may be released from activated MPs, thereby leading to the propagation of inflammatory responses (Wang et al., 1999; Andersson et al., 2000). In vivo, the administration of blocking antibodies to amphoterin enhanced the survival of rodents subjected to conditions mimicking that of overwhelming septic shock (Wang et al., 1999). These findings support the concept that amphoterin played a role in the generation of inflammatory mediators and was not merely a bystander in immune/inflamed milieu. Studies are underway to elucidate the precise contributions of amphoterin–RAGE as well as the interaction of amphoterin with other binding species, such as toll-like receptor-4 and toll-like receptor-2 (TLR-4 and TLR-2) (Park et al., 2004). However, recent studies do suggest that in amphoterin-enriched environments, such as arthritis, RAGE blockade plays important roles (Hofmann et al., 2000; Pullerits et al., 2003).

The precise roles for RAGE and TLR in transducing the inflammatory effects of amphoterin remain to be fully elucidated.

Linking these diverse ligands: RAGE and signal transduction

Experimental evidence supports the premise that RAGE-dependent modulation of gene expression and cellular properties is dependent on signal transduction. The signal transduction pathways downstream of RAGE activation in a wide array of cell types are diverse. Depending on the acuteness/chronicity of ligand stimulation, distinct signaling pathways may be activated. All of the ligands identified to date have been shown to cross compete and bind at the RAGE V-type Ig domain (Hofmann et al., 1999). AGEs, S100/calgranulins, and amphoterin may activate cell types intimately involved in macro-/microvascular disease initiation/progression, such as EC, SMC, MP, lymphocytes, neurons, and podocytes. Signaling cascades activated upon ligand–RAGE interaction include pathways such as p21ras, erk1/2 (p44/p42) MAP kinases, p38 and SAPK/JNK MAP kinases, rho GTPases, phosphoinositol-3 kinase, and the JAK/STAT pathway; downstream consequences such as the activation of the key transcription factors, NF-kB and cAmp response element binding protein (CREB) have also been reported (Lander et al., 1997; Deora et al., 1998; Hofmann et al., 1999; Huttunen et al., 1999, 2002; Kislinger et al., 1999; Huang et al., 2001).

Importantly, ligand stimulated RAGE activation results in the generation of ROS, at least in part via the activation of NADPH oxidase (Wautier et al., 2001). Definitive roles for RAGE-mediated activation of NADPH oxidase were demonstrated in studies in NADPH oxidase null mice. Specifically, we found that monocytes retrieved from NADPH oxidase null mice, when compared with wild-type monocytes, failed to display increased generation of tissue factor upon incubation with AGEs (Wautier et al., 1994). Studies are underway to delineate if additional mechanisms, distinct from NADPH oxidase, underlie the potent ability of RAGE to generate ROS.
In this context, it is essential to indicate that the cytosolic domain of RAGE is critical for RAGE-dependent signal transduction and modulation of gene expression and cellular phenotype. In studies both in vitro and in vivo, studies have indicated that the deletion of the cytosolic domain of RAGE causes a dominant negative (DN) effect which abrogates ligand-mediated RAGE signaling and function. For example, when DN RAGE is expressed in vivo in SMC, cells of MP lineage, neurons of the central or peripheral nervous system or CD4 lymphocytes, RAGE-mediated signaling is effectively suppressed, thereby strikingly modulating injury-triggered outcomes in diverse settings (Hofmann et al., 1999; Kislinger et al., 1999; Taguchi et al., 2000; Sakaguchi et al., 2003; Yan et al., 2003; Arancio et al., 2004; Rong et al., 2004; Cataldeghirmen et al., 2005).

Such studies indicate that RAGE is a signal transduction receptor for multiple classes of proinflammatory ligands. Our findings support the premise that the ligand-RAGE axis stimulates EC and other cell types to augment cellular perturbation in a diverse array of disease settings characterized by ligand accumulation.

**RAGE and implications for the pathogenesis of type 1 diabetes: nearly full circle**

Based on the findings that RAGE appeared to play key roles in euglycemic inflammation, we hypothesized that RAGE might play a role in autoimmune disease, such as that observed in type 1 diabetes. To test this premise, we studied the role of RAGE in a model of adoptive transfer of diabeticogenic spleen cells into NOD/SCID mice (Chen et al., 2004). Both RAGE and S100 were expressed on islet cells with an inflammatory infiltrate in sections of pancreata from diabetic NOD/SCID mice that had received a transfer of splenocytes, but not from control NOD/SCID mice. In addition to RAGE expression in endocrine cells, other studies demonstrated that RAGE was also expressed at least in a population of T cells (CD4+ and CD8+) and B cells (Chen et al., 2004).

To test the potential role of RAGE in mediating autoimmune diabetes in this model, we transferred splenocytes from a diabetic NOD donor into NOD/SCID mice; NOD/SCID mice were treated with either sRAGE or mouse serum albumin. Treatment with sRAGE significantly reduced the rate of transfer of diabetes. By day 36 after transfer, 22 of 24 (92%) control animals but only 2 of 25 (10%) mice treated with sRAGE were diabetic. In parallel, the expression of key cytokines, IL-1beta and TNF-alpha, was significantly reduced in the sRAGE-treated islets compared with vehicle-treated animals. The expression of IL-10 was strikingly increased in sRAGE-treated mice islets, along with increased transforming growth factor (TGF)-beta, compared to vehicle-treated animals (Chen et al., 2004).

Further, in the context of our proposed model of AGEs and RAGE triggering a vicious cycle of injury with multiple points of amplification, glucose, AGEs, and oxidative stress augment injury to islets and beta cells (El-Assaad et al., 2003; Robertson, 2004; Wu et al., 2004). Certainly, in the context of islet transplantation, stopping the cycle of AGE generation and oxidative stress, in part via RAGE, may diminish injury to newly transplanted islets, possibly delaying diabetes.

Interestingly, when preactivated diabeticogenic BDC2.5 cells were injected into mice, sRAGE had no effect on preventing diabetes. Such observations require that we “re-open” the issue of potential roles for RAGE in the adaptive immune response. Studies to rigorously address this hypothesis are underway at this time.

Taken together, these findings suggest that it will be critical to dissect each potential component of RAGE-dependent mechanisms in autoimmunity. Specifically, do roles exist for RAGE in both the adaptive immune response and/or the amplification of immune/inflammatory injury in autoimmunity targets such as islets? Will there be beneficial roles for RAGE blockade in attenuating rejection after allogeneic islet transplantation? These concepts must be addressed by instituting RAGE blockade at distinct time points either alone or in combination with classical immunosuppressive agents in experimental models of allogeneic islet transplantation. Such studies are underway at this time.

Indeed, AGEs, too may be found on aging lymphocytes (Poggioli et al., 2002, 2004). Although at the very least such modifications may represent “biomarkers” of aging, the more intriguing hypothesis is certainly that AGE-modification of lymphocyte cell surface molecules may either enhance or blunt the impact of antigen presentation on T-cell responses. In this context, recent hints to the implications of AGEing of DC suggest that such glycation may promote DC development but impair their ability to stimulate primary T-cell responses (Price et al., 2004). Certainly, altering the imprint of presented antigen may either enhance or suppress proper presentation to the T cell. Likely, such processes depend on the specific site/time/modification in question. Taken together, these observations in lymphocytes and DC suggest that AGE-mediated immune modulation may redirect immune responses in aging and diabetes. The potential links to RAGE in such processes provide for the development of fascinating hypotheses on the role of RAGE in the adaptive immune response.

**AGE and AGEing: an old story with new twists**

The AGEing hypothesis of aging was prompted by multiple observations that aged tissues are characterized by the accumulation of a variety of types of AGEs, from CML adducts; to carboxyethyl (CEL) lysine adducts; methylglyoxal; pentosidine; and others (Ahmed et al., 1997; Schleicher et al., 1997; Odetti et al., 1998). In addition to proteins and lipids, evidence exists that DNA may also undergo AGE modification. It is speculated that the modification of DNA has profound implications for both regulatory and perhaps epigenetic components of the aging process (Baynes, 2002). The issue of the role of AGEs as a cause and/or biomarker in the maladaptive aspects of the aging process is not a settled issue (Baynes, 2001; Kirkland, 2002). The intriguing studies of Sell and colleagues suggested, perhaps, at least a little of both. In their experiments, they tested the hypothesis that the longitudinal determination of the rate of glycation/glyoxidation of skin collagen would predict longevities in ad libitum versus caloric-restricted mice. They biopsied C57BL/6Nnia male mice at age 20 months and at natural death. Levels of skin furosine, CML adduct, and pentosidine...
were determined. Caloric restriction significantly increased life span in these animals versus ad-libitum feeding. In parallel, skin levels of the three types of AGEs were sharply reduced in caloric-restricted fed animals and correlated significantly with longevity (Sell et al., 2000). Such considerations may suggest that the levels of skin AGEs are a yardstick for assessing the extent of AGEing in the vasculature.

Evidence suggests that AGEs form during natural aging, especially as a consequence of exposure of long-lived proteins to even homeostatic levels of glucose. As our population develops even further degrees of insulin resistance with aging, it is likely that hyperglycemia/glucose-intolerance itself will further propel AGE generation in aging tissues. In addition to increased AGE formation, aging appears to be associated in part with reduced AGE defenses. For example, glyoxalase I, a cytosolic enzyme that functions to decrease glycation reactions, displays decreased activity with aging (Thornalley, 2003). Further, Kil and colleagues have shown that aging-associated glycation-induced inactivation of NADP (+)-dependent isocitrate dehydrogenase results in decreased activity of this enzyme, and, in parallel, the perturbation of cellular antioxidant defense mechanisms (Kil et al., 2004). Such considerations support the premise that in aging, injury-provoking mechanisms may be enhanced, in the face of slowly deteriorating anti-injury defense mechanisms. These considerations lead us to consider what are the key tissue/organ targets of AGEs in aging?

The brain and the heart, of course AGE–RAGE and the brain

AGEs increase in the brain during normal aging. Interestingly, experimental evidence supports the premise that AGEs are further increased in the brain in the presence of vascular or Alzheimer’s dementia (Yan, Chen et al., 1994; Dei et al., 2002; Jono et al., 2002; Bar et al., 2003; Girones et al., 2004). Further, in diabetes and Alzheimer’s disease, evidence suggests that CML accumulation is greater than that seen in Alzheimer’s disease alone (Girones et al., 2004). The relationship between diabetes, cognitive decline, and Alzheimer’s disease is still under active investigation, but some studies suggest that diabetes may be associated with an increased risk of developing Alzheimer’s disease, along with enhanced decline in some cognitive systems (Arvanitakis et al., 2004; Luchsinger et al., 2004). The intimate link between vascular and Alzheimer’s dementia is underscored by the observation that stroke is increased in subjects with Alzheimer’s disease, especially in the presence of known vascular risk factors (Honig et al., 2003). Connecting the dots may, ultimately, highlight unifying roles for AGEs in neuronal stress thus exacerbating aging and neurodegenerative processes in the brain. Indeed, central roles for altered inflammatory mechanisms in the diabetic-aged brain, in part secondary to AGEs, may amplify neuronal stress. In this context, hyperglycemic db/db mice subjected to transient cerebral hypoxia/ischemia, demonstrated the decreased expression of anti-apoptotic molecules such as bfl-1 in microglia, decreased glial activation upon reperfusion, and increased tissue injury suggesting an impaired immune response analogous to defective wound healing (Zhang et al., 2004).

Further, multiple studies suggest that AGEs are directly neurotoxic to cultured neurons (Yan, Chen et al., 1994; Takeuchi et al., 2000). AGEs and their precursors (methylglyoxal and glyoxal) may increased the aggregation and cytotoxicity of intracellular amyloid-beta carboxy-terminal fragments (Woltjer et al., 2003). Taken together, these considerations underscore the premise that AGEs may be central to the exacerbation of dementia and enhanced predilection of stroke. In this context, both AGEs and Aβ are signal transduction ligands of RAGE. The link between Aβ–RAGE interaction and Alzheimer pathology has been studied in experimental systems. The overexpression of mutant amyloid precursor protein (APP) and neuronal RAGE in transgenic mice accelerates hippocampal neuronal loss and decline in long-term potentiation and behavior (Arancio et al., 2004).

RAGE interacts with Aβ as well as other beta-sheet fibrils, such as prion peptide (Yan et al., 2000). The settings of diabetes and Alzheimer’s disease are further complicated by the premise that in Alzheimer’s disease subjects, the pancreas (islets), too, may be affected by amyloid deposition. Janson and colleagues provided evidence to support the premise that Alzheimer’s disease may be a risk factor for diabetes. Upon review of the Mayo Clinic Alzheimer disease patient registry, the authors found that islet amyloid was increased in Alzheimer compared to control subjects and that the slope of the increase of fasting plasma glucose concentration was greater in Alzheimer versus control individuals (Janson et al., 2004). Together, such data support the hypothesis that vulnerability to type 2 diabetes and Alzheimer’s disease may share a key common link—the RAGE ligand amyloid.

Interestingly, another group of RAGE ligands, the S100 family, was highly expressed in Corpora amylacea in human brain. Corpora amylacea (also called polyglucosan bodies) are hallmarks of normal brain aging. Hoyaux and colleagues examined the expression of 10 distinct S100 proteins in these bodies by immunohistochemistry (S100A1, S100A2, S100A3, S100A4, S100A5, S100A6, S100A8, S100A9, S100A12 and S100B). Of these 10, 9 were detected in Corpora amylacea. Three of these (S100A8, S100A9, and S100A12, the latter, EN-RAGE), linked importantly to inflammation, were most highly expressed in neurons as well as inflammatory cells (Hoyaux et al., 2000). Thus, the ligand-RAGE axis may be a part of quite a tangled web in the aging brain—linked to the complications of aging, diabetes, and excess accumulation of Aβ.

AGE-RAGE, aging, and the heart

The aging heart is susceptible to glycation processes; consequences of which may include various degrees of ventricular dysfunction, such as increased ventricular and vascular stiffness, and altered regulation of vascular tone (Lakatta and Levy, 2003). Potential roles for RAGE in the cardiac dysfunction of AGEing are suggested by the studies of Simm and colleagues. These authors reported that in human subjects undergoing cardiac surgery, an age-dependent increase in RAGE protein in the atria was observed, with the highest levels observed in the senescent population and the lowest levels observed in the youngest children (Simm et al., 2004). Correlation analyses revealed that the
degree of RAGE expression was associated with reduced heart function in these subjects. Although the potential mechanistic link between these observations remains to be elucidated, these experiments, nonetheless, underscore the observation that RAGE is up-regulated at sites of tissue stress in an array of disorders, including the aging human heart.

**Hypotheses and perspectives**

Recent evidence suggests that AGEing is not solely a facet of being “old.” Rather, in addition to natural aging, acute stresses may trigger rapid AGE generation. As depicted in Figure 1, we hypothesize that stimulated by oxidative stress and ROS generation, inflammatory stimuli, physical injury, bouts of intermittent (or sustained) hyperglycemia, AGE formation is likely a key first step in a broad array of injury settings. Once formed, such AGE foci create a nidus for the amplification of stress pathways. Particularly in aging, for example, reduced anti-AGE defenses likely contribute to smoldering accumulation of these species. Specific consequences of AGE accumulation are the up-regulation of RAGE itself and the attraction of inflammatory cells, such as polymorphonuclear leukocytes, MP, and lymphocytes. Certainly, the observation that RAGE is a counter-receptor for Mac-1 integrins underscores key roles for this axis, directly or indirectly (the latter, via the up-regulation of chemokines and adhesion molecules) in generating the inflammation scaffold in AGEd tissues. Such inflammatory cells, normally serving homeostatic mechanisms such as removal of infectious substances or necrotic debris, take on new roles in this inflammatory cascade. For example, release of S100/calgranulins and/or amphoterin from such cells triggers a new wave of inflammatory and cell stress reactions. In an autocrine and/or paracrine manner, engagement of these species with the signal transduction receptor RAGE (as well, perhaps, as other binding sites such as certain TLRs) generates a second wave of cell perturbing substances. Indeed, one consequence of ligand–RAGE interaction is the further generation of ROS; such ROS may beget further AGE generation, inflammation, and ROS production. Such a third wave may feed back to sustain the cycle of stress in a wide range of cell types, such as EC, SMC, MP, PBMC, podocytes, neurons, and glia, and, thus, eventually cause tissue dysfunction and irreparable damage.

The testing of these premises will require agents to target AGEs and/or their signal transduction receptor RAGE, first in preclinical models, and then in clinical trials.

**How do we break the cycle?**

**AGEing with grace?**

To break the cycle of AGE formation and protein cross-linking, many strategies are in various stages of development to either block AGE formation or breakdown established AGE cross-links in the tissues. The first agent studied in the context of agents to prevent AGE formation was aminoguanidine (or pimagedine). Based on extensive preclinical efficacy in animal models in which AGEs are prevalent (Edelstein and Brownlee, 1992; Ulrich and Cerami, 2001), pimagedine was administered to 690 subjects with type 1 diabetes, nephropathy, and retinopathy. The estimated glomerular filtration rate progressed more slowly in the pimagedine-treated group, and the degree of proteinuria was significantly lower in the pimagedine-treated group (Bolton et al., 2004). However, the trial failed to meet significance in the primary endpoint (doubling of serum creatinine); yet, notwithstanding this finding, this trial was the first to support the AGE hypothesis and its role in diabetic complications in human subjects based on changes in secondary endpoints.

The next level of anti-AGE strategies involves the AGE cross-link breakers. The prototypic agent, ALT-711, has already been tested in human clinical trials. After 56 days of treatment, ALT-711 resulted in improved total arterial compliance in aged humans with vascular stiffening (Kass et al., 2001; Susic et al., 2004). In addition, newer agents, such as pyridoxamine, an inhibitor of advanced glycation end products (AGE-RAGE) and a vicious cycle of cellular perturbation and tissue injury: implications for aging, inflammation, neurodegeneration, and diabetic complications. We hypothesize that stimulated by oxidative stress and reactive oxygen species (ROS) generation, inflammatory stimuli, physical injury, bouts of intermittent (or sustained) hyperglycemia, AGE formation is likely a key first step in a broad array of injury settings. Once formed, such AGE foci create a nidus for the amplification of stress pathways. In addition to increased formation, decreased removal/detoxification of AGES may contribute to stress in aging, for example, reduced anti-AGE defenses likely contribute to smoldering accumulation of these species. Specific consequences of AGE accumulation are the up-regulation of RAGE itself, and the attraction of inflammatory cells, such as polymorphonuclear leukocytes, MP, and lymphocytes. Such inflammatory cells, normally mediating homeostatic mechanisms, such as removal of infectious substances or necrotic debris, take on new roles in this inflammatory cascade. For example, release of S100/calgranulins and/or amphoterin from such cells triggers a new wave of inflammatory and cell stress reactions. In an autocrine and/or paracrine manner, engagement of these species with the signal transduction receptor RAGE generates a second wave of cell perturbing substances. Indeed, one consequence of ligand–RAGE interaction is the further generation of ROS; such ROS may beget further AGE generation, inflammation, and ROS production. Such a third wave may feed back to sustain the cycle of stress in a wide range of cell types, such as EC, SMC, MP, PBMC, podocytes, neurons, and glia, and, thus, eventually cause tissue dysfunction and irreparable damage.

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reactions are on the horizon. This target has shown efficacy in preclinical models, to date (Onorato et al., 2000).

**ROS and mitochondrial stress: roles for benfotiamine and thiamine**

Based on Brownlee’s hypothesis that glucose triggers three major pathways of hyperglycemic damage in the tissues (hexosamine, AGE formation, and diacylglycerol pathways), he hypothesized that lipid-soluble thiamine derivatives might inhibit these pathways. In rats with diabetes, the long-term administration of benfotiamine resulted in decreased vascular abnormalities in the retina and attenuated activation of NF-κB (Hammes et al., 2003). Further, other studies extended these findings to nephropathy; the administration of either thiamine or benfotiamine to diabetic rats attenuated albuminuria (Babaei et al., 2003). These studies, a test of concept of the glucose-triggered common pathway model of diabetic complications, provide potential new avenues for therapy in human subjects with diabetes. Large-scale clinical trials will be needed to fully test these concepts.

**Are we really what we eat?**

Recent studies by Vlassara and colleagues have suggested that diets high in AGEs, such as those exposed to high temperatures, may accelerate the consequences of natural aging and diabetes (Hofmann et al., 2002a; Goldberg et al., 2004). When tested in human subjects, the reduction of inflammatory mediators was observed in human diabetic subjects consuming low-AGE food (Vlassara et al., 2003). Further, the restriction of glycotoxins was found to reduce excess levels of AGEs in human subjects with renal failure (Uribarri et al., 2003).

Taken together, these observations strongly suggest that lifestyle modification, including diet, may exert potent influences on inflammation, aging, and the consequences of diabetes.

**Stopping RAGE: a good policy?**

Lastly, as extensively discussed, studies from our laboratory and others have highlighted central roles for the ligand/RAGE axis in the complications of diabetes, inflammation, dementia, and Alzheimer’s disease. Our first studies have extensively employed sRAGE, the extracellular ligand-binding domain of RAGE. Importantly, the studies described above by using homozygous RAGE null mice strongly support the premise that the chief target of sRAGE is RAGE itself. However, a critical question to address is, what are the potential consequences of antagonizing RAGE? Although homozygous RAGE null mice develop and age normally and display normal fertility, it remains possible that compensatory pathways have been engaged during the development of RAGE null mice, thereby, possibly, masking critical innate and/or adaptive response functions of this receptor. In this context, studies demonstrating that sRAGE does not block diabetes upon adoptive transfer of BDC2.5 preactivated T cells into recipient mice suggest that RAGE contributes importantly, in some manner yet to be determined fully, in the adaptive immune response.

Distinct from adaptive immunity, our studies have uncovered a role for RAGE in the response to acute nerve crush in euglycemic animals. The administration of sRAGE or blocking F(ab′)2 fragments derived from anti-RAGE IgG delays axonal regeneration in wild-type mice subjected to acute crush to the sciatic nerve (Rong et al., 2004a). Histological and molecular analysis revealed that macrophage infiltration and, thus, Wallerian degeneration, were reduced in RAGE-blocked mice. To further dissect the impact of macrophage or neuronal RAGE, transgenic mice were developed that expressed DN RAGE in either macrophages or peripheral neurons. Compared to littermate controls, regeneration was delayed in either transgenic mouse, especially in double transgenic mice expressing DN RAGE in both macrophages and peripheral neurons (Rong et al., 2004b).

These studies are the first to uncover innate roles for RAGE in a response to injury. As antagonism of RAGE nears the clinic, it will be essential to carefully monitor the impact of chronic RAGE blockade in human subjects. Certainly, however, all therapeutic agents have potential for some degree of toxicity. Definitive answers to these questions will require definitive long-term trials in many subjects.

**Blocking AGEing—to a fountain of youth and wellness?**

Recent studies have elucidated the wide array of settings in which AGEs may be generated in the absence or presence of frank disease and pathology. In particular, contrary to earlier theories, AGEs may be generated rapidly and be the first to arrive at foci of injury, inflammation, or trauma. It is our hypothesis that even fleeting AGE deposition imparts a first and early signature to the tissues that may be, if not checked naturally or pharmacologically, transduced into chronic cellular perturbation. In this new view, it is highly likely that AGEs serve homeostatic purposes, perhaps in clearance of microbes or quenchers of biochemical species destined to cause mischief. Indeed, the observation that AGEs accumulate in less complex organisms, such as *Drosophila melanogaster* and *Escherichia coli*, may suggest, at least in part, homeostatic properties of these species (Oudes et al., 1998; Mironova et al., 2001). In this very context, exciting new studies have elucidated that an S100 family member, S100A7, protects skin from *E. coli* infection (Glaser et al., 2005).

Although the balance of the evidence suggests that AGE species may trigger maladaptive responses in the organism, there is also a reason to suspect that exclusive roles for AGEs in pathology may be too simple a premise.

**Acknowledgments**

This work was supported by grants from the USPHS, the Juvenile Diabetes Research Foundation, American Diabetes Association (SIV), and the American Heart Association (RR). AMS is a recipient of a Burroughs Wellcome Fund Clinical Scientist Award in Translational Research.
Abbreviations

Aβ, amyloid-beta peptide; AGE, advanced glycation end product; AR, aldose reductase; CML, carboxymethyl lysine; DC, dendritic cells; 3-DG, 3-deoxyglucosone; DN, dominant negative; EC, endothelial cells; IL, interleukin; MP, mononuclear phagocytes; NF, nuclear factor; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; SMC, smooth muscle cells; sRAGE, soluble RAGE; stz, streptozotocin; TLR, toll-like receptor.

References


