REVIEW
RAGE biology, atherosclerosis and diabetes

Drazenka Pongrac BARLOVIC*, Aino SORO-PAAVONEN† and Karin A. M. JANDELEIT-DAHM‡§
*Clinical Department of Endocrinology, Diabetes and Metabolic Diseases, University Medical Centre Ljubljana, Zaloska 7, Ljubljana, Slovenia, †Division of Nephrology, Department of Medicine, Helsinki University Central Hospital, Helsinki, Finland, ‡Diabetes Division, Baker IDI Heart and Diabetes Institute, 75 Commercial Road, Melbourne, Australia, and §Department of Medicine, Monash University, Melbourne, Australia

ABSTRACT
Diabetes is characterized by accelerated atherosclerosis with widely distributed vascular lesions. An important mechanism by which hyperglycaemia contributes to vascular injury is through the extensive intracellular and extracellular formation of AGEs (advanced glycation end products). AGEs represent a heterogeneous group of proteins, lipids and nucleic acids, irreversibly cross-linked with reducing sugars. AGEs are implicated in the atherosclerotic process, either directly or via receptor-mediated mechanisms, the most extensively studied receptor being RAGE (receptor for AGEs). The AGE–RAGE interaction alters cellular signalling, promotes gene expression and enhances the release of pro-inflammatory molecules. It elicits the generation of oxidative stress in numerous cell types. The importance of the AGE–RAGE interaction and downstream pathways leading to injurious effects as a result of chronic hyperglycaemia in the development, progression and instability of diabetic atherosclerotic lesions has been amply demonstrated in animal studies. Moreover, the deleterious link of AGEs with diabetic vascular complications has been suggested in many human studies. In the present review, our current understanding of their role as an important mediator of vascular injury through the various stages of atherosclerosis in diabetes will be reviewed and critically assessed.

INTRODUCTION
Macrovascular disease is the major cause of morbidity and mortality in diabetes [1]. Recent findings from the follow-up of two large clinical trials in patients with Types 1 and 2 diabetes have shown that the level of glycaemic control is an important determinant of long-term cardiovascular outcomes. Follow-up of both the DCCT (Diabetes Control and Complications Trial) [2] and the UKPDS (U.K. Prospective Diabetes Study) [3] trials have shown that superior glycaemic control in the trials was associated subsequently with improved cardiovascular outcomes

Key words: advanced glycation end-product (AGE), atherosclerosis, diabetes, receptor for advanced glycation end-products (RAGE), vascular injury.
Abbreviations: ACE, angiotensin-converting enzyme; ADAM10, a disintegrin and metalloprotease 10; AGE, advanced glycation end product; apoE, apolipoprotein E; CML, Nε-(carboxymethyl)lysine; DCCT, Diabetes Control and Complications Trial; EPC, endothelial progenitor cell; ERK, extracellular-signal-regulated kinase; HbA1c, glycated haemoglobin; HMGB1, high-mobility group box protein 1; IL, interleukin; KO, knockout; LDL, low-density lipoprotein; LDLR, LDL-receptor; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-κB; NOS, NO synthase; eNOS, endothelial NO synthase; PAI-1, plasminogen activator inhibitor-1; P38K, phosphoinositide 3-kinase; PKC, protein kinase C; RAGE, receptor for AGEs; esRAGE, endogenous secreted RAGE; RAS, renin-angiotensin system; sRAGE, soluble RAGE; TGF-β1, transforming growth factor-β1; VCAM-1, vascular cell adhesion molecule-1.

1 These authors contributed equally to this work.
Correspondence: Professor Karin A.M. Jandeleit-Dahm (email karin.jandeleit-dahm@bakeridi.edu.au).
well after the differences in glycaemic control between intensively treated and conventionally treated groups were lost.

In diabetes, atherosclerosis is often accelerated with a wide distribution of vascular lesions [4]. It still remains controversial as to whether diabetes-associated atherosclerosis represents a specific disease rather than an accelerated form of atherosclerosis [5]. Recently, there has been evidence that diabetes-associated atherosclerosis is indeed a unique form of vascular disease with a more prominent inflammatory phenotype [6].

The pathogenesis of vascular damage in diabetes is multifactorial and involves complex interactions among many homoeostatic systems. Hyperglycaemia is thought to contribute to vascular injury, including atherosclerosis, through at least four different mechanisms [7]. During normoglycaemia, the major pathway of glucose metabolism is the glycolytic pathway, with other pathways of glucose metabolism relatively suppressed. However, during hyperglycaemia, in cells that are unable to reduce glucose uptake, such as occurs in endothelial cells, glucose metabolism shifts from the glycolytic pathway to other pathways. These involve the increased flux via the polyol pathway, increased intracellular formation of AGEs (advanced glycation end-products), the activation of the intracellular signalling molecule PKC (protein kinase C) and increased flux through the hexosamine pathway [7]. In diabetes, these altered metabolic pathways, driven by chronic hyperglycaemia, interact with haemodynamic factors, including activation of the RAAS (renin–angiotensin–aldosterone system) as well as the endothelin and urotensin systems in mediating vascular injury. It is considered that metabolic pathways act independently and in concert with altered haemodynamics, to activate nuclear transcription factors such as NF-κB (nuclear factor κB) and other intracellular second messengers, including PKC and MAPK (mitogen-activated protein kinase). These activated pathways are associated with an overproduction of superoxide ions by the mitochondrial electron-transport chain and other pathways, including NADPH oxidase, which produces a cascade of deleterious metabolic events, ultimately leading to damage of the vessel wall [7].

In the present review, we aim to elucidate in detail the mechanisms which explain how increased plasma glucose exerts its effect on the vasculature as well as contributing to development and progression of atherosclerosis with a particular emphasis on the role of increased AGE formation.

**ATHEROSCLEROSIS**

Atherosclerosis is considered to be a chronic inflammatory disease, developing over years and proceeding through many steps [8]. Atherosclerosis in the diabetic milieu appears to occur as a result of complex interactions among a range of risk factors, and it has remained difficult to separate the effects of hyperglycaemia from other proatherogenic factors. Thus it still remains unclear whether the complex interaction of adverse metabolic and haemodynamic pathways in the diabetic milieu or hyperglycaemia contribute to the advanced atherosclerosis observed in diabetes. In *vitro* multiple mechanisms, whereby hyperglycaemia might be atherogenic, have been elucidated. A high-glucose milieu leads to activation of NF-κB in endothelial cells, which then results in enhanced expression of a variety of pro-inflammatory genes [9]. In addition, monocytes grown in high-glucose conditions also show evidence of increased expression of inflammatory cytokines, such as IL (interleukin)-1β and IL-6 [10]. Although much of the work has been devoted to show a specific role of glucose in atherosclerosis development *in vivo*, it has been difficult to dissect the relative contribution of high glucose from the effects of diabetes-induced dyslipidaemia in animal models. In one of these preclinical studies, the causative role of hyperglycaemia was demonstrated using a model with viral destruction of pancreatic β-cells in the transgenic LDLR [LDL (low-density lipoprotein)-receptor]-deficient mouse, thus mimicking Type 1 diabetes [11]. In this model, the induction of diabetes accelerated initiation of the atherosclerotic lesion in association with increased macrophage accumulation and fatty streak formation. In addition, accelerated intraplaque haemorrhage in advanced lesions was detected. The effect of diabetes on lesion initiation was not dependent on lipid levels and was most likely a consequence of hyperglycaemia. Moreover, intensive insulin therapy prevented diabetes-initiated atherosclerotic lesions. However, in advanced lesions, hyperglycaemia alone, without hyperlipidaemia, was not sufficient to cause intraplaque haemorrhage and lesion disruption [11], further demonstrating the complex interaction between lipid and glucose-related factors on the development and progression of the atherosclerotic plaque in diabetes.

It has been suggested that the initiation of atherosclerosis is closely linked to endothelial dysfunction [12]. The endothelium acts as a complex receptor–effector structure, counteracting different physical or chemical stimuli by synthesizing a variety of molecules that act as agonists or antagonists to maintain homoeostasis between vasodilator and vasoconstrictor stimuli. When this balance is disturbed, the endothelium becomes leaky, defective and vulnerable to invasion by inflammatory cells and lipids, which represent the key steps in the formation of the atherosclerotic plaque [13]. The availability and activity of NO, an endothelium-derived vasodilator substance, plays a pivotal role in the protection of the endothelium. In diabetic arteries, endothelial dysfunction seems to occur as a result, first, of the direct effect of hyperglycaemia and, secondly, as a result of selective
insulin resistance, specific to the PI3K (phosphoinositide 3-kinase) pathway [14]. Hyperglycaemia inhibits production of NO in arterial endothelial cells and stimulates the production of PAI-1 (plasminogen activator inhibitor-1) [15]. Pathway-selective insulin resistance, as observed in diabetes, may contribute to decreased endothelial NO production and increased proliferation of vascular smooth muscle cells and production of PAI-1 [14].

Atherosclerosis is further characterized by the adhesion of monocytes to endothelial cells, followed by their transmigration into the subendothelial space along a chemotactic gradient. One mechanism whereby high glucose may enhance this process is through the activation of the key pro-inflammatory nuclear transcription factor, NF-$\kappa$B, which leads to the expression of multiple inflammatory genes, including adhesion molecules [e.g. VCAM-1 (vascular cell adhesion molecule-1)], which facilitate monocyte adhesion to endothelial cells [9]. Monocytes then differentiate into intimal macrophages, which transform into foam cells by lipid uptake. Foam cells accumulate in the vessel wall, resulting in accelerated fatty streak formation. Subsequently, these fatty streak lesions develop into advanced lesions, characterized by smooth muscle cell accumulation, necrotic core formation and lipid accumulation as well as the formation of a fibrous cap. Some of these advanced lesions eventually become unstable and rupture, thus triggering thromboembolic events, which result in the clinical manifestations of cardiovascular disease such as myocardial infarction and stroke.

**FORMATION OF AGEs**

AGEs form during an irreversible post-translational modification of proteins, lipids or nucleic acids. They undergo a chain of chemical reactions, the process known as the Maillard or browning reaction. Indeed, this reaction represents the same chemistry that is involved in the browning of foods and tobacco during their processing [16,17]. The initial chemical reaction between reducing sugars or sugar-derived products and amino groups on proteins, lipids or nucleic acids to form AGEs is known as early glycation. It is a reversible non-enzymatic reaction by which enolized sugars form Schiff bases with available amine residues and then undergo rearrangement to form more stable Amadori products (Figure 1). The best known of these Amadori products are HbA$_{1c}$ (glycated haemoglobin) and fructosamine, which are useful markers of glycaemic control. Neither fructosamine nor HbA$_{1c}$ are AGEs. However, these Amadori products are able to undergo further dehydration, oxidation, rearrangement and fragmentation reactions that eventually lead to the formation of AGEs, the irreversibly cross-linked heterogeneous fluorescent derivatives of sugars and macromolecules. This spontaneous rearrangement is normally slow, often taking months to years. Nevertheless, the presence of oxidative stress, metal ions and other catalysts can substantially increase the post-Amadori formation of AGEs.

Glucose is the major sugar in biological systems. It predominantly exists in a stable unreactive ring form. However, the metabolism of glucose known as glycolysis results in the formation of a number of reactive products that have the potential to interact with protein residues to rapidly form AGEs. The best known of these are the glucose-derived dicarbonyls (methylglyoxal, glyoxal and 3-deoxyglucosone) that are generated intracellularly during glycolysis (Figure 1) [18,19]. Reactive dicarbonyls can also be generated from ketones, lipids and other
metabolic pathways, suggesting that hyperglycaemia and the resulting glycolytic flux is not a prerequisite for AGE formation [20]. Indeed, AGEs are found not only in diabetes, but also in a diverse range of other contexts, such as the normal aging process, renal failure [21], amyloidosis and chronic inflammation [22,23]. Increased production of reactive dicarbonyls or reduced detoxification by the glyoxalase system [24] or endogenous scavengers leads to a state of ‘carbonyl stress’, which is increasingly considered to be the major driving force for AGE formation and accumulation.

The spontaneous chemistry of AGE formation is also crucially dependent on time. Over a person’s lifespan, the amount and variety of AGE-modified tissue progressively increases, due in part to the time-dependence of the Maillard reaction but also reduced protein turnover associated with chronological aging and the resistance of AGE-modified proteins to proteolytic digestion. Proteins and other molecules that have a long half-life are more likely to be modified by exposure to glucose and glucose-derived products and are more likely to be modified to a greater extent [20]. Some common examples of AGE-modified proteins include extracellular matrix proteins, myelin, cartilage and lens crystallins. In the plasma, albumin has the longest half-life of all circulating proteins and consequently is a major target for modification [25]. Interestingly, it is possible to approximate the ‘age’ of any protein by its degree of AGE modification. Formation of AGEs may thus be one of the ways our body identifies those targets that are ready for turnover, but spares those that were synthesized more recently, even though the structure or function may be similar.

Finally, whether a protein will become AGE-modified or not also depends on the availability of residues capable of activation with sugars. For example, proteins with abundant lysine and arginine residues in externally accessible locations are most vulnerable to AGE modification. Equally, within any protein, these sites are the most susceptible. For example, on serum albumin, an externally located arginine residue (410) that is responsible for much of its enzymatic activity as well as drug transport is readily modified by methylglyoxal. By contrast, internal arginine residues are rarely modified in vivo [26]. Some AGEs have intrinsic fluorescence, meaning that fluorescence can be used as a surrogate marker for the presence of AGE modifications. It must be remembered that AGEs are a chemically heterogeneous group of compounds with only about 25 AGEs having been fully characterized. Many more, probably the majority, remain to be fully defined. The hydromimidazole AGEs, derived from glyoxal, methylglyoxal and 3-deoxylglucosone, appear to be quantitatively the most common detectable AGEs. CML [N\(^\text{\text{cis}}\)-carboxymethyl]lysine], is the simplest and best characterized AGE, derived predominantly from the carbonyl modification of lysine [27]. CML is also the main epitope for recognition by most commercially available antibodies used for the detection and quantification of AGEs. Other more complex AGEs form ‘cross-links’ both between and within modified proteins, such as pentosidine, MOLD (methylglyoxal-lysine dimer) and GOLD (glyoxal-lysine dimer). These cross-links have the potential to produce important changes in protein structure and function. For example, AGE modification of vascular collagen leads to structural alterations, including changes in packing density and surface charge, manifested as increased stiffness, reduced thermal stability and resistance to proteolytic digestion, which cumulatively result in decreased compliance of the vasculature [28]. Heterotypic interactions between matrix proteins and homotypic interactions required for polymeric self-assembly are disturbed by AGE modifications. Cell–matrix interactions may also be disrupted by matrix glycation, contributing to changes in cellular adhesion [29], altered cell growth and loss of the epithelial phenotype. AGE-induced covalent cross-linking may also lead to trapping of local macromolecules, such as LDLs in the arterial wall [30]. Apart from affecting extracellular proteins, other important targets for AGE modification appear to be those closest to the site of generation of reactive dicarbonyls, the mitochondrion. These include proteins of the respiratory chain and mitochondrial DNA, which appear to be particularly vulnerable to AGE modification [31].

The precise identity of the AGEs that specifically contribute to the development of vascular complications has not been clearly determined. However, it is likely that there is an aggregate effect in vivo, rather than an effect dependent on individual structures. Moreover, at least some of the injury that is mediated by AGEs, for example in diabetes, occurs through the activation of multiligand AGE receptors that recognize a range of chemical structures [e.g. RAGE (receptor for AGEs)]. AGEs contribute negative charge and hydrophobicity to target proteins, so that pattern recognition of topological features on AGE-modified proteins may contribute to a diffuse recognition process involving a wide range of ligands. Although it is possible that one structural moiety has the greatest pathogenic potential, it is more likely that, given the broad chemical heterogeneity of AGEs, the extent of modification may be more important than the presence of any one chemically distinct AGE [31].

AGEs can be eliminated by their undergoing cellular proteolysis, producing AGE peptides and AGE adducts bound to single amino acids [32]. While the latter, after being released in plasma, can be excreted directly in the urine, AGE peptides are endocytosed by proximal tubular cells and subsequently degraded by the endolysosomal system into AGE amino acids. It is believed that AGE amino acids are then exported back into the tubular lumen for subsequent excretion [34].
Larger, extracellularly derived AGEs cannot pass through the glomerular basement membrane and must first be degraded by peripheral macrophages into these smaller AGE-containing moieties [34].

### RAGE AND ITS LIGANDS

RAGE is a multiligand receptor of the immunoglobulin superfamily of cell-surface molecules, originally described as having a pivotal role in diabetes and other metabolic disorders characterized by AGE accumulation [35,36]. RAGE is expressed on many cells including endothelial cells, mononuclear phagocytes, lymphocytes, vascular smooth muscle cells and neurons [35,36]. RAGE is induced by reactions known to initiate inflammation, and its biological activity is dependent on its ligation by various ligands [37]. In addition to binding with AGEs, RAGE interacts with HMGB1 (high-mobility group box protein 1), members of the S100/calgranulin family, β2-integrin Mac, amyloid-β peptide and β-sheet fibrils [37–42]. RAGE acts as a pattern recognition receptor for these ligands released by inflamed, stressed and damaged cells. Increased expression of both cell-surface RAGE and accumulation of its ligands are observed in a range of disorders characterized by chronic inflammation, such as inflammatory bowel disease, rheumatoid arthritis, atherosclerosis, amyloidoses, Alzheimer’s disease and the vascular complications of diabetes [43].

RAGE is composed of a large extracellular part, a transmembrane domain and a 43-amino-acid-long cytoplasmic tail [44] (Figure 2). The extracellular domain of the RAGE protein includes a short signalling sequence for binding to the cell membrane and two C- and one V-type immunoglobulin-like domains. The ligand binding site has been identified to be in the V-domain, whereas the cytosolic tail mediates intracellular signalling. More recently, it has been suggested that RAGE may form dimers and multimers for ligand binding to occur [44]. A range of splice variants of murine and human RAGE have been identified, including soluble isoforms [45]. A complete classification and identification has been published previously [46,47]. Most of the splice variants or RAGE lead to removal of the transmembrane domain to produce soluble variants with most of those being degraded at the mRNA level. Only one of these splice variants was found to be secreted, the so-called RAGE splice variant I or esRAGE (endogenous secreted...
RAGE)/sRAGE3 [48,49]. An alternative mechanism of forming soluble circulating RAGE from the receptor is by proteolytic cleavage mediated by ADAM10 (a disintegrin and metalloprotease 10), producing sRAGE (soluble RAGE) [50]. Thus sRAGE, which comprises only the extracellular part of full-length RAGE, is either produced by proteolytic shedding or by alternative splicing (esRAGE) [50,51].

The measurement of circulating RAGE is usually performed by two different commercially available assays. The first assay measures the total sRAGE pool in serum and plasma using a monoclonal antibody raised against the whole extracellular human sRAGE protein (Quantikine assay, R&D Systems) [45]. The second assay specifically measures RAGE/RAGEv1 using a specific antibody against the unique C-terminus of RAGEv1 (B-bridge International). However, esRAGE represents only a fraction of the overall pool of soluble RAGE. It remains to be elucidated if the ratio between both types of circulating RAGE differs between disease states and which parameter is a better and more reliable measurement of cardiovascular disease. A study by Humpert et al. [52] has suggested that soluble RAGE, but not esRAGE, is associated with albuminuria in Type 2 diabetes.

The role of sRAGE in biological systems is not yet completely understood. sRAGE is thought to act as an AGE decoy receptor as it binds AGEs competitively and may aid in their secretion [53]. On the other hand, recent studies suggest a more active involvement of sRAGE in the pathobiology of vascular dysfunction [54]. As discussed later in more detail, administration of exogenous sRAGE in various experimental models has been shown to suppress the pro-inflammatory actions of membrane-bound RAGE. Recombinantly produced extracellular ligand-binding domain of RAGE, termed sRAGE, functions as a decoy for the cell-surface receptor acting by binding ligands in the circulation and preventing them from binding to the cell-surface-bound receptor avoiding RAGE activation and signalling.

The RAGE–ligand interaction triggers activation of NF-κB and other signalling pathways through stimulation of p21ras, ERK (extracellular signal-regulated kinase) 1/2, p38 MAPK, SAPK (stress-activated protein kinase)/JNK (c-Jun n-terminal kinase), Rho GTPases, PI3K and JAK (Janus kinase)/STAT (signal transducer and activator of transcription) pathways. Subsequently, expression of inflammatory cytokines is increased, leading to an inflammatory response with associated cellular migration and proliferation [38] (Figure 2). In fact, ligation of RAGE causes a positive feed-forward loop, in which inflammatory stimuli activate NF-κB, which induces RAGE expression, followed again by NF-κB activation [55]. NF-κB up-regulates multiple cellular signalling cascades, such as NADPH oxidase, CDC42, RAC-1, the MAPKs, p21ras, ERKs p38 MAPK and PKC [56,57,59,60]. This causes increased production of numerous growth factors and cytokines including VCAM-1, ICAM-1 (intercellular adhesion molecule-1), E-selectin, TGF-β1 (transforming growth factor-β1), CTGF (connective tissue growth factor), PDGF (platelet-derived growth factor), TNF-α (tumour necrosis factor-α), IL-1 and IL-6 [61–66], all of these molecules having been implicated in the development and progression of a variety of diabetic complications.

**AGE/RAGE INVOLVEMENT IN THE ATHEROSCLEROTIC PROCESS**

A large body of experimental evidence supports the integral contribution of AGE–RAGE interactions to all major stages associated with the development and progression of atherosclerosis. In addition, particularly AGEs, together with HMGB1 and S100/calgranulins, have been shown to accumulate in plaques also in the absence of diabetes [67,68], suggesting that AGE/RAGE signalling may represent a ‘final common pathway’ in linking inflammatory disorders with accelerated atherosclerosis.

In the atherosclerotic process, RAGE expression is particularly evident in endothelial cells of the aorta, but also in monocytes/macrophages and medial smooth muscle cells, albeit to a lower extent [69]. Moreover, in the plaques of mice genetically lacking RAGE, there was a significant reduction of macrophage content.

**Endothelial dysfunction**

Endothelial dysfunction is a priming event in atherosclerosis. An endothelium-derived vasodilator substance, NO, is one of the key protective molecules of the endothelium. Bioavailability and activity of NO has been demonstrated to be reduced by AGEs [70]. Nevertheless, the role of AGEs in endothelial dysfunction has been clearly demonstrated in human diabetes, where levels of serum AGEs in patients with Type 2 diabetes were negatively associated with the extent of endothelium-dependent and endothelium-independent vasodilatation [71].

Several mechanisms have been suggested to explain this association. AGE–associated induction of oxidative stress may quench and inactivate endothelium-derived NO [72] and uncouple NOS (NO synthase) activity. AGEs may also directly reduce eNOS (endothelial NOS) activity through receptor-mediated phosphorylation of serine residues in eNOS [72] and increase degradation of eNOS mRNA [73]. In addition, AGEs may impair endothelial balance by reducing the endothelial production of PGI2 [prostaglandin I2 (prostacyclin)] [74] and enhancing the expression of endothelin-1 [75].
**Induction of endothelial hyperpermeability**

AGEs increase endothelial permeability to macromolecules [76,77]. This appears to be dependent on activation of RAGE [78,79], as it can be blocked by RAGE antagonists, sRAGE and introduction of dominant-negative RAGE into endothelial cells [69]. Subsequent NF-κB-dependent induction of VEGF (vascular endothelial growth factor) may also be important [80].

**Migration of monocytes into the vascular intima**

AGE-modified proteins have been shown to promote sequestration of monocytes/macrophages into the vessel wall. Indeed, AGEs have been shown to be chemotactic for human blood monocytes both in vitro and in vivo [81,82]. For example, AGEs induce monocyte migration across the endothelial cell monolayer [81], through the RAGE-dependent induction of the NF-κB pathway.

**Intravascular fat accumulation**

AGEs are also significantly involved in deleterious lipid accumulation within the vessel wall. AGE modification of lipoproteins contributes to atherogenic dyslipidaemia. For example, glycation of the LDLR and plasma lipoproteins results in impaired cholesterol uptake [81]. Similarly, glycation of ApoA1 (apolipoprotein A1) impairs cholesterol and phospholipid efflux from plaques. Furthermore, AGE modification of oxidized LDLRs in human monocyte-derived macrophages [83] leads to enhanced oxidized LDL uptake. It was also demonstrated that AGE–LDL induces pro-inflammatory cytokine production in endothelial cells and macrophages through the TLR4 (Toll-like receptor 4) pathway [84]. Within the atherosclerotic plaques, AGEs also significantly affect phenotypic conversion of smooth muscle cells to foam cells [85].

**EPCs (endothelial progenitor cells)**

AGEs also may modify the functions of EPC function [86], with a range of both positive and negative effects. Recent studies have demonstrated AGE-/RAGE-dependent promotion of apoptosis of EPCs and inhibition of their migration [86]. By contrast, the interaction of RAGE with one of its well-described ligands, HMBG1, was shown to be beneficial during vascular tissue repair by enhancing integrin-dependent homing of EPCs to ischaemic locations [87].

**Oxidative stress**

Oxidative stress is a key mediator of atherogenic changes in the vasculature. It is increased in proportion to the accumulation of AGEs. AGEs increase the expression and activity of NADPH oxidase, an important source of vascular oxidative stress in diabetes. In addition, AGEs are able to deplete cellular antioxidant systems such as glutathione peroxidase [88]. Glycation of antioxidants such as Cu/ZnSOD (superoxide dismutase) also contributes to the decline in antioxidant activity. AGEs can also directly enhance the formation of free radicals, possibly involving binding and activation of transition metal ions [59]. Furthermore, also in EPCs, overexpression of oxidative stress ignited through RAGE resulted in increased apoptosis in these cells [89].

**Activation of coagulation**

AGE–RAGE interaction induces a procoagulant state in endothelial cells, resulting from reduced thrombomodulin activity in parallel with increased tissue factor expression [90]. AGEs also increase expression of tissue factor by monocytes [91]. Interestingly, it has been shown that endothelial cells from Type 2 diabetic patients despite optimal glycaemic control (HbA1c < 6.0 %) exhibited a prothrombotic phenotype when compared with endothelial cells from subjects without diabetes, and these effects were shown to be mediated through the AGE–RAGE pathway [92].

**The RAS (renin–angiotensin system)**

Increased activity of the RAS has been strongly associated with the pathogenesis of vascular complications. Interruption of the RAS with drugs such as ACE (angiotensin-converting enzyme) inhibitors and ARAs (angiotensin receptor antagonists) are currently the mainstay of clinical intervention for both the prevention and treatment of diabetic complications. Beneficial effects, beyond those achieved by blood pressure control alone, have been observed in numerous experimental and clinical trials in diabetes. Interestingly, AGEs induce up-regulation of key components of the RAS in the vasculature, and this induction can be prevented by AGE inhibition [93]. Moreover, it has been shown that ACE inhibition reduces AGE accumulation and increases sRAGE levels [93]. Nevertheless, our group has shown that overexpression of RAGE or treatment with AGE decreases cell-surface AT1Rs (angiotensin II type 2 receptors) [94].

**AGE/RAGE in in vivo models**

*In vivo*, a few studies have shown the reduction of AGE accumulation to be effective in attenuating different components of vascular dysfunction and injury. Our group has previously explored AGE inhibition in an experimental model of accelerated atherosclerosis associated with diabetes. In this model, plaque accumulation was attenuated by 30 % by treatment with alagebrium chloride and by 40 % with aminoguanidine. Both drugs also demonstrated the ability to reduce vascular AGE accumulation, collagen I, III and IV deposition and were associated with reduced profibrotic cytokine expression [95]. Furthermore, the
effects of AGEs in development of diabetic heart disease in Sprague–Dawley rats have been studied [96]. Treatment with alagebrium chloride decreased left ventricular mass in these animals in association with decreased BNP (brain natriuretic peptide) gene expression. In addition, alagebrium completely prevented cardiac collagen III mRNA and protein up-regulation [91].

With respect to the activation of RAGE, data acquired in the streptozotozin-induced diabetic apoE−/− mouse clearly show that RAGE activation has a central role in the formation and progression of atherosclerotic lesions and in the maintenance of pro-inflammatory and prothrombotic pathways contributing to diabetes-related atherosclerosis [64,97–100]. In these mice, RAGE protein and mRNA expression is increased in the vascular endothelium [64,99]. We [64] and others [66,100] have recently shown that in diabetic RAGE−/−/apoE−/− double KO (knockout) mice, the absence of RAGE was associated with a significant attenuation of atherosclerotic plaque accumulation. The reduction in atherogenesis is associated with decreased accumulation of macrophages and T-lymphocytes, reduced aortic expression of the NF-κB subunit p65 and decreased expression of inflammatory cytokines and adhesion molecules, including VCAM-1, MCP-1 and certain NADPH oxidase subunits such as gp91phox, p47phox and rac-1. Furthermore, pathway expression analysis revealed that the TGF-β and focal adhesion pathways play a significant role in diabetes-mediated formation of atherosclerotic plaques and in the vasculoprotective effect conferred by RAGE deletion. In particular, ROCK-1 (rho-associated protein kinase-1) of the TGF-β pathway has been identified to mediate diabetes-accelerated atherosclerosis [66]. In addition to reducing atherosclerotic lesions, competitive inhibition of RAGE by exogeneously administered sRAGE once atherosclerosis was already established, results in a decrease in mean atherosclerotic lesion area and number of complex lesions with no evidence of lesion progression subsequent to sRAGE administration in diabetic apoE KO mice [98].

In addition, in non-diabetic models of atherosclerosis, RAGE ligands have been shown to participate in the acceleration of atherosclerosis. In RAGE and apoE double KO mice as well as in apoE KO mice with blocked RAGE signalling, atherosclerosis was significantly reduced compared with apoE KO mice [69]. JNK was recognized as the main signalling pathway of RAGE-mediated inflammatory signals in this model. In another hyperlipidaemic model, the non-diabetic LDLR-deficient mice, RAGE inactivation inhibited atherosclerosis through reduced oxidized LDL-induced pro-inflammatory responses and oxidative stress [101].

In both the diabetic and non-diabetic context, inhibition of RAGE using sRAGE and anti-RAGE antibodies [anti-RAGE F(ab′)2] has been shown to suppress neointimal expansion in mice following arterial denudation, suggesting that RAGE blockade would be beneficial in the prevention of systemic atherosclerosis [102,103]. Competition of RAGE ligand binding by sRAGE has been shown to suppress NF-κB activation and the expression of pro-inflammatory cytokines, resulting in reduced inflammation, reduced activity of matrix-degrading proteases and increased levels of interstitial collagen [98,104]. Importantly, treatment with sRAGE had no effect on levels of glucose, insulin or lipids [105].

Interestingly, data from various experimental models suggest that protection from diabetic micro- and macro-vascular changes afforded by sRAGE is more obvious in wild-type mice than in RAGE−/− animals [97,98,102,103]. Therefore one could speculate that sRAGE binds ligands that otherwise would interact with other cell-surface pro-inflammatory receptors.

In addition, it seems that RAGE–ligand interactions are very complex ones, and it cannot be excluded that the RAGE system may exert some beneficial effects in atherosclerosis as well as it does in nerve regeneration [106]. Thus, in experiments with mice globally deficient in RAGE, the potential reparative roles of RAGE in atherosclerosis could be concealed [101].

The complexity of the role of RAGE in the biological setting is also evident from human studies in different vascular disease states. In the settings of increased cardiovascular risk such as end-stage renal disease and diabetes, both Types 1 and 2, increased levels of AGES and sRAGE were demonstrated [107,108]. In addition, in patients with heart failure, higher sRAGE concentration was associated with ischaemic disease and heart failure severity [109]. All these data suggest that sRAGE is a marker of increased activation of ligand–RAGE axis. On the other hand, many studies have pointed out that decreased sRAGE levels are associated with increased extent of coronary artery disease and also with higher incidence of cardiovascular events, such as myocardial infarction. For example, recent data from the large Dallas Heart Study [110] including 2571 subjects have shown that lower levels of sRAGE are independently associated with a greater prevalence of coronary atherosclerosis. In addition, in diabetes, lower levels of sRAGE were correlated with severity of diabetic complications [111,112]. These results suggest that sRAGE is an endogenous protection factor against the occurrence of atherosclerosis and other vascular complications. Moreover, it was shown that certain polymorphisms in the RAGE gene are strongly associated with higher sRAGE levels [113], implicating a complex genetic regulation of sRAGE levels and suggesting that sRAGE may not merely be a marker of a disease state but also a potential target in the pathobiology of the atherosclerotic process.

These findings clearly illustrate the need for further studies of the complex involvement of sRAGE in the
development of vascular disease states. As yet, no controlled clinical trials have been completed testing sRAGE in humans. Nevertheless, the first clinical trial testing RAGE inhibition is currently underway using an alternative strategy involving the administration of a novel RAGE inhibitor, PF 04494700. The efficacy and safety of this compound is clinically being initially studied in patients with moderate to severe Alzheimer’s disease, although it would ultimately be of interest to explore such a therapy in the context of diabetes.

RAGE and metabolic memory

There is a phenomenon known as ‘hyperglycaemic memory’ best described in the DCCT/EDIC (Epidemiology of Diabetes Interventions and Complications) trial where previous periods of improved glycaemic control continue to afford benefits on diabetic complications despite individuals returning to normal, often worse, glycaemic control [2,3]. The underlying explanation for this phenomenon remains unexplained, but it has been proposed that sustained cellular activation as a result of hyperglycaemia and oxidant stress triggers AGE generation and then, via sustained RAGE activation, including within the vasculature, there is prominent low-grade inflammation, and this injury is self-perpetuating as a result of the feed-forward loop linking RAGE to inflammatory gene expression.

CONCLUSIONS

Although RAGE and its ligands are clearly present at the onset and progression of diabetes-associated vascular disease, there are, as yet, no clinical or experimental data to definitively prove that RAGE is the key factor in the end-organ pathology observed in human diabetes. Furthermore, the clinical utility of AGE inhibition or sRAGE application for the prevention of cardiovascular disease remains to be established. Nevertheless, there is a large body of evidence to suggest that AGE–RAGE activation plays an important role in the development of atherosclerotic complications of diabetes.

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