The Receptor RAGE in Vascular and Cerebral Dysfunctions

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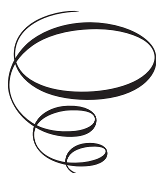
Edited by

Jean-Luc Wautier

Cambridge Scholars Publishing



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INTRODUCTION

THE RECEPTOR RAGE IN VASCULAR AND CEREBRAL DYSFUNCTIONS

JEAN-LUC WAUTIER

Advanced Glycation End products (AGE) are compounds which result from a non-enzymatic reaction between carbohydrates and proteins. They were first described in 1912 by an expert in amino acid chemistry, Louis Camille Maillard, who observed that the formation of these reactive substances in nature was dependent on sugar concentration and length of incubation. AGEs were first named as non-enzymatically glycated products, and glycated hemoglobin was found to be a useful biomarker in diabetes mellitus.

During the three last decades numerous investigations have explored the role of AGE and their binding with the Receptor for Advanced Glycation End products (RAGE). RAGE is the main cell-surface molecule which is implicated in the toxicity of AGE. The RAGE gene is present on locus 6p21.3, and is next to the CHM class III protein family. RAGE can bind a wide range of endogenous molecules including AGEs; the high mobility group box-1 (HMGB-1), which is also called amphoterin-c; β-amyloid peptide; and S100 calgranulins. RAGE is a member of the immunoglobulin superfamily that contains three Ig-like domains, one variable and two that are constant in the extracellular part; a single transmembrane domain; and one short cytosolic tail. First described as a cell receptor, RAGE is now known to generate several isoforms produced through alternative splicing or post-translational modifications. A publication summarizing the work of many laboratories showed that extensive splicing of RAGE transcripts led to as many as 20 splice variants. In endothelial cells, only three isoforms of RAGE were detected at significant levels: N-truncated (Nt-RAGE), full length (FL-RAGE, usually called RAGE), and endogenous secretory (esRAGE). Other than splicing, soluble RAGE (sRAGE) can also be produced following FL-

RAGE proteolysis and may act as a decoy, which will prevent the engagement of ligands in RAGE. With the exception of lung tissues, where the constitutive expression of FL-RAGE is abundant, RAGE is expressed at low levels in most other tissues, including normal brain tissue.

In Chapter One, Jean-Luc Wautier and Marie-Paule Wautier describe AGE, the receptors for AGE products, and their influence on human health. The deleterious effects of AGE products appear to have major consequences in diabetes as they contribute to the development of retinopathy and nephropathy, as well as vascular complications. AGE products are present in food and absorbed by the intestine but are also formed in the body. AGE products accumulate during aging and, alongside retinopathy, nephropathy, and vascular disease, they have a deleterious effect on protein structure of bones and muscles.

In Chapter Two, Ravichandran Ramasamy, Alexander Shekhtman, and Ann Marie Schmidt observe that diabetic polyneuropathy is a major cause of foot ulcerations and may lead to the amputation of digits or limbs. N(epsilon)-(carboxymethyl)lysine (CML)—AGE ligands accumulated in human obese, rather than lean, adipose tissue and RAGE expression is upregulated. They describe the fact that DIAPH1-formin is a partner for the RAGE cytoplasmic tail (ctRAGE) and participates in the signaling and modulation of gene expression. DIAPH1 is a formin molecule linked to the actin cytoskeleton. Formins are affect Rho GTPase signaling. RAGE overexpression in melanoma cells results in an independent ligand signaling receptor. This discovery opens up a new possible strategy to block the interaction of ctRAGE and DIAPH1.

In Chapter Three, Thibault Teissier, Rachel Litke, Solenne Taront-Dezitter, Steve Lancel, Chantal Fradin, and Eric Boulanger consider the aspects of nutrition and inflammaging during aging. Aging and age-related diseases share some basic mechanistic pillars that largely converge on inflammation. AGE and RAGE participate in various steps of inflammation. Genetic and epigenetic variations of RAGE are implicated in senescence. AGE present in food may accumulate during life and participate in sarcopenia. Mitochondria have a predominant role in cell life and mitochondrial functions can be altered by AGE-RAGE interactions leading to apoptosis. RAGE contribution to senescence may involve mTOR (mammalian Target of Rapamycin) and ER stress (Endoplasmic Reticulum stress).

In Chapter Four, Varun Kumar, Peter Nawroth, and David Stern have focused on a new function of RAGE. RAGE appears to be a protein that is essential for normal aging and resistance against reactive oxygen intermediate mediated cellular damage. This new discovery demonstrates that beside the deleterious consequences of RAGE activation, RAGE could have beneficial effects, as RAGE might play a role in DNA repair.

CHAPTER ONE

PROGRESS IN THE COMPREHENSION OF THE ROLE OF AGE AND THE RECEPTORS IN HUMAN PATHOLOGY

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Introduction

Diabetes mellitus and vascular disorders are two major causes of mortality and are also responsible for severe disabilities. Clinical investigations have been conducted in several countries and risk factors—including genetics, diet, obesity, and biological parameters—have been identified. During the last three decades it was discovered that one of the consequences of hyperglycemia was the formation of Advanced Glycation End products.

Advanced glycation End products (AGE) result from a chemical reaction between the carbonyl group of reducing sugars and the nucleophilic NH2 from a free amino acid from a protein: lysine and arginine are the main reactive amino acids on proteins. Following this first step, a molecular rearrangement occurs, which leads to the rearrangement of Amadori, resulting in the formation of Maillard products. AGE can react with structural proteins or receptors.

Glycation can cause the eye lens to cloud and it can also alter the skeleton by inducing reactions in the crosslinking proteins. Specialized receptors (RAGE, Galectin 3) bind AGE products. This binding causes the formation of reactive oxygen species (ROS), which have a deleterious effect because they are powerful oxidizing agents. ROS also play the role of intracellular messengers, altering the cell functions which leads to end organ alterations. This is especially true at the level of endothelial cells: for instance, the attachment of AGE products to the RAGE receptor causes an increase in vascular permeability (Wautier et al., 1994, Wautier et al., 1996, Chappey et al., 1997).

AGE binding to RAGE on endothelium and on monocytes-macrophages leads to the production of cytokines and growth factors, to the expression of adhesion molecules, and to the synthesis of procoagulant activity. Diabetic retinopathy is related to the excessive secretion of vascular endothelial growth factor (VEGF). AGE RAGE receptor-binding causes the synthesis and secretion of VEGF.

Increased permeability and facilitation of leukocyte migration, as well as production of reactive oxygen species, cytokines, and VEGF suggest that AGE could be an element from a cascade of reactions responsible for diabetic angiopathy and the vascular damage observed during aging and chronic renal failure.

History

In 1912 Louis Camille Maillard discovered that high glucose concentration leads to a chemical reaction with proteins (Figure 1.1)

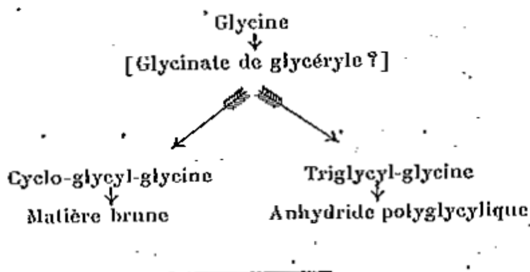

Figure 1.1: Copy of the original illustration of the Maillard reaction
(Maillard LC, 1913. Genese des matières proteiques et des matières humiques. Masson edit. Paris.)

This is a chemical reaction resulting in the binding of a carbohydrate to the free NH2 of an amino-acid (lysine). It is a non-enzymatic reaction, which is time, temperature, and sugar concentration dependent in the presence of oxygen or oxidants.

The initial description of melanoidin formation by LC Maillard in 1912 was very simple (Wautier et al., 2014). Maillard products arise from a reaction between free amino groups of proteins, mostly lysine and arginine, and carbohydrates. The rest of the reaction was then described by Amadori and it became known as the Amadori Heyns rearrangement. Maillard products are therefore referred to as chemical structures which are not completely identified. The sugar protein complex was followed by a series of further rearrangements, dehydration, and condensation to form irreversible end products and Advanced Glycation End products (AGE) which may be fluorescent and yellow-brown in color; some of these products can form stable intermolecular and intramolecular cross-links (Ulrich and Cerami, 2001).

In vivo, the amount of AGE on a protein has been found to be dependent on the inherent reactivity of specific amino groups as determined by their microenvironment, the glucose concentration, and the half-life of the protein. Realization of the importance of Maillard-like reactions in vivo began in the mid-1970s with the study of hemoglobin A1c (HbA1c) (Cerami and Ulrich, 2001, Wautier and Guillausseau, 1998): a naturally occurring minor human hemoglobin that is elevated in diabetic patients. HbA1c is known to be a post-translational glucose adduct with the N-terminal valine amino group from the hemoglobin β-chain, in which the glucose was thought to be attached via a nonenzymatically formed Schiff base structure. In fact, the carbohydrate in HbA1c is attached as a 1-deoxy-1-fructosyl residue to the N-terminal valine nitrogen, which has been derived from an initially formed Schiff base via an Amadori rearrangement. HbA1c is used for assessing long-term metabolic control in diabetic patients and provides a measure of the average integrated blood glucose concentration for a 28-day period approximately.

Glycation adducts are formed by the reaction of proteins with glucose-reactive α-oxoaldehydes, such as glyoxal, methylglyoxal, and 3-deoxyglucosone, and other saccharide derivatives (Brownlee et al., 1988). The initial Schiff base adducts, which are formed from glucose and lysine and N-terminal amino-acid residues, rearrange to form fructosamine. Fructosamine degradation and the direct reaction of α-oxoaldehydes with proteins form many AGE products. (Figure 1.2).

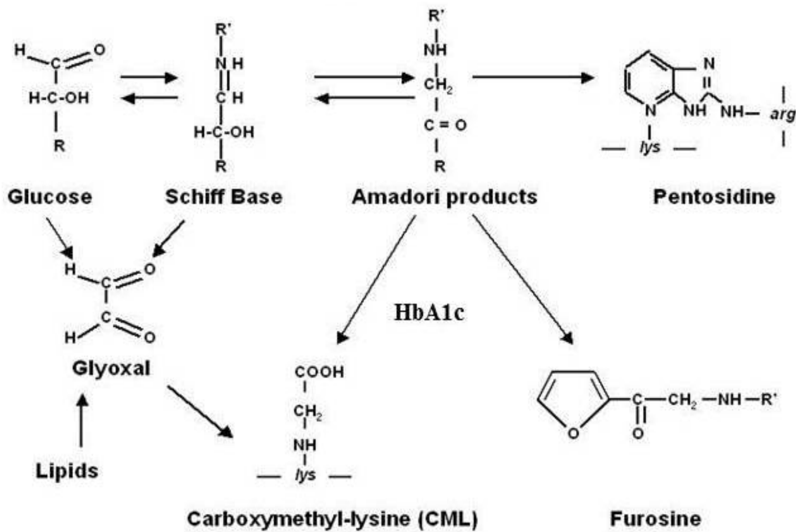

Figure 1.2: Advanced Glycation End products (AGE)
Different pathways generating AGE products: including the Maillard reaction, Amadori rearrangement, glycolysis, and oxidation.

Advanced Glycation End products

The first observation was the glycation of constitutive proteins, like collagen or elastin, that resulted in vascular or articular disorders. Glycation affects the interactions of collagen with cells and other matrix components, and causes the formation of glucose-mediated intermolecular cross-links. These cross-links decrease the critical flexibility and permeability of the tissues and reduce turnover (Paul and Bailey, 1996).

Today we know that AGE may result from the Maillard reaction or from glycolysis and oxidative pathways. All of these reactions have steps that involve glycation, oxidation, and intra and intermolecular rearrangement. The biochemistry of glycoxidation products, and some products that form from the degradation of glucose, such as glyoxal or methylglyoxal (MG), are also recognized to generate AGE products following their interaction with the appropriate substrates (Westwood and Thornalley, 1995, Shipanova et al., 1997). Such glycoxidation may occur in the extracellular compartment, but also takes place in a more rapid and extensive manner intracellularly (Brownlee, 2001). N (epsilon)-(carboxymethyl) lysine

(CML) (Ikeda et al., 1996), MG-derived AGE, and pentosidine (Sell and Monnier, 1989) are the best chemically characterized AGE compounds found in humans.

AGE products are spontaneously formed in nature and are dependent on heating and oxygen. We find them in food and they are absorbed by our intestines, thereby becoming transformed in the body and excreted by the kidneys (Koschinsky et al., 1997). AGE products are also endogenous and they can be partly degraded by glyoxalase and eliminated by the kidneys, but they accumulate with aging. About 10 to 30% of AGE products present in our diet are absorbed. The deleterious effects of AGE products on human health have been observed during aging, diabetes, and kidney disease, and these last two pathological states are often associated (Schleicher et al., 1997, Makita et al., 1991).

Oxidative reactions may be increased by oxidative stress arising from mitochondrial dysfunction and the activation of NADPH oxidase (Schmidt et al., 1994). Some AGE products are cross-linked, for example, bis (lysyl) imidazolium salts may denature proteins and confer resistance to proteolysis. When AGE are formed at critical sites in enzymes or proteins, they may be associated with enzyme inactivation (Wautier and Guillausseau, 2001).

Cross-linked AGE, GOLD [glyoxal-derived lysine dimer, 1, 3-di(*N*ε-lysino imidazolium salt]; MOLD [methylglyoxalderived lysine dimer, 1,3-di(*N*ε-lysino)-4-(methyl-imidazolium salt]; DOLD [3-deoxyglucosone-derived lysine dimer, 1, 3-di(*N*ε-lysino)-4 (2, 3, 4-trihydroxybutyl)imidazolium salt]; and pentosidine may alter protein structure and functions.

AGE receptors

The most recent discovery was that AGE may have several cellular receptors: AGER1 (oligosaccharyl transferase-48) and AGER2 (80K-H) are ubiquitous receptors involved in oligosaccharide transfer and Protein Kinase C (PKC) metabolism, respectively. AGER3, which is present on a monocyte macrophage, is considered to be a scavenger receptor. The AGER3 KO mice developed an accelerated nephropathy. When AGER3 was sequenced it was found to have homology with galectin-3. Galectin-3 reacts with various carbohydrates (Vlassara et al., 1995). Finally, AGER3 was found to be the same molecule as galectin-3 (Figure 1.3). The receptor for AGE (RAGE), isolated from the bovine lung, was first described as including two polypeptides, one lactoferin-like component (80 KDa), and

another polypeptide (35 KDa) (Schmidt et al., 1992). Subsequently RAGE was identified as a 45-50 KDa molecule (Wautier et al., 1996). This discovery of AGE receptors led to a trend in research which has allowed us to better understand how AGE may perturb normal physiology.

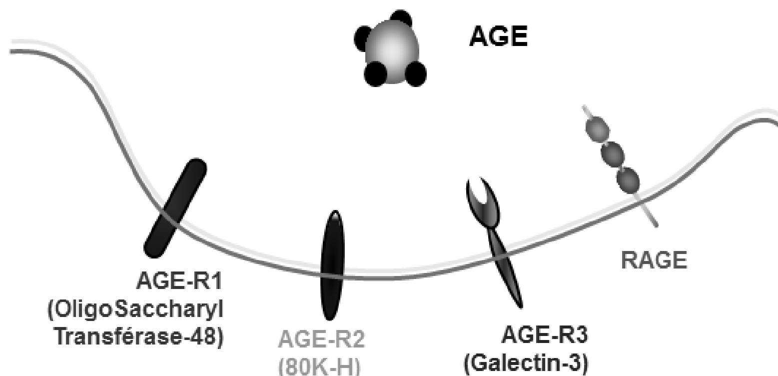

Figure 1.3: AGE receptors
Several membrane molecules able to bind AGE have been described: AGER1 (oligosaccharyl transferase-48), AGER2 (80K-H), AGER3 (galectin-3), and RAGE.

Receptor for AGE: RAGE

RAGE is the main cell-surface molecule implicated in the toxicity of AGE. The RAGE gene is present on locus 6p21.3, next to the CHM class III protein family. RAGE can bind a wide range of endogenous molecules, including AGE and the high mobility group box-1 (HMGB-1), which are also known as amphoterin-c, β-amyloid peptide, and S100 calgranulins. RAGE is a member of the immunoglobulin (Ig) superfamily that contains three Ig-like domains: one variable (V) and two constants (C1 and C2) in the extracellular part. It also consists of a single transmembrane domain, and one short cytosolic tail (Neeper et al., 1992) (Figure 1.4).

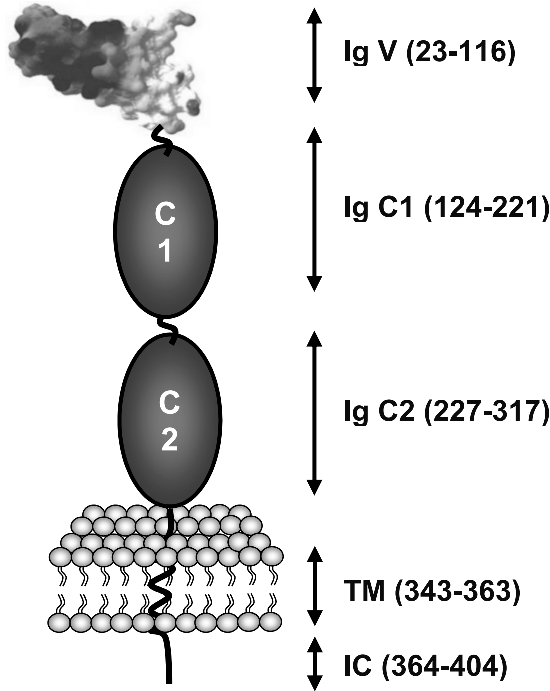

Figure 1.4: Structure of RAGE
Structure of a full-length RAGE including three Ig-like domains: the variable (Ig V) domain and two constant (Ig C1 and Ig C2) domains in the extracellular part. It also consists of a single transmembrane domain (TM) and one short cytosolic tail (IC).

First described as a single cell receptor, RAGE is now acknowledged to generate several isoforms produced through alternative splicing or post-translational modifications. A publication summarizing the work of many laboratories showed that extensive splicing of RAGE transcripts led to as many as 20 splice variants (Hudson et al., 2008). In endothelial cells, only three isoforms of RAGE were detected at significant levels: N-truncated (Nt-RAGE), full length (FL-RAGE, usually called RAGE), and endogenous secretory (esRAGE). Other than by splicing, soluble RAGE (sRAGE) can also be produced as a consequence of FL-RAGE proteolysis (Galichet et al., 2008) and may act as a decoy, thereby preventing RAGE engaging the ligands (Figure 1.5). With the exception of lung tissues, where constitutive expression of FL-RAGE is abundant, RAGE is

expressed at low levels in most other tissues, including normal brain tissue.

An unexpected finding was that methylglyoxal human serum albumin (MG-HSA) and N(epsilon)-(carboxymethyl)lysine human serum albumin (CML-HSA)—two major AGE present in vivo and binding to the same receptor—differentially regulated the expression of RAGE isoform transcripts. MG-HSA stimulated the expression of mRNA for all three isoforms of RAGE found in endothelial cells, whereas CML-HSA only stimulated transcripts for FL- and Nt-RAGE isoforms, without affecting esRAGE mRNA expression levels. In both cases, MG-HSA and CML-HSA stimulated RAGE expression by interacting with RAGE itself. However, MG-HSA enhanced esRAGE expression, thereby potentially implicating a negative feedback loop. This is because soluble RAGE thus generated may act as a decoy intercepting the interaction of ligands with cell surface RAGE, thereby limiting RAGE mediated cellular activation (Grossin et al., 2009). Factors involved in the regulation of RAGE isoform expression could be important in rendering a vascular bed more or less vulnerable to the effect of RAGE ligands. FL-RAGE and ligand interaction set up a positive mechanism that can accelerate disease progression. However, soluble forms of RAGE provide significant inhibition to these positive feedback mechanisms, since these forms of RAGE contain functional ligand binding domains but lack the cellular signaling domains (Wautier et al., 2017).

With the finding that RAGE may exist as three splice variants in humans, the evaluation of the role of the receptor in pathology has become more complex. The soluble isoform esRAGE, acting as a decoy of AGE, limits RAGE activation and the deleterious consequences. The number of RAGE splice variants was recently enlarged to up to more than twenty variants but the exact role of each variant remains unclear. These splice variants reveal a complex dual or synergistic interplay in RAGE physiology. The distribution of these splice variants may differ according to the organ considered and some of them are probably not synthesized. To date, the majority of these splice variants have not been detected as proteins. Soluble RAGE (sRAGE) found in human blood is the product of two constitutive mechanisms: alternative splicing (esRAGE) and proteolysis of RAGE. The cleavage of RAGE, upregulated by calcium, is achieved by several metalloproteinases, including ADAM10 and γ-secretase. It is still unclear whether the pathophysiological roles of esRAGE and sRAGE are distinct in different clinical situations and which organ or tissue produces sRAGE and esRAGE. Many works reported the higher incidence of

complications in patients with the lowest blood levels of sRAGE and/or esRAGE in the following conditions (either related or not to AGE): type 1 diabetes, type 2 diabetes, renal failure, atherosclerosis, metabolic syndrome, essential hypertension, aging, Alzheimer's disease, and vascular dementia. Serum levels of sRAGE are positively correlated with the inflammatory markers Tumor Necrosis Factor-α (TNF-α) and Monocyte Chemoattractant Protein-1 (MCP-1) in type 2 diabetic patients, making sRAGE a potential biomarker of inflammation (Grossin et al., 2010).

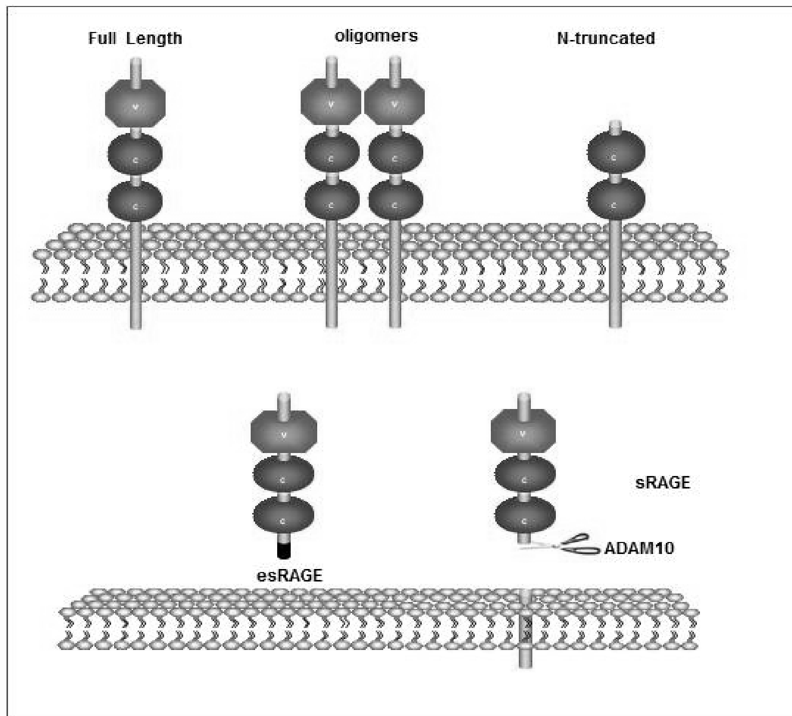

Figure 1.5: Isoforms of RAGE
An illustration of full length RAGE, oligomerized full length RAGE (oligomers), N-truncated RAGE, endogenous soluble RAGE (esRAGE), and soluble RAGE (sRAGE) resulting from FL-RAGE proteolysis by ADAM10.

RAGE polymorphisms

RAGE gene polymorphisms are currently being studied in human subjects for their potential role in the modulation of sRAGE levels. Among the polymorphisms of RAGE is the -429 T/C polymorphism in the gene promoter, where patients carrying C allele have significantly higher esRAGE levels (Park L 1998). However, this polymorphism was associated with a higher incidence of retinopathy in type 2 diabetic patients (Yan SD 1994). The Gly82Ser polymorphism inhibits the *N*-glycosylation in position 81 in the V domain of RAGE and results in the lower affinity of RAGE for AGE products and a lower sRAGE blood level. Those subjects with a S/S homozygosity have a higher cardiovascular risk and lower sRAGE levels, compared with those bearing only one G allele (G/S) (Yonekura H 2003). This observation was confirmed with esRAGE which has been found to be associated with increased levels of TNF-α and IL-6 in blood in Chinese patients with type 2 diabetes mellitus.

The binding of AGE to the receptor RAGE

The link between glycated proteins and vascular disease has been investigated for three decades (Brownlee et al., 1988). The first relationship was established in animal models, where infusion of glycated proteins induced nephropathy. Accumulation of AGE has been linked to cellular perturbation in diabetes (Peppa and Vlassara, 2005), renal failure, amyloidosis, and inflammation (Schmidt et al., 2001) .

The binding of glycated proteins, plasmatic or cellular, to the receptor RAGE was directly demonstrated in vitro. In vivo, in diabetic rats, the blockade of glycated proteins that were bound to RAGE prevented an increase in vascular permeability and oxidant stress. The infusion of recombinant soluble RAGE in hyperlipidemic diabetic rats protected them from the development of accelerated atherosclerosis (Wautier et al., 1996) (Figure 1.6).

Several studies have shown that specific AGE products—including N-epsilon (carboxymethyl)lysine (CML)-adducts of proteins, the most prevalent AGE found in vivo—interact with RAGE to activate signal transduction pathways, ultimately leading to the expression of proinflammatory genes. The interaction of RAGE ligands (AGE, β-amyloid peptide, S100 calgranulins) with RAGE initiates a cascade of signal transduction events involving, $p21^{ras}$, p44/p42 MAP kinases, and NF-kB. The activation of NADPH oxidase by AGE-RAGE interaction contributed, at least in part,

to the generation of Reactive Oxygen Species (ROS) (Wautier et al., 2001).

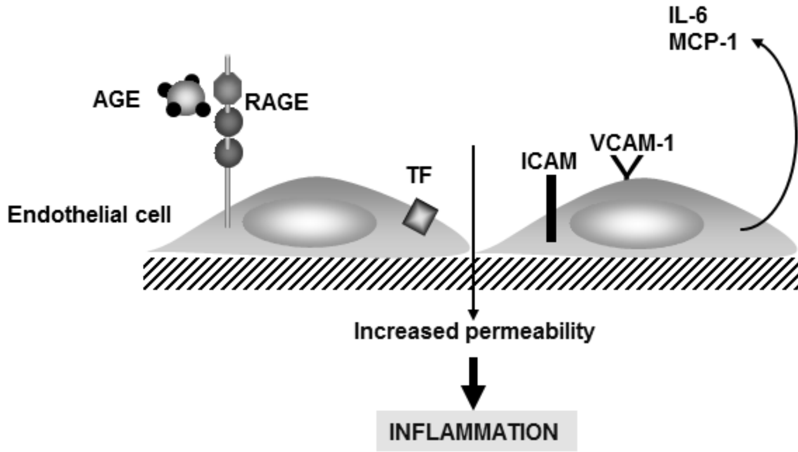

Figure 1.6: Endothelial dysfunction induced by Advanced Glycation End Products (AGE)
The engagement of endothelial RAGE induced Tissue Factor (TF) production; Intercellular Cell Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) expression; IL-6; Macrophage Chemoattractant protein-1 (MCP-1) release; and an increase in vascular permeability.

The transient transfection of a form of RAGE, which lacked the intracellular domain but possessed the extracellular and transmembrane components, into endothelial cells or murine BV2 macrophages preserved the ability to bind ligands; however, it imparted a "dominant negative" (DN) effect upon the cellular ligation of CML-adducts. Specifically, the CML-mediated activation of NF-kB by CML-ovalbumin was markedly suppressed in DN-RAGE-transfected cells versus mock-transfected cultured cells that carried the vector on their own. This supports the concept that RAGE is a signal transduction receptor for AGE products such as CML-adducts.

RAGE-mediated modulation of ERK—for instance, the activation of ERK, altering its subcellular localization/substrate specificity, and cross-talking with other intracellular signals—may result in the sustained activation of

cells via its downstream effectors, such as NF-kB. Thus, a key challenge raised by these observations was to identify the precise molecular mechanism(s) by which ROS were generated due to the cellular ligation of RAGE by AGE.

RAGE activation and cellular consequences

Retinal complications in diabetes typically have two components: macular edema, due to the leakage of macromolecules (such as lipoproteins) into the retinal layers, and progressive capillary closure related to micro thrombosis. Biochemical alterations such as oxidative stress, activation of protein kinase C, and the formation of Advanced Glycation End products have been detected as a response of the retina to hyperglycemia. Capillary closure leads to non-perfused hypoxemic retinal areas (ischemic retinopathy) which, in turn, induces the secretion of vascular endothelial growth factor (VEGF) and the development of new vessels (proliferative retinopathy) (Wautier and Guillausseau, 1998). The relationship between the extent of retinal ischemia in Proliferative Diabetic Retinopathy (PDR) and angle neovascularization was explored using panoramic fundus fluorescein angiography and 360-degree fluorescein Gonio angiography. The study revealed that retinal nonperfusion in the mid periphery, and capillary occlusion in the radial peripapillary capillaries, temporal raphe, and optic disk were risk factors for angle neovascularization (Hamanaka et al., 2001). VEGF, a potent vascular permeability and proangiogenic factor, has various isoforms, with VEGF165 or VEGF-A being the predominant form in humans. VEGF-A exerts its important action on vascular endothelial cells through two specific cell surface receptor tyrosine kinases: VEGF-receptor 1 (VEGF-R1 [Flt-1]) and VEGF receptor -2 (VEGFR-2 [Flk-1/KDR]). VEGFR-2 has been reported to transduce the major signals for angiogenesis (Haque et al., 2015).

The role of chronic hyperglycemia in the development of diabetic retinopathy has been established both in type 1 diabetes mellitus and in type 2 diabetes mellitus by the Diabetes Control and Complications Trial (Guillausseau et al., 1998). Glycated hemoglobin has been used for years to monitoring diabetic treatment. Moreover, a positive correlation has been established between the level of hyperglycemia as indicated by elevated glycated hemoglobin (HbA1c) and the prevalence and severity of retinal lesions (Chase et al., 1989) .

AGE, Reactive Oxygen Species, and gene regulation: a pivotal role for NADPH oxidase

A key consequence of the interaction of AGE with RAGE is the generation of Reactive Oxygen Intermediates (ROS). This can be either prepared in vitro (such as AGE- or CML-modified adducts of proteins) or those formed endogenously in vivo (such as AGE-$ß_2$Microglobulin [AGE-$ß_2$M]); it can also occur when AGE is formed on the surface of diabetic red blood cells, or immunoisolated from the serum of patients with diabetes or renal failure. In vitro and/or in vivo, AGE-RAGE interaction resulted in the generation of thiobarbituric acid reactive substances (TBARS), increased mRNA for heme oxygenase-1, enhanced nuclear translocation of NF-kB, increased endothelial expression of Vascular Cell Adhesion Molecule-1 (VCAM-1), and increased endothelial permeability (Schmidt et al., 1995, Wautier et al., 1994, Wautier et al., 1996, Yan et al., 1994).

In each of these cases, the effects of AGE were mediated by RAGE, as indicated by suppression in the presence of anti-RAGE IgG or by soluble (s)RAGE and the extracellular ligand-binding domain of the receptor. Generation of ROS and enhanced oxidant stress were potent factors initiating signal transduction and altered gene expression, as AGE-RAGE-mediated effects were inhibited in the presence of antioxidants such as N-acetyl-cysteine (NAC), probucol, or vitamin E. Consistent with this concept, in Vascular Smooth Muscle Cells the AGE-RAGE-mediated activation of ERK 1/2 kinases was enhanced in the presence of glutathione depletion.

Previously, Lander and colleagues (Lander et al., 1996) demonstrated that ROS generated in the cellular milieu directly activated $p21^{ras}$; in those experiments the cysteine at position 118 was a critical residue that was responsive to ROS. Consistent with the concept that AGE-RAGE-mediated generation of ROS was the key stimulus that led to the activation of signal transduction pathways, RAGE-bearing PC12 cells stably-transfected to express mutant $p21^{ras}$, in which cys118 was mutated to a serine, which displayed a complete suppression of activation of ERK 1/2 kinases upon exposure to AGE-albumin. In contrast, during AGE-mediated activation of ERK 1/2 kinases in PC12 cells overexpressing wild-type $p21^{ras}$ it remained intact (Lander et al., 1997). Therefore, a key challenge raised by these observations was to identify the precise molecular mechanism(s) by which ROS were generated due to the cellular ligation of RAGE by AGE.

To test the concept that NADPH oxidase was a central target of AGE-RAGE interaction in the cellular milieu by which ROS was generated

within the cell, we studied the effects of AGE on RAGE-bearing Human Vein Endothelial cells (HUVEC) and in vivo-derived murine macrophages (Wautier et al., 2001). Our previous studies demonstrated that red blood cells obtained from human diabetic subjects (DRBC) bore cell surface AGE and these AGE engaged cell surface RAGE on HUVEC to induce generation of a range of markers of oxidant stress, including thiobarbituric acid reactive substances (TBARS), as well as activation of NF-kB and induction of monolayer hyperpermeability. All of these effects were inhibited by the blockade of RAGE or antioxidants such as probucol. We dissected the specific molecular pathways underlying the AGE-mediated enhanced generation of ROS. First, we assessed the ability of in vivo-derived AGE on the surface of diabetic red blood cells to stimulate extracellular generation of H_2O_2 by HUVEC. Compared with nondiabetic red blood cells (NRBC), the incubation of HUVEC with DRBC resulted in a ≈3.4-fold increase in extracellular generation of H_2O_2. These effects were due to RAGE, as demonstrated by sRAGE's suppression of DRBC-mediated generation of H_2O_2. Antioxidants—including catalase, superoxide dismutase (SOD), probucol and N-acetylcysteine (NAC)—inhibited the DRBC-mediated generation of ROS in the extracellular milieu. In addition to the extracellular space, it was essential to examine the biochemical consequences of AGE-RAGE interaction in the intracellular environment, as our studies suggested that the generation of ROS initiated a cascade of intracellular signaling events. The binding of DRBC to HUVEC induced a ≈2.3-fold increase of intracellular formation of ROS compared with NRBC. These effects in DRBC were inhibited in the presence of sRAGE and by diphenyliodonium (DPI) with the latter strongly suggestive of a role for NADPH oxidase in the generation of intracellular ROS. However, the antioxidants catalase and SOD had no significant effect on the DRBC-mediated generation of intracellular H_2O_2, which is consistent with their inability to penetrate living cells. Furthermore, the addition of L-NMMA (Monomethylated L-arginine, inhibitor of nitric oxide synthase) did not affect the ability of DRBC to generate intracellular H_2O_2, which is consistent with the lack of direct involvement of nitric oxide (NO) in AGE-RAGE-mediated generation of intracellular ROS.

Our previous studies suggested that the incubation of HUVEC with AGE albumin, or AGE immunoisolated from the plasma of patients with diabetes, resulted in the increased transcription and translation of VCAM-1. To test the concept that the generation of ROS was a critical step in this process, we incubated HUVEC with DRBC. Compared with the incubation of an EC monolayer with NRBC, the exposure of the cells to DRBC resulted in a ≈1.8-fold increase in the cell surface expression of VCAM-1. That