The Receptor for Advanced Glycation End Products: Mechanisms and Therapeutic Opportunities in Obesity and Diabetes

Del Pozo CH,1 Alexander Shekhtman2, Ravichandran Ramasamy1, and Ann M Schmidt*1
1Diabetes Research Program, Division of Endocrinology, NYU Langone Medical Center, New York, NY, USA
2Department of Chemistry, University at Albany, 1400 Washington Avenue, Albany, New York, NY, USA

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*Corresponding author: Ann Marie Schmidt, Department of Medicine, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA, Tel: 212-263-9444, E-mail: annmarie.schmidt@nyumc.org.

Abstract

The Receptor for Advanced Glycation Endproducts (RAGE) plays important roles in the pathogenesis of metabolic disorders, including obesity, type 2 diabetes and diabetic complications. RAGE is recruited through the increased production and decreased removal of its ligand families in obese and diabetic tissues, which are driven by such metabolic stresses as high fat feeding, hyperglycemia, inflammation and oxidative stress. In this review, we summarized the state of knowledge regarding RAGE ligand binding to the extracellular domains of RAGE; the mechanism of RAGE signal transduction through DIAPH1 and its consequences in obesity and diabetic complications; and the implications for human subjects, from biomarkers to therapeutic interventions. This body of work supports targeting the RAGE/DIAPH1 signaling axis in metabolic disorders.

Keywords: Advanced Glycation End products; Obesity; Diabetes; Immunoglobulin; Hyperglycemia

Introduction

The Receptor for Advanced Glycation Endproducts (RAGE) transduces the biological effects of a diverse group of binding molecules, or ligands. RAGE was first identified for its ability to bind the advanced glycation end products, or AGEs, which accumulate in diverse settings such as diabetes, inflammation, oxidative stress, aging, and ischemia/reperfusion injury [1,2]. In addition to AGEs, distinct ligand families of RAGE, including the S100/calgranulins, high mobility group box 1 (HMGB1), amyloid-peptide (A)[3–6], and other ligands such as mac-1, phosphatidylserine (PS) and lysophosphatidic acid (LPA)[7–9] have been identified. The identification of these non-AGE ligands of RAGE expanded the possible milieu in which the biology of RAGE plays pathobiological roles, such as in sterile inflammation and autoimmunity, neurodegeneration (such as Alzheimer’s disease (AD) and amyotrophic lateral sclerosis (ALS)), cancer and metabolic disorders [10–13]. In homeostasis, in rodents, other species and in human subjects, the expression of RAGE is low in all tissues except for the lung. However, in disease, and particularly in the distinct conditions in which RAGE ligands accumulate, the expression of RAGE is higher compared to age-matched or non-diseased controls. Key questions arise as to how is it possible that such diverse ligands bind RAGE and, further, how do these diverse moieties transverse signals via the receptor? In this review, we will consider the current state of knowledge in these key areas and we will present examples of diseases of metabolic dysfunction in which RAGE appears to play contributory mediating roles. Finally, we will address the status of antagonizing RAGE and prospects for clinical translation.

We begin with a review of the complexity of the RAGE domains with respect to ligand binding and how these diverse ligands mediate signal transduction.

RAGE – Structure and Ligand Engagement

RAGE is a member of the immunoglobulin superfamily of cell surface molecules. Its extracellular region is composed of three immunoglobulin like domains, one V (variable) type domain, followed by two C (constant) domains. These domains are followed by a single hydrophobic transmembrane domain and lastly by a highly charged, short cytoplasmic domain, which has been shown to be essential for RAGE-mediated signal transduction [14]. Most of the ligands of RAGE bind to the V-type immunoglobulin domain. However, a number of studies have suggested that the first two extracellular domains (V-C1) form an integrated unit to facilitate ligand binding [15].

Xue and colleagues used NMR spectroscopy to identify three distinct surfaces on the V-domain capable of binding the RAGE ligand, AGEs: (1) strand C and loop CC, (2) strand C, strand F and loop FG, and (3) strand A’ and loop EF[16]. These authors suggested that although the binding affinities for AGEs were low (µM range) for isolated V-domain, constitutive receptor oligomerization facilitated the recognition of AGE-modified proteins with affinities less than 100 nM.

Park and colleagues prepared a 1.5Å crystal of the V-C1 domains [17]. These authors identified that the V-C1 domains contained two key “patches” that were responsible for ligand engagement. First, a large basic patch is required for the effects of binding one of the S100/calgranulins, S100B, and second, a negatively charged patch binds AGE proteins [17]. Interestingly, these authors reported that distinct molecules, including double stranded DNA and double stranded RNA, bound RAGE in these extracellular domains. In a distinct study, Koch and colleagues prepared a 1.85Å crystal of the V-C1 binding domains; their data illustrated that the V-C1 domains functioned as an integrated structural unit [18]. They found that V-C1 contained a large positively charged electrostatic surface, which was consistent with the acidic or negative charges of many of the ligand families. Self-association of V-C1 was also shown by these authors in their models [18].

Xue and colleagues examined a solution structure of a carboxy ethyl lysine (CEL) peptide (a specific type of AGE) complex with the V-domain. The studies revealed that the CEL peptide fit into a positively charged cavity on the V-domain and that peptide backbone atoms made specific contact with this domain [19]. Recently, Xue and colleagues further illustrated that distinct AGEs, such as hydroimidazolones, bound specifically to the V-domain, with nM affinity via multiple contacts with a positively charged surface of the V-domain [20].

Yatime and Andersen showed that the homodimerization of the RAGE molecule was multimodal, that is, in addition to oligomerization of V-C1, additional sites for oligomerization were also proposed in the transmembrane domains through a conserved...
GxxG motif [21]. These patterns of oligomerization were linked mechanistically to both ligand binding and to signal transduction.

Taken together, this published work illustrates putative mechanisms by which distinct ligand families might engage the same receptor. Indeed, very early studies by Xie and colleagues highlighted that oligomeric forms of ligands might be preferred ligands for RAGE; these researchers showed, through high resolution NMR, that hexameric forms of RAGE ligand S100A12 were the likely RAGE binding forms of this S100, in a process that was calcium-dependent [22]. The work described above considered the extracellular and transmembrane domains of RAGE. In the section to follow, we present the current state of understanding the RAGE intracellular domain, as it is essential for the effects of RAGE ligands on signal transduction.

**RAGE Cytoplasmic Domain and Signal Transduction**

The cytoplasmic domain of RAGE is short and essential for RAGE ligands to stimulate signal transduction. Indeed, in cultured cells and in vivo, using novel transgenic mice in which the cytoplasmic domain of RAGE was deleted in a cell-specific manner, although RAGE ligands still bound the extracellular domains, they were no longer able to initiate RAGE signaling in a range of cell types, such as macrophages, T-lymphocytes, neurons, endothelial cells and smooth muscle cells [23–27]. Two key points may be made regarding these findings: First, multiple different ligands of RAGE were tested in these models, such as AGEs, members of the S100/calgranulin family, and AGE; in each case, RAGE signaling by these ligands required the cytoplasmic domain. Second, these studies, albeit that they illustrated the essential role of the RAGE cytoplasmic domain in RAGE-mediated functions, did not identify the proximate mechanisms by which this domain of RAGE triggered signal transduction.

To address this critical issue, a yeast two hybrid assay was performed in order to identify molecules that bind the cytoplasmic domain of RAGE as a first step to identifying RAGE-dependent signal transduction effector pathways. The results of this experiment, probing for candidate molecules in a lung library, revealed DIAPH1 (diaphanous-1), a member of the form in family, as a putative effector for RAGE signaling [28]. Multiple confirmatory studies, including immunoprecipitation and immunofluorescence microscopy confirmed that in vitro and cellular systems, RAGE bound DIAPH1, particularly its FH1 (or formin homology) domain 1. Signaling and functional studies were performed, which showed that small interference RNA (siRNA) knockdown of DIAPH1, which had no suppressive effect on RAGE expression, blocked RAGE ligands S100B or carboxy methyl lysine (CML)-AGE-mediated activation of the Rho GTPases rac1 and cdc42 [28]. In functional studies, siRNA-knockdown of DIAPH1 in transformed cells blocked RAGE ligand-mediated cell migration, but had no effect on the RAGE ligand-mediated effects of fetal bovine serum (10%), which, in each case, RAGE signaling by these ligands required the cytoplasmic domain. Second, these studies, albeit that they illustrated the essential role of the RAGE cytoplasmic domain in RAGE-mediated functions, did not identify the proximate mechanisms by which this domain of RAGE triggered signal transduction.

Studies in distinct cell types were performed to test the importance of DIAPH1 in RAGE signal transduction. In smooth muscle cells, RAGE and DIAPH1 are expressed. Previous work addressing roles for RAGE in smooth muscle cell responses to neointimal injury (induced in mice by femoral artery endothelial denudation) revealed that genetic (global deletion of Ager – the gene encoding RAGE) or transgenic-mediated expression of cytoplasmic domain-deleted RAGE in smooth muscle cells resulted in significant protection from endothelial denudation injury. This is, the intima/media ratio was greatly reduced [27]. These concepts were tested in mice globally devoid of Diaph1 or their littermate controls. In those studies, quite analogous to results seen with Ager deletion, deletion of Diaph1 significantly reduced intima/media ratio after arterial injury [29]. Primary murine aortic smooth muscle cells were isolated from wild type mice or mice devoid of Diaph1. These studies revealed that RAGE ligand S100B triggered smooth muscle cell migration through a process that was DIAPH1-dependent. The signal transduction mechanisms were traced to S100B/ DIAPH1-dependent membrane translocation of c-Src, which caused activation of Rac1 and redox phosphorylation of AKT/glycogen synthase kinase 3β, all processes that were essential for smooth muscle cell migration. Experiments in other cell types supported that DIAPH1 was required for RAGE ligand signal transduction.

In macrophages, it was previously shown that deletion of Ager resulted in protection from hypoxia-mediated upregulation of Egr1, a critical transcription factor involved in pro-inflammatory and pro-thrombotic tissue responses in hypoxic conditions [30]. A key question that arose from these findings was what was the RAGE ligands generated in this environment, which were responsible for stimulating RAGE? It was shown that hypoxia resulted in a time dependent increase in release of AGEs into cellular supernatant [30]. It was thus logical to ask, does DIAPH1 play roles in hypoxia- or AGE-mediated upregulation of Egr1 in the hypoxic state? To address this question, Xu and colleagues retrieved macrophages from wild type and mice devoid of Diaph1 and showed that DIAPH1 was required for hypoxia-mediated upregulation of Egr1 through a pathway including activation of protein kinase C βI, ERK1/2 (extracellular regulated kinase) and c-Jun Nterminal kinase signaling [31]. Other cell types in which RAGE ligands were shown to require DIAPH1 for signal transduction were human thyroid cancer cells (S100A4) and microglia (S100B) [32,33].

These studies underscored the need to identify the specific mechanisms by which the RAGE cytoplasmic domain bound DIAPH1. Work by Shekhtman’s laboratory revealed that amino acids 2-15 of the RAGE cytoplasmic domain were ordered, and contained the amino acid residues required for binding to DIAPH1’s FH1 domain [34]. They showed that the interaction surface of the RAGE cytoplasmic domain with the FH1 domain of DIAPH1 consists of a hydrophobic patch formed by the methylene groups of Arg5 and Gln6, and that this is contiguous with a positively charged surface formed by Arg4 and Arg5 [34]. To provide further proof of this relationship, these investigators mutated Arg5 and Gln6 to alanine residues and tested the double mutant. They reported that the double mutant, in contrast to the wild type, bound the FH1 domain of DIAPH1 only weakly, at best.

An essential test of this concept was whether mutation of Arg5 and Gln6 in vivo, in RAGE and DIAPH1-expressing cells, would suppress the effects of RAGE ligands. These experiments were carried out in primary murine aortic smooth muscle cells and revealed that whereas treatment with RAGE ligand S100B stimulated phosphorylation of Akt and cellular migration in the wild type cells, introduction of the double mutant blocked these effects [34]. However, the double mutant exerted no suppressive effect on macrophage, PDGF-BB, with respect to promotion of smooth muscle cell proliferation or migration. These key findings indicated that the introduction of the double mutant did not broadly block smooth muscle cell functions.

Recently, Xue and colleagues showed that two soluble RAGE monomers (extracellular domains) orient head-to-head and form an asymmetric dimer with the carboxy terminus through a process that then recruits DIAPH1, thereby activating signal transduction triggered by RAGE ligands [35].

In summary, extensive evidence is emerging suggesting that the interaction of the RAGE cytoplasmic domain with DIAPH1
is important and essential for RAGE ligand-mediated signal transduction. Further studies are required to determine if DIAPH1 is essential for RAGE effects in all cell types, or, whether there is a cell-restricted pattern.

In the sections to follow, we present a review of the studies linking RAGE and these ligand families to disease states. Because of the diversity of RAGE actions in disease, we have focused this review on RAGE and metabolic diseases.

**RAGE and Obesity – The Unexpected yet Fascinating Link**

A first question in *in vivo* studies was whether or not RAGE and its ligand families are expressed in the relevant disease tissue. For many years, the study of RAGE and AGEs was largely restricted to potential roles in diabetic complications. In the past few years, however, it has become apparent that RAGE and its ligand families are expressed in human and murine obese adipose tissue. Gaens and colleagues first showed that in human “fatty liver,” increased accumulation of CML-AGE epitopes was evident, in parallel with increased expression of RAGE and a host of inflammatory markers such as PAI-1, IL8 and IL6 [36]. Further, it was shown that in human obese adipose tissue, both CML-AGE adducts and RAGE expression were higher than that observed in lean adipose tissue, with expression evident in adipocytes, adipose tissue macrophages and endothelial cells [37]. Intriguingly, it was observed that CML-AGE levels in plasma were actually lower in obese versus lean subjects and experiments in animal models suggested that the AGE adducts were actually “trapped” in the obese adipose tissue, and presumably, more accessible to cell surface RAGE [37]. Other studies, particularly in the Cohort on Diabetes and Atherosclerosis Maastricht Study and Hoorn Study affirmed these findings and linked CML-AGE levels to central obesity [38].

Non-AGE RAGE ligands have also been linked to human obesity; elevated blood levels of S100B were associated with increased adipose tissue mass [39]. In other studies, higher levels of HMGB1 (blood) were associated with obesity in children (vs. the lean state controls) [40]. In vitro, it was shown that inflammation stimulates the release of HMGB1 from adipocytes, thereby, possibly, potentiating adipose inflammation [41].

Studies in *vivo* affirmed these findings in mice fed high fat diet (60% kcal/fat). Even before the development of diabetes, metabolic tissues displayed increased concentrations of AGE adducts and HMGB1 [42]. Genetic deletion of Ager prevented the deleterious effects of high fat diet on reduced energy expenditure, weight gain, adipose tissue inflammation, and insulin resistance. In contrast, Ager deficiency had no effect on genetic forms of obesity caused by impaired melanocortin signaling. The effects of RAGE were attributed, at least in part, to myeloid RAGE expression, as hematopoietic deficiency of Ager imparted partial protection against high fat diet-induced inflammation and weight gain in high fat feeding [42]. Finally, in adult RAGE-expressing mice, treatment with soluble RAGE, the extracellular ligand-binding domain of RAGE that binds RAGE ligands and blocks their interaction with and activation of the cell surface RAGE, reduces weight gain in mice fed high fat diet (either at onset of high fat diet, or; three weeks into the course of high fat feeding) [42].

Taken together, these data indicated that RAGE plays unexpected and key roles in obesity. Although studies in Ager null mice demonstrated significant effects on prevention of obesity in high fat feeding, the reconstitution of lethally irradiated wild type mice with Ager null bone marrow was significantly, but overall less effective than the global deletion. Studies are actively in progress to identify the distinct RAGE-expressing cells that play significant roles in the response to high fat diet.

**RAGE Spares No Tissue in Diabetes: RAGE and Macro/Microvascular Complications in Diabetes**

**Macrovascular Complications**

One of the chief causes of morbidity and mortality in diabetes, either type 1 or type diabetes is acceleration of atherosclerosis, with consequent heart attacks and strokes [43]. Burke and colleagues performed a meticulous analysis of hearts and coronary arteries from human subjects with type 2 diabetes versus control individuals. These authors reported that atherosclerotic lesions from diabetic subjects had larger necrotic cores and greater total and distal plaque load [44]. The type 2 diabetic subjects’ lesions had higher content of T lymphocytes and macrophages, as well as HLA DR expression. Further, macrophage infiltration was not dependent on levels of cholesterol or subject age, suggesting that unique diabetes-specific mechanisms underlie these epidemiologic and pathologic findings. Finally, Burke and colleagues showed that expression of RAGE and one of its inflammatory ligands EN-RAGE (or S100A12) was significantly higher in diabetic vs. non-diabetic subjects and was associated with apoptotic macrophages and smooth muscle cells [44]. Other studies localized HMGB1 [45] and AGEs to human atherosclerotic plaques, particularly in regions of plaque vulnerability [46].

The role of RAGE in diabetic atherosclerosis has been addressed extensively in animal models, using multiple modalities of interruption of the ligand – RAGE axis. First, sRAGE was administered to diabetic (insulin-deficient) Apoe null mice fed normal chow in which accelerated atherosclerotic plaques developed, in parallel with increased concentrations of AGE ligands. Administration of sRAGE to diabetic (and non-diabetic) mice was associated with reduced atherosclerotic lesion area, without any effect on levels of glucose, thereby suggesting unique mechanisms that were operative, at least in part, in the diabetic milieu [47]. The finding of the benefits of sRAGE even in non-diabetic mice is not surprising, as the ligands of RAGE, on account of such factors as inflammation and oxidative stress, also accumulate in vascular lesions. Second, genetic deletion of Ager was tested. Soro-Paavonen and colleagues tested the same atherosclerosis-prone mouse model and showed that deletion of Ager in diabetic mice in that background reduced atherosclerosis, in parallel with reduced leukocyte lesion content, lower oxidative stress and reduced levels of RAGE ligands [48]. In a third strategy, Koulios and colleagues performed lethal irradiation of diabetic (streptozotocin) ApoE null mice or ApoE null mice devoid of Ager, followed by reconstitution with either Ager expressing or Ager null bone marrow. The results demonstrated that both compartments – bone marrow and non-bone marrow – were required for the effects of RAGE in diabetic atherosclerosis [49]. Fourth, Harja and colleagues generated transgenic mice in which the cytoplasmic domain RAGE was deleted, primarily in endothelial cells, but not exclusively (as driven by the pre-proendothelin 1 promoter). When these mice were bred into the ApoE null background, even without diabetes, atherosclerosis was suppressed compared to RAGE-expressing controls [25]. Finally, Bu and colleagues performed Affymetrix arrays on aortas retrieved from non-diabetic or diabetic ApoE null vs. ApoE null / Ager null mice and reported that a key RAGE-dependent pathway was found to be the ROCK1 branch of the transforming growth factor-beta pathway, particularly in smooth muscle cells [50].

In addition to coronary atherosclerosis, other studies have examined the potential role of RAGE and its ligands in stroke. In human unilateral cerebral infarction, increased expression of
RAGE was noted in the ischemic region; the same pattern of RAGE expression was noted in rats after middle cerebral artery occlusion [51]. In animal models, expression of cytoplasmic domain-deleted RAGE in neurons [52], global deletion of Ager [53], transgenic expression of endogenous secretary (a form of soluble) RAGE [53], blockade of HMGB1 [54], or administration of FPS-ZM1 (RAGE inhibitor) [54] beneficially modulated cerebral damage in rodent models of stroke.

Taken together, these considerations suggest that evidence from human tissues places RAGE and its ligands in coronary atherosclerosis and brain tissue in stroke and that blockade of this axis may be of benefit in limiting disease pathology and functional derangements.

**Microvascular Complications**

RAGE has been studied in many of the microvascular complications of diabetes. In this review, we will focus on complications in the kidney, heart and eye.

**Diabetic Nephropathy**

Multiple studies place RAGE and its ligands in the diabetic kidney and indicate that such localization is not restricted to the diabetic state [55,56]. D’Agati and colleagues established that the podocyte was a principal cell type expressing RAGE in the human kidney, which was upregulated in the diabetic state [56]. Multiple studies in animal models have addressed the role of RAGE in the diabetic kidney and suggest, collectively, that blockade of this axis may be beneficial in diabetic kidney disease.

First, Yamamoto and colleagues developed a transgenic mouse model of diabetes in which RAGE was overexpressed in vascular cells; this resulted in enlargement of the kidney and glomerular hypertrophy, increased mesangial expansion and glomerulosclerosis, and increased albuminuria compared with controls. This was the first RAGE overexpression study to demonstrate the role of the receptor in advancing diabetic kidney disease [57]. Further, in that study, an AGER inhibitor (OPB-9195) attenuated nephropathy in the RAGE overexpression model. Second, in db/db mice, in which severe obesity and hyperglycemia are evident, administration of sRAGE diminished the pathological and functional indices of nephropathy. Additional studies in mice devoid of Ager (C57BL/6) attenuated nephropathy [58]. Third, Inagaki and colleagues developed a triple transgenic mouse model with overexpression of meglin, iNOS and RAGE; the triple transgenic demonstrated the highest degrees of glomerular sclerosis vs. the single or double transgenic mice [59]. Fourth, others confirmed the benefits of Ager deletion in the diabetic kidney and suggested that administration of low molecular weight heparin in might be a treatment for diabetes associated nephropathy [60]. Fifth, administration of anti-RAGE neutralizing antibodies showed protection against renal complications of diabetes in insulin resistant diabetic db/db mice and in streptozotocin-induced insulin deficient diabetic mice [61,62].

Finally, work in the OVE26 mouse model of diabetic nephropathy provided further support for the role of RAGE in this disorder [63]. This mouse model develops loss of glomerular function, as well as the typical pathological changes in the diabetic kidney and albuminuria [64]. Reiniger and colleagues bred the OVE26 mouse into the FVB Ager null background; compared to Ager-expressing mice, the diabetic mice devoid of Ager showed significantly lower in OVE26 renal cortex of OVE26 mice devoid of Ager [63]. Importantly, Reiniger and colleagues showed that the levels of the AGE precursor, methylglyoxal, were significantly lower in OVE26 mouse kidney devoid of Ager; they traced the mechanism to higher levels of glyoxalase 1 (GLO1), which detoxifies the AGE precursors, in the Ager null kidney [63]. These authors showed that increased transcription of the genes for Serpine1, Tgb1, Tgfb1 and Col4a1 observed in the diabetic Ager expressing OVE26 renal cortex was significantly reduced in the kidney cortex of OVE26 mice devoid of Ager. Further, they reported that ROCK1 activity was significantly lower in Ager null OVE26 mice compared with OVE26 Ager-expressing kidney cortex [63].

Taken together, these data implicate RAGE in the pathogenesis of functional and pathological derangements in the diabetic kidney and suggested that blockade of the receptor might be protective in this complication.

**Diabetic Myocardium**

Induction of diabetes in the rat resulted in a time-dependent increase in AGE accumulation and RAGE expression in the heart [65] and thus suggested that the RAGE axis might contribute to diabetic cardiac dysfunction. Candido and colleagues linked AGEs to diabetic myocardial dysfunction; using ALT-711, an AGE cross link breaker, they showed that this treatment restored optimal collagen properties in the diabetic heart [66].

In the context of RAGE, first, Bucciarelli and colleagues showed that diabetic mice devoid of Ager were protected from ischemia-reperfusion injury in the isolated perfused heart model [26] and further illustrated that transgenic expression of cytoplasmic domain-deleted RAGE in endothelial cells or macrophages was also protective in the diabetic heart against ischemia/reperfusion. In the RAGE antagonized hearts, lower levels of AGEs were also identified post-ischemia/reperfusion. A key observation was the reduction in markers of cell death in the hearts of the diabetic Ager null mice after injury [26]. Second, in db/db mice, Nielsen and colleagues used a RAGE antibody and showed that treatment of db/db mice prevented the reduction in systolic function and the development of increased left ventricular chamber stiffness. In parallel, they found that expression of collagen genes was reduced significantly by the RAGE antibody [67]. Third, other studies probed the role of the RAGE ligand S100B in the diabetic myocardium and showed that deletion of S100B protected the diabetic heart from the adverse effects of induced myocardial infarction [68].

In summary, these representative studies linked the RAGE ligand/RAGE axis to the pathogenesis of innate damage to the diabetic myocardium as well as to the superimposed adverse effects of ischemia/reperfusion injury.

**Diabetic Retinopathy**

Diabetes is a leading cause of blindness and current therapeutic efforts in diabetes fail to modify the key underlying mechanisms that lead to retinopathy and loss of eyesight [69]. In experimental diabetic retinopathy, RAGE was found to be highly expressed in glial cells [70].

A number of investigations sought to identify if RAGE plays roles in the pathogenesis of diabetic retinal disorders. First, Barile and colleagues administered sRAGE to type 2 diabetic db/db mice devoid of Ape; these investigators showed that long-term treatment with sRAGE provided significant protection against the neurovascular perturbations that occur in early diabetes [71]. Second, an inhibitor of RAGE ligand HMGB1 prevented retinal vascular permeability [72] and inflammatory and proangiogenic signals in a diabetic animal model [73]. Third, using mice devoid
of Ager, McVicar and colleagues showed that these mice, compared with Ager-expressing controls in diabetes, demonstrated less vascular permeability, leukostasis and activation of microglia. Further, formation of cellular capillaries, but not pericyte loss, was reduced in diabetic Ager null mice [74]. Hence, these data provided support for the premise that RAGE might affect the pathogenesis of diabetic retinopathy, at least in part through neural, vascular and glial-dependent mechanisms. Studies in tissue targeted Ager deleted mice will be the next key steps in identifying the proximate RAGE-dependent mechanisms.

**Other Microvascular Complications and RAGE**

In addition to roles for RAGE in complications of the kidney, heart and eye, RAGE has also been implicated in diabetes in the following settings, impaired wound healing, neuropathy, peripheral arterial disease, periodontitis, and erectile dysfunction, as examples [75–79]. It is important to note that the protective effects of sRAGE on acceleration of impaired wound healing in diabetic db/db mice indicated that blocking RAGE did not subvert homeostatic mechanisms underlying dermal healing.

**Tracking RAGE in Human Subjects**

As discussed above, extensive evidence from human subject diseased tissue localizes RAGE and its ligands to the affected site. However, in practice, diseased tissue is not always readily accessible, especially in a prospective and repetitive manner. How then, may RAGE activity be traced in human subjects?

In fact, soluble forms of RAGE may be detected in human subject plasma or serum. There are two forms of sRAGE that may be detected; they are distinct by their sequences and by the sources of their production. The first form of sRAGE is produced from the naturally-expressed cell surface RAGE through the actions of matrix metalloproteinases (MMPs) and A-Distintegrin and Metalloprotease (ADAM)-10 [80,81]. The second, endogenous secretory (es) RAGE is formed from the actions of pre-mRNA alternative splicing [82]. In general, the cleaved form of sRAGE represents about 80% of the total sRAGE identified in circulation.

In diabetes, a number of studies have suggested links between the circulating levels of these soluble RAGEs and the diabetic state and/or the presence or absence of complications. A recent review of published studies on sRAGEs in diabetes reveals the following highlights: lower levels of sRAGE were found in type 2 diabetic patients with mild cognitive impairment vs. matched control individuals, in parallel with higher levels of serum AGE-peptide [83]; in youth with type 1 diabetes, levels of sRAGE and esRAGE declined over a five year period, independent of gender, diabetes or puberty stage and a positive association was noted with carotid intima-media thickness [84]; in type 2 diabetic subjects enrolled in the ADVANCE trial (Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation), increased levels of sRAGE were associated with new or worsening renal disease over the following five years and higher levels of AGES were also associated with adverse renal outcomes [85]; and in type 1 and 2 diabetic subjects, skin autofluorescence was positively associated with levels of sRAGE, but not in control subjects [86].

In addition to cross-sectional or prospective studies on levels of sRAGE and esRAGE in diabetic human subjects, other studies have reported on the fate of sRAGEs after therapeutic interventions. Examples of recent studies in this context include: in subjects with type 2 diabetes, treatment with pioglitazone suppressed RAGE expression in peripheral blood mononuclear cells and increased levels of sRAGE and esRAGE [87]; levels of sRAGE increased in morbidly obese subjects who experienced weight loss after bariatric surgery [88]; and in type 2 diabetic subjects, treatment with atorvastatin resulted in increased levels of sRAGE and esRAGE [89]. Others summarized the results of multiple studies in which cardiovascular drugs and nutraceuticals modulated levels of sRAGE [90].

Much work needs to be done to fully establish the ability of sRAGE and/or esRAGE as bona fide biomarkers of diabetes, diabetes complications and the response to therapeutic interventions. It is very possible; however, that consideration of the degree of renal dysfunction and the relation of sRAGE levels to RAGE ligand levels will be superior to identify specific markers of the state of the RAGE ligand/RAGE axis in human subjects.

**Therapeutic Opportunities –Paths to Targeting RAGE in the Clinic**

Experiments in animal models have used a variety of general strategies to antagonize RAGE and ligand/RAGE interaction. For example, experiments using soluble RAGE in mice suggested that sequestration of the RAGE ligands blocks their binding to and activation of the cell surface receptor and mimic the effects observed by genetic deletion of Ager in a given complication. Other approaches targeting the extracellular domains, with such strategies as RAGE-directed antibodies [61,62] or small molecule antagonists [6,91] have also been tested in animal models.

Recent work based on the discovery that the RAGE cytoplasmic domain bound the formin, DIAPH1, and that this interaction was essential for RAGE-mediated signaling led to the screening of a > 58,000 small molecule library to seek inhibitors of this interaction. As recently reported, 13 such molecules were identified, which bear nanomolar affinities and that bind directly to the RAGE cytoplasmic domain and not to DIAPH1. These 13 small molecules demonstrate efficacy in blockade of RAGE ligand-stimulated cellular signaling and modulation of gene expression and functional properties in cultured immune and vascular cells. Further, in the isolated perfused diabetic heart, many of the compounds imparted significant benefit on suppression of reduced cardiac function after ischemia/reperfusion [92].

In addition to strategies targeting RAGE itself, as noted above, multiple approaches to target RAGE’s ligands, such as antagonism of AGEs [93,94], HMGB1[54,72] and S100/calgranulins [95,96], have also been reported. Ongoing and future research should uncover the feasibility and optimal means of antagonizing RAGE in the clinic.

**Summary and Perspectives**

The Figure summarizes current knowledge of the complexities of RAGE signaling and its impact on metabolic stress in obesity and types 1 and 2 diabetes. Although many years of research were dedicated to understanding how RAGE and its ligands contributed to diabetic complications, the fascinating observations regarding the accumulation of RAGE ligands in obesity underscored that the receptor played roles in the development of insulin resistance and the pathogenesis of type 2 diabetes [37–40,42]. Furthermore, in type 1 diabetes, evidence supports that the RAGE ligands/RAGE axis contribute to pancreatic insulitis and cell damage [97,98]. These findings, as well as the prominent roles for S100/calgranulins and HMGB1 in inflammation, broadened the sphere of RAGE actions. Hence, in both types 1 and 2 diabetes, deleterious RAGE ligands are already accumulating prior to the diagnosis of hyperglycemia, thus, perhaps, triggering the very earliest manifestations of macro- and microvascular perturbation in the course of metabolic dysfunction. In this context, also notable in the biology of RAGE is its apparent ability to contribute to regulation of AGE levels. Although the
precise mechanisms by which RAGE downregulates Glo1 have yet to be identified, it is apparent that once RAGE is activated, it contributes to a feed forward cycle of continued production and accumulation of its ligand families.

One of the most significant challenges in identifying new drug targets is the determination of the overall safety profile in targeting a given pathway. These considerations are valid in the context of RAGE. To date, accruing evidence appears to tip the balance in favor of overall safety and efficacy of blocking the receptor. First, homozygous Ager null mice are viable and display normal reproductive capacity and they do not have a reduced life span. Second, multiple studies in rodents have shown that long term blockade of the receptor (sRAGE or RAGE antibodies or small molecules, as cited above), impart no adverse consequences on the animal's overall health and longevity. Third, a plethora of studies have tested infection models. In murine models of cecal ligation and puncture and in certain forms of pneumonia, deletion of Ager or RAGE blockade is protective, with overall improved host survival [99–101]. However, other studies suggested that increased bacterial outgrowth and dissemination ensued upon E. coli abdominal sepsis in RAGE-blocked animals [102]. In this context, it will be important to continue to study multiple modalities of blocking RAGE in these critical models of innate responses to stress. It is also necessary to note that the means by which these pathogens were introduced in the rodent may not be fully akin to the means and time course for which they infect human subjects.

Finally, as has been extensively noted and identified in one of the earliest reports on RAGE [14], RAGE is highly expressed in the lung, even in homeostasis and without any evidence of disease. A large body of work has been published suggesting that soluble RAGE may be a biomarker for RAGE activity in lung fluids (bronchoalveolar lavage fluid) and that blockade of RAGE may be beneficial in models of acute lung injury, respiratory distress syndromes and chronic airway diseases [103,104]. It should be noted, however, that Englert and colleagues reported distinct effects of Ager blockade/deletion in murine models of asbestosis (harmful) vs. bleomycin induced injury (protective) [105].

All issues considered, although extensive work has been done in the preclinical models and in the human subject probing the role of the RAGE axis as a biomarker, there is no substitute for long-term exposure of the human subject to antagonism of the ligand/RAGE axis. We posit that the body of accumulating evidence supports that testing the anti-RAGE strategy in the human subject in carefully controlled and performed studies may well be worth the effort. The answers to these critical questions are eagerly anticipated.

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