Published in final edited form as:

Trends Endocrinol Metab. 2014 January ; 25(1): . doi:10.1016/j.tem.2013.08.002.

Unlocking the Biology of RAGE in Diabetic Microvascular Complications

Michaele B. Manigrasso, Judyta Juranek, Ravichandran Ramasamy, and Ann Marie Schmidt

Diabetes Research Program, Division of Endocrinology, New York University School of Medicine, 550 First Avenue, New York, New York, USA 10016

Abstract

The discovery of the receptor for advanced glycation endproducts (RAGE) set the stage for the elucidation of important mechanisms underpinning diabetic complications. RAGE transduces the signals of advanced glycation endproducts, pro-inflammatory S100/calgranulins and high mobility group box 1 (HMGB1), and is a one of a family of receptors for lysophosphatidic acid (LPA). These ligand tales weave a theme of vascular perturbation and inflammation linked to the pathogenesis of the chronic complications of diabetes. Once deemed implausible, this concept of inflammatory cues participating in diabetic complications is now supported by a plethora of experimental evidence in the macro- and microvasculature. We review the biology of ligand-RAGE signal transduction and its roles in diabetic microvascular complications, from animal models to human subjects.

Keywords

diabetes; ligands; inflammation; complications; receptor for AGE

The Problem of Diabetes – One for the AGES

Diabetes is emerging as one of the most challenging health problems of the 21st century. Stimulated by the rising rates of obesity, type 2 diabetes (T2D) is becoming a worldwide epidemic with life-threatening consequences. The International Diabetes Foundation has estimated that over 371 million people had diabetes in 2012, and this number is expected to grow to 552 million people by 2030 [1]. Evidence also supports a rise in the incidence of type 1 diabetes (T1D) [2, 3]. Irrespective of the precise etiology of hyperglycemia, the final common pathway for elevated glucose levels is the development of chronic complications [4–5]. Subjects with diabetes have an increased risk of developing a number of serious health concerns; prevention of vascular complications continues to be a key goal. While the exact mechanisms underlying the pathogenesis of vascular damage in diabetes have yet to be completely elucidated and are multifactorial, dysfunction of the vascular endothelium is regarded as a fundamental inciting factor. Results from both T1D and T2D patients have indicated that hyperglycemia plays a critical role in the progression of neuropathy,

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Address correspondence to: Ann Marie Schmidt MD, Diabetes Research Program, Division of Endocrinology, New York University School of Medicine, 550 First Avenue, Smilow 901C, New York, N.Y. 10016, (tel) 212 263 9444, annmarie.schmidt@nyumc.org.

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nephropathy, retinopathy and accelerated atherosclerosis and is an independent risk factor for the development of vascular complications [4, 5].

What are the consequences of hyperglycemia that account for these data? Numerous studies have reported a direct correlation between advanced glycation endproducts (AGEs) and the development and progression of diabetic vascular disease and complications. AGEs (see glossary) are a diverse group of compounds that are generated through the non-enzymatic glycation or glycooxidation of proteins, lipids and nucleic acids [6], with the reactive carbonyl methylglyoxal, considered a major AGE precursor [7]. AGE production is directly accelerated by hyperglycemia. Furthermore, AGE formation is often associated with an increase in reactive oxygen species (ROS) [8]. While AGEs accumulate slowly in both plasma and the tissues during aging [9–11], they are markedly increased in patients with diabetes [12] and may be utilized as biomarkers in the clinic as an indicator of yet-to-be-revealed and current complications [13–17].

In addition to putative roles as biomarkers, AGEs may contribute actively to complications. For example, AGEs, as measured by skin auto fluorescence, were associated with impaired anti-oxidative functions of HDL [18]. The major AGE precursor, methylglyoxal, mediates oxidative stress and impairs nitric oxide (NO)-dependent vasorelaxation and upregulates inflammatory markers in rats fed this AGE precursor [19].

Microvascular complications of diabetes – implications for ligand-RAGE interaction

Diabetic Nephropathy

Diabetic nephropathy, characterized by the development of proteinuria and a decline in glomerular filtration rate, represents the major cause of end stage renal disease in the Western world [20]. Due to the diversity of cell populations within the glomerulus and the tubulointerstitium of the kidney, the development and progression of diabetic nephropathy is highly complex, of variable course in the individual subject, and one that typically progresses over a long period of time. One of the earliest changes in the diabetic kidney is the development of hyperfiltration. As a consequence, the kidney filters increased amounts of glucose, proteins, growth factors and cytokines, which then trigger multiple pathological pathways, such as those which mediate fibrosis and inflammation.

In diabetes, the kidney is an important site for AGE accumulation and was one of the first diabetic tissues in which AGE accumulation was observed [21]. Additionally, the kidney contributes to increased levels of AGEs in the serum of diabetic patients since it is the major site for AGE clearance [22]. Previous reports have shown a correlation between the levels of skin collagen AGEs and the development and progression of diabetic nephropathy in T1D patients [23]. Similar results were observed in T2D patients where skin autofluorescence was shown to be a strong indicator of microvascular renal complications [24]. An intriguing recent observation from the Joslin Medalist study subjects (individuals with T1D for >50 years) suggested that certain specific AGEs such as N-formylkynurenine protein adduct residue and N(ω)carboxymethylarginine free adduct were actually decreased in subjects with long-term T1D, particularly those with kidney complications [25]. The authors speculated that perhaps the enhanced ability to metabolize these AGEs was linked mechanistically to the pathogenesis of kidney disease and microalbuminuria. These considerations suggest that specific AGEs might have distinct effects in the pathobiology of diabetic kidney function decline.

RAGE is expressed by a number of cell types in the kidney, such as podocytes and endothelial cells [26–28] and studies have shown the beneficial effects of RAGE deletion in

delaying the progression of diabetic renal disease. Indeed, RAGE deletion was found to be beneficial in attenuating diabetic renal disease in OVE26 mice, a transgenic model of severe early-onset T1D, as measured by reduction in glomerulosclerosis, podocyte effacement and glomerular basement membrane thickening [29]. In parallel, improved renal function, as measured by inulin clearance, was observed in the RAGE null vs. RAGE-expressing OVE26 mice [29]. This work showed that levels of methylglyoxal were significantly lower in the diabetic RAGE null OVE26 vs. the RAGE-expressing control diabetic kidneys, even in the face of equivalent degrees of hyperglycemia. These data revealed for the first time that RAGE action suppresses levels of glyoxalase 1 in kidney, thereby reducing the protective anti-AGE roles of this enzyme. Glyoxalase 1 is a key enzyme that detoxifies the major AGE precursor MG; hence, RAGE-dependent regulation of this enzyme, at least in the diabetic kidney, may provide a RAGE-dependent mechanism for amplification of glycation and oxidative damage in this tissue.

Although earlier studies with the anti-AGE agent aminoguanidine (or pimagidine) failed to show statistically significant benefit in human diabetic kidney disease in Phase III clinical trials [30], the anti-AGE therapeutic approach remains an active area of research and development. For example, the use of a DNA aptamer raised against AGEs was shown to block the progression of experimental diabetic nephropathy. Kaida and colleagues screened a DNA aptamer directed against AGEs *in vitro*, and examined its effects on renal injury in KKAY/Ta mice, an experimental animal model of T2D [31]. Their data suggest that continuous administration of the AGEs-aptamer could protect against experimental diabetic nephropathy by blocking the AGEs-RAGE axis and could possibly be a feasible and promising therapeutic strategy for the treatment of diabetic nephropathy.

Because dysregulated glucose control contributes to vascular complications in diabetes, a common way of preventing these effects is to regulate glucose levels by pharmacological intervention. Metformin, an oral antidiabetic drug in the biguanide class and first-line drug of choice for the treatment of T2D, was shown to inhibit advanced glycation end products (AGEs)-induced renal tubular cell injury by suppressing ROS generation via reducing RAGE expression through AMP-activated protein kinase (AMPK) activation. It was concluded that metformin may protect against tubular cell injury in diabetic nephropathy by blocking the AGEs-RAGE-ROS axis [32].

Another recent study examined the correlation between dyslipidemia and tubular damage and found that pravastatin, a cholesterol lowering medication, could block the AGEs-RAGE-elicited tubular cell injury *in vitro* [33]. This suggests that preventing dyslipidemia in diabetic patients could inhibit the typically observed AGEs-induced apoptosis and the resulting serum asymmetric dimethylarginine (ADMA) generation in tubular cells by suppressing RAGE expression via inhibition of geranylgeranyl diphosphate (GGPP) synthesis. Therefore, the use of statins may exert beneficial effects on tubular damage in diabetic nephropathy by blocking the AGEs-RAGE axis.

Distinct studies have suggested pathological roles for S100- and HMGB1-RAGE interaction (Box 1) in diabetic nephropathy. In mesangial cells, S100B increased expression of Tgf- β 1 and fibronectin and activated p38 MAP kinase, ERK1/2 kinase and NF- κ B [34]. Others showed that hyperglycemia induced by streptozotocin in rats upregulated renal expression of HMGB1 in glomerular and tubular epithelial cells in parallel with increased RAGE expression and activated NF- κ B [35]. In human subjects, the examination of the S100 member fibroblast specific protein 1 (FSP1) in diabetic kidney was performed in 109 T2D subjects who underwent kidney biopsy. The findings revealed that the appearance of FSP1 particularly in the podocyte was associated with more severe clinical and pathological indices of diabetic nephropathy [36]. The authors suggested that this occurred due to the

induction of podocyte detachment via the process of epithelial-mesenchymal transition phenomena.

Much more is to be learned about the roles for RAGE in diabetic nephropathy. Despite the consideration that animal models of nephropathy may have limitations with respect to clinical application, the data nevertheless suggest that RAGE and its ligands may contribute to diabetic nephropathy. If RAGE blockade achieves clinical translation, it will be important to discern if its effects enhance the benefits of angiotensin converting enzyme inhibitors or angiotensin receptor blocking agents. This remains an open question.

Diabetic retinopathy

In line with the increase in numbers of subjects impacted by diabetes, diabetic retinopathy has become the most common microvascular complication associated with diabetes and a leading cause of blindness among adults aged 20–74 years [37, 38]. It is characterized by a spectrum of lesions within the retina, including changes in permeability, capillary microaneurysms, capillary degeneration and increased formation of new blood vessels [37]. Clinically, diabetic retinopathy is distinguished by the nonproliferative and proliferative diseases stages.

Diabetic retinopathy develops over many years and almost all patients with T1D and most with T2D display retinal lesions after 20 years of hyperglycemia [38,39]. While multiple pathways have been linked mechanistically to the pathogenesis of diabetic retinopathy, including polyol pathway flux, activation of diacylglycerol (DAG)-PKC pathway, increased expression of growth factors, oxidative stress, hemodynamic or retinal blood flow changes, renin–angiotensin system activation and sub-clinical inflammation, accelerated AGE formation and the AGE-RAGE interaction has recently become increasingly investigated [40,41].

In concert with findings from studies in other diabetic tissues, RAGE and its ligands, AGEs, S100/calgranulins and HMGB1 are upregulated in the human diabetic eye (vitreous and epiretinal membranes) [21]. RAGE is expressed in retinal glial cells in the inner retina, thereby implicating RAGE as a major player in the recruitment of immune cells that induce inflammation as retinopathy progresses [42].

A recent report in Tunisian diabetic patients showed that serum AGEs, sRAGE (Box 1) and pentosidine levels (a biomarker for AGEs) were increased in those with retinopathy, and their findings suggest that increased AGE-RAGE activity and increases in pentosidine may initiate and exacerbate the severity of diabetic retinopathy [43].

Other implications of the deleterious effects of RAGE in diabetic retinopathy include the breakdown of the blood-retinal barrier and increased leukostasis, characteristic clinical symptoms of diabetic retinopathy, all of which were attenuated by treatment with soluble RAGE in RAGE-overexpressing mice [44], suggesting that blocking AGE bioactivity may be effective for the treatment of diabetic retinopathy.

HMGB1 has also been shown to activate inflammatory signaling pathway components and disrupt the retinal vascular barrier in the diabetic retina. These findings support that in the diabetic retina, HMGB1 interacts with RAGE and activates ERK(1/2) and NF- κ B to generate an inflammatory response and disturb retinal vascular barrier. In vivo, when diabetic rats were treated with the HMGB1 inhibitor glycyrrhizin, activation of NF- κ B was attenuated and occludin expression was downregulated [45]. These studies implicate HMGB1 as a potent mediator of vascular permeability in the retina and suggested pathogenic roles for RAGE.

Inhibition of RAGE was shown to block the development of important lesions of diabetic retinopathy and also suppressed the development of sensory allodynia in diabetes [46]. Studies in which soluble RAGE was administered to T2D apolipoprotein E null mice in the db/db background revealed an attenuation of neuroretinal dysfunction and prevention of vascular capillary abnormalities [47].

Interestingly, earlier studies reported high levels of RAGE expression in the retinal Müller glia in diabetic mice. When retinal glial MIO-M1 cells were cultured in the presence of high glucose, RAGE and S100B were upregulated, in parallel with increased RAGE signaling via the MAPK pathway; processes which stimulated cytokine responses via RAGE, as demonstrated by RAGE siRNA knockdown experiments [42].

Taken together, these findings indicate that RAGE may be an important therapeutic target to inhibit the development of vascular and neural complications of diabetes. Further, these findings in retinal Müller glia suggest that the role of RAGE in diabetic retinopathy may be, at least in part, related to inflammatory and glial cell dysfunction. Local delivery of RAGE antagonists into the eye has yet to be tested in animal models. It will be of interest to determine if the maximal benefit of blockade of this axis might be achieved by local as well as systemic intervention.

Diabetic Neuropathy

More than half of all individuals with diabetes will eventually develop neuropathy and it is estimated that some populations have as much as a 15% lifetime risk of undergoing a lower extremity amputation [48]. Diabetic neuropathy affects both the somatic and autonomic divisions of the peripheral nervous system, with damage to the spinal cord and higher central nervous system [49, 50]. Diabetic patients with neuropathy present variable degrees of symptoms and disease progression is clinically characterized by the development of vascular abnormalities, including capillary basement membrane thickening and endothelial hyperplasia. Advanced diabetic neuropathy due to nerve fiber deterioration is characterized by altered sensitivities to vibrations and thermal thresholds, and increased pain seriously impeding the quality of life [49,50]. Consistent with other diabetic complications, the duration of diabetes and the lack of glycemic control are major risk factors for the development of neuropathy [4, 5].

The localization of AGEs has been examined in both human subjects and in experimental models of diabetic nephropathy. In human diabetic patients, AGEs were found in the vascular endothelial cells, pericytes, basement membrane, axons and Schwann cells [51]. Similar findings have been reported in STZ-induced diabetic rats [52].

RAGE has been localized to the endothelial cells of both the peri- and endoneurial blood vessels and the interaction between AGEs and RAGE has been implicated in the progression of diabetic neuropathy [53]. Studies investigating this link show that when AGE binds to RAGE in the endothelial cell, this activates a number of inflammatory and vascular cell adhesion factors further implicating RAGEs role in the development of diabetic neuropathy [54]. Levels of these soluble advanced glycation end product-receptors and other soluble serum markers may be useful as indicators of diabetic neuropathy in the foot [13].

Dauch et al. recently reported that Langerhans cells located in the skin and associated with painful diabetic neuropathy are positive for RAGE expression and they hypothesize that diabetes-induced RAGE expression might contribute to the increased subepidermal Langerhans cell populations in db/db mice. They speculated that this increased RAGE expression is important for the homing of maturing dendritic cells to lymph nodes where they physically interact with and activate T lymphocytes [55].

In experimental models, a recent study has shown the role of HMGB1 in the primary afferent nerve contributed to the development of neuropathic pain after nerve injury. The authors conclude that blocking HMGB1/RAGE signaling might be a promising therapeutic strategy for the management of neuropathic pain [56]. Recent work has provided convincing evidence that deletion of the RAGE gene attenuates the debilitating effects of diabetes in the peripheral nerve [57]. In vitro, incubation of primary sensory neurons with S100 generates oxidative stress and results in increased caspase-3 activation and nuclear DNA degradation, processes that are prevented by treatment of the cells with the anti-oxidant alpha-lipoic acid [58].

The peripheral nervous system of the diabetic subject is further vulnerable to thermal and pressure injuries [59, 60] and impaired wound healing. It was recently reported that in diabetic mice, deletion of RAGE resulted in significantly higher myelinated fiber densities and conduction velocities consequent to superimposed acute sciatic nerve crush compared to wild-type control animals [61]. By immunofluorescence staining, significantly higher numbers of fibers positive for HMGB1 and carboxymethyllysine (CML)-AGE epitopes were present in sciatic nerve at baseline between wild-type diabetic versus non-diabetic mice. Interestingly, HMGB1 and CML-AGE epitopes in the fibers were 2–3 fold higher in the post-crushed nerve vs. the uninjured nerve, in a manner not dependent on the presence or absence of diabetes or of RAGE. Hence, these data support the hypothesis that acute injury to the nerve results in generation of additional RAGE ligands. Such data suggest that multiple “hits” of RAGE ligand generation in a RAGE-expressing environment may contribute to the development of chronic cellular stress.

Indeed, in the study of Juranek and colleagues, closer examination of the cell populations in the nerve tissue suggested that ligand-RAGE interaction altered the profile of macrophages infiltration into the crushed nerve [61]. Specifically, in wild-type mice, diabetes resulted in a significant increase in total number F4/80-positive macrophages/region of interest at the crush site, which tended to be even higher in the diabetic RAGE null mice. In the perineurium, diabetes had no effect on the number of F4/80 macrophages, but absence of RAGE in the non-diabetic or diabetic state was associated with significantly higher F4/80-positive macrophages per region of interest, compared to their respective wild-type controls. When the state of “polarization” of these macrophages was examined, that is, the M1 vs. M2 state, a higher percentage of M2 macrophage markers was found that in diabetic RAGE null mice, compared to the diabetic wild-type mice, paralleled with reduced percentage of M1 markers, compared to wild-type diabetic mice in the nerve sections post-crush [61]. Bone marrow transplantation experiments confirmed that in wild-type mice, reconstitution with RAGE null vs. wild-type bone marrow, particularly in the diabetic state, resulted in improved myelinated fiber densities and sensory and motor conduction velocities [61]. Further, reconstitution of the wild-type diabetic mice with RAGE null bone marrow resulted in the expression of higher M2 vs. M1 macrophage markers in the crushed nerve tissue. Taken together, these data suggest that deletion of RAGE re-programs macrophage signatures to that of an overall anti-inflammatory/tissue repair phenotype vs. tissue damage-provoking profile. Consistent with this premise, in vitro, incubation of wild-type bone marrow derived macrophages with CML AGE ligand increased M1 (CD86) expression and decreased M2 (arginase 1) expression, that was prevented by deletion of RAGE in the macrophages [61]. The precise mechanisms underlying these observations are currently under study at this time but these findings underscore the concept that RAGE blocks adaptive and repair-stimulating mechanisms in the injured diabetic peripheral nerve.

It is critical to note that studies testing the role of RAGE upon acute superimposed nerve crush in diabetic mice did not identify negative consequences. These experiments, modeling the response to superimposed thermal or pressure injuries such as those that occur in human

subjects with profound neuropathic symptoms in long-standing diabetes, strongly suggest that blockade of RAGE is not likely to suppress healing and repair responses in chronic neuropathy.

Concluding remarks and future perspectives

In this review, we have discussed updates on how ligand-RAGE interaction contributes integrally to the pathophysiology of diabetic microvascular complications. Despite the progress that has been made in discerning the role of RAGE in diabetes, no doubt many questions remain (“Outstanding Questions” box). Although beyond the scope of this review, it is well-established that RAGE ligands accumulate to accelerated degrees in diabetic atherosclerosis and that blockade or genetic deletion of RAGE suppresses acceleration of vascular disease and inflammation in that setting [62]. From the first studies demonstrating reduction of accelerated atherosclerosis in STZ-induced diabetic mice devoid of ApoE treated with soluble RAGE or subjected to RAGE deletion [63], to recent work in human subjects suggesting that levels of soluble RAGEs may be biomarkers of cardiovascular disease in diabetic subjects [64–66], the evidence points strongly to roles for RAGE in this disorder. For example, Colhoun and colleagues showed that in the CARDS study, levels of total sRAGE and endogenous secretory (es) RAGE were associated with incident coronary heart disease [66].

In the macro- and microvascular complications of diabetes, the question arises, what is the role of inflammation? As discussed above, recent data suggest that at least in the diabetic peripheral nerve, RAGE action might suppress tissue repair signatures of macrophages [61]. Although the concept of “M1” vs. “M2” polarization is an oversimplification in biology, the data do underscore the possibility that ligand-RAGE interaction may innately suppress anti-inflammatory/repair-stimulating gene expression patterns in macrophages.

How does RAGE action in diabetic complications begin? How soon must we intervene in diabetes? We believe the answer is – as soon as hyperglycemia is discovered! We hypothesize that in foci of high glucose, the deliberate and inevitable generation and accumulation of AGEs marks the inciting and very early event in macro- and microvessels. Once AGEs bind RAGE, a host of pro-inflammatory events ensues in endothelial cells and inflammatory cells, leading to the influx of inflammatory cells to such foci. In addition to their multiple modes of inflammatory damage, these inflammatory cells may be akin to “Trojan horses,” in that once drawn into the sites vulnerable to complications, their release of S100/calgranulins and HMGB1 exacerbates and sustains pro-inflammatory damage, vascular permeability and dysfunction, at least in part via RAGE (Figure 1). Intriguingly, in the atherosclerotic plaque, findings reveal that RAGE deletion/blockade reduced the numbers of inflammatory cells in the vasculature. However, in the injured diabetic sciatic nerve there were just as many if not more macrophages in the RAGE null diabetic tissue. Perhaps is not the “numbers” that count – but, rather, their gene expression profiles. Hence, in the diabetic injured nerve, although there were more macrophages, their inflammatory signatures were polarized toward an M2 phenotype- that is possibly more anti-inflammatory and destined to signal repair and remodeling. How and by what means RAGE suppresses “M2”/anti-inflammatory gene expression patterns remains to be determined. Such investigations, actively underway, will further our understanding of RAGE’s contribution to inflammatory mechanisms as they relate to diabetic complications – and, likely, beyond.

It is important to acknowledge that AGEs represent a diverse group of compounds; some of these are fluorescent and others not; some are generated primarily through methylglyoxal pathways (such as methylglyoxal hydroimidazolones (MG-HI) and some are generated through 3-deoxyglucosone (3-DG). Which one or ones are precise ligands for RAGE

remains an open question; however, multiple laboratories have shown strong evidence that at least certain AGEs signal through RAGE. Key issues for consideration is the source, concentration and degree of modification of the *in vitro*-prepared AGEs used in many studies and provide strong rationale for experimental testing of naturally-derived AGEs in studies, such as those retrieved from diabetic urine or plasma, as examples.

Why are AGEs relevant? The fascinating concept of “hyperglycemic metabolic memory” which emerged from the long term DCCT/EDIC and UKPDS studies suggested that subjects exposed to intensive vs. conventional glycemic control in T1D and T2D, respectively, experienced significantly fewer complication many years after the study ended and the glycemic control between the two groups had long become largely indistinguishable [67]. Roles for ROS, epigenetic changes, and AGEs have been ascribed to this phenomenon. In this context, the skin tissue of diabetic subjects may hold key insights into hyperglycemic metabolic memory. As noted earlier in this review, Genuth and colleagues identified carboxy methyl lysine in skin collagen as a key predictor of future risk of 10 year progression of retinopathy and nephropathy in the DCCT/EDIC study [23]. Monnier and colleagues showed that glucosepane identified from skin collagen in this study was strongly associated with risk of nephropathy, retinopathy and neuropathy in T1D despite adjustment for glycosylated hemoglobin [68]. Do glucosepane levels underlie, at least in part, hyperglycemic memory? Does glucosepane bind RAGE? Indeed, many open questions remain in this intriguing area of diabetes complications.

Lastly, a new question that arises from recent work is as follows: what is, if any, the role of the newly-identified RAGE ligand, LPA, in diabetic complications? Relatively little is known at this time about LPA and its possible impact in diabetes. Interestingly, in the diabetic retina, El-Asrar and colleagues showed that levels of LPA and autotaxin (a chief enzyme responsible for LPA production *in vivo*) were higher in diabetic proliferative retinopathy vitreous samples compared to non-diabetic controls [69]. Aranda and colleagues recently studied the effects of diabetes and LPA in an ex vivo assay in which neovessels are sprouted from retinal explants retrieved from either non-diabetic or diabetic mice. Although diabetes was found to have no effect on formation of neovessels, diabetes prevented LPA-mediated regression of the neovessels [70]. By what mechanism(s) did this occur? It is known that LPA binds to a number of distinct receptors, such as those of the G-protein coupled receptor families [71]. Is it plausible that RAGE might contribute to the failure of LPA-induced regression in diabetes? Studies are underway to discern the impact of diabetes and RAGE on LPA impact on the vascular and inflammatory responses in diabetes.

In conclusion, extensive data in animal models and human subjects place the multi-faceted families of RAGE ligands and RAGE squarely in diabetic tissues; compelling data in animals using soluble RAGE and other inhibitors or RAGE deletion underscore that the relationship is not solely that of biomarker but likely of mechanism. What about mDia1? Studies are in progress to discern the precise expression patterns and potential functions of mDia1 in diabetes complications. If such studies are successful, then an entirely new class of “intracellular-based” RAGE signaling antagonists may be on the horizon. Time will tell; stay tuned.

Acknowledgments

The authors gratefully acknowledge funding from the United States Public Health Service and the JDRF. The authors are grateful to Ms. Latoya Woods for her assistance in the preparation of this manuscript.

Glossary

Advanced glycation end products (AGEs)	a heterogeneous group of compounds generated through the non-enzymatic glycation or glycoxidation of proteins, lipids and nucleic acids. They are the result of a series of complex biochemical reactions that involve the formation of Amadori products, glyceraldehyde-3-phosphate, and the reactive carbonyl methylglyoxal.
Autofluorescence	the natural emission of light by cellular structures such as mitochondria and lysosomes when they have absorbed light. Human skin autofluorescence can be used to measure the level of advanced glycation end-products, which are often elevated in several human diseases.
Calgranulin	an S-100 calcium-binding protein that is expressed in multiple cell types, including renal epithelial cells. Calgranulin may function in vivo as an inhibitor of calcium oxalate kidney stone formation.
High-mobility group protein B1 (HMG-1)	an important chromatin protein that belongs to high mobility group and. In the nucleus HMGB1 interacts with nucleosomes, transcription factors and histones to reorganize DNA, facilitate binding of other proteins and regulate transcription
Mouse diaphanous homolog 1 (mDia1)	the mouse version of the diaphanous homolog 1 of <i>Drosophila</i> . mDia1 belongs to the protein family called the formins and is a Rho effector. It plays a role in stress fiber and filopodia formation, phagocytosis, and activation of serum response factor.
Receptor for advanced glycation end products (RAGE)	also known as pattern recognition receptor is a transmembrane receptor of the immunoglobulin super family. REAGE has a number of ligands and RAGE activation via its ligands is pro-inflammatory.
Reactive oxygen species (ROS)	chemically reactive molecules and natural byproducts of the normal metabolism of oxygen, such as oxygen ions and peroxides. ROS have important roles in cell signaling and homeostasis and increased ROS levels are detrimental to the cell.

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Box 1**RAGE and RAGE ligands**

At least certain of the family of heterogeneous AGEs bind the receptor for AGE or RAGE. AGE-RAGE interaction mediates oxidative stress generation, evokes vascular inflammation, macrophage and platelet activation, and stimulates migration of inflammatory cells into AGE-laden foci in diabetes, thereby contributing to the development and progression of diabetic vascular complications [72–73]. RAGE is a member of the immunoglobulin superfamily; its extracellular domain is the site of ligand engagement; this domain consists of one V-type Ig domain (where most of the ligands bind) and two C-type domains. There is a single transmembrane spanning domain and a highly charged, short cytoplasmic domain that is essential for RAGE signal transduction. AGE-RAGE interaction in multiple cell types, such as endothelial cells and monocytes/macrophages, incites activation of NF- κ B, a central transcription factor of the immune/inflammatory response [72]. RAGE may also exist as a soluble truncated form - generated either from cell surface proteolytic cleavage or by alternative splicing mechanisms.

Besides AGEs, RAGE binds non-AGE ligands such as S100/calgranulins and HMGB1 [72]. A chief signature of S100/calgranulins and HMGB1 is in autoimmunity and chronic inflammation [74]. As discussed below, multiple studies link S100s and HMGB1 to diabetes and its complications and suggest that the measurement of these molecules may mirror the state of cellular stress and perturbation in distinct complications [75–77]. It is important to note that S100s and HMGB1 may interact with distinct receptors beyond RAGE, such as molecules of the toll receptor family, toll-like receptors 2 and 4 [78].

Beyond AGEs, S100/calgranulins, and HMGB1, RAGE binds mac-1, lipopolysaccharide, and C1q [79–82]. RAGE also binds amyloid- β peptide and other amyloid forms [83]. The recent discovery that RAGE is a newly identified receptor for lysophosphatidic acid (LPA) suggests key roles for RAGE in vascular perturbation and in tumor biology [84]. Indeed, this discovery brings full circle the implications of RAGE in fundamental tumor biology, as S100P and HMGB1 are directly linked to tumor migration, proliferation and invasiveness.

Box 2**RAGE Signaling - roles for an intracellular effector molecule, mouse diaphanous homolog 1 (Dial)**

How does RAGE signal? There are various theories on the precise mechanisms by which RAGE mediates signal transduction, including receptor oligomerization [85, 86]. In the absence of the RAGE cytoplasmic domain, in cultured cells or in transgenic mice, RAGE ligands are unable to evoke signaling [87]. The interaction of the RAGE cytoplasmic domain (in a manner requiring amino acids R5/Q6) with the FH1 domain of the formin molecule, mDia1, is essential for RAGE signaling and functional changes (proliferation and migration) stimulated by RAGE ligands in smooth muscle cells [87–88]. mDia1 is a member of the formin family and plays key roles in actin binding (with implications for cellular migration) and in cellular signaling, such as the family of Rho GTPase signaling effectors. Experimental evidence suggests key roles for mDia1 in macrophages; when these cells are exposed to hypoxia, deficiency of mDia1 prevents upregulation of the transcription factor Egr1 (which is also a RAGE-dependent phenomenon) [89]. In transformed cells, knock down of mDia1 prevents RAGE ligand-stimulated activation of cdc42 and rac1 and cellular migration [87]. These data suggest that mDia1 plays pivotal roles in the signal transduction biology of RAGE ligands. Studies are underway in diabetic animals to identify if and by what mechanisms mDia1 is essential for the development of diabetic complications.

Box 3**Outstanding Questions**

- What is the role of inflammation in macro- and microvascular complications?
- How does RAGE action in diabetic complications begin? How soon must we intervene in diabetes?
- How and by what means RAGE suppresses “M2”/anti-inflammatory gene expression patterns?
- What is, if any, the role of the newly-identified RAGE ligand, LPA, in diabetic complications?
- By what mechanism(s) diabetes prevents LPA-mediated regression of the neovessels
- How does RAGE contribute to the failure of LPA-induced regression in diabetes?
- What is the precise expression patterns and potential functions of mDia1 in diabetes complications?
- Do RAGE ligands and RAGE play important roles in the phenomenon of “hypoglycemic metabolic memory?”

Highlights

- RAGE is a multi-ligand receptor whose families of ligands accumulate in diabetes.
- RAGE contributes to the pathogenesis of the microvascular complications of diabetes.
- Levels of soluble RAGEs may serve as biomarkers for diabetes and its complications.

Figure 1. The ligands of RAGE and the complications of diabetes - one ligand at a time?

We hypothesize that the key trigger to activation of RAGE in diabetes is hyperglycemia-mediated generation of AGEs. When AGEs interact with RAGE, they stimulate generation of reactive oxygen species (ROS); ROS contribute to further generation of AGEs. Once AGE-RAGE interaction is set in motion, upregulation of inflammatory cell adhesion molecules and chemokines results; the recruitment of inflammatory cells to the vessel wall results in inflammatory cell activation and release of RAGE ligands, S100/calgranulins and HMGB1. These molecules sustain the inflammatory response by (1) increasing vascular permeability and endothelial dysfunction; and (2) polarizing macrophages to a predominant “M1” vs. “M2” paradigm. Such a shift increases inflammatory/tissue-damaging signals (M1) and suppresses repair / remodeling (M2) signatures. Together, these forces converge to contribute to the macro-and microvascular complications of diabetes.