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Review

The mechanism by which dietary AGEs are a risk to human health is *via* their interaction with RAGE: Arguing against the motion

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We are interested in the regulation of intracellular calcium and the various diseases associated with an altered regulation of this second messenger. More recently, we also became interested in pathologies involving the Ca2+-binding S100 proteins and AGEs and their association with the multifunctional Receptor for Advanced Glycation Endproducts (RAGE).

Keywords: AGEs / Biacore / Calcium / RAGE / S100 proteins

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This article focuses on contra arguments about "Dietary AGEs and ALEs interact with RAGE".
Introduction: http://dx.doi.org/10.1002/mnfr.200700017
Pro arguments: http://dx.doi.org/10.1002/mnfr.200700008

1 Introduction

For many years our research has focused on the regulation of intracellular calcium and the various diseases associated with altered regulation of this second messenger [1, 2]. More recently, we also became interested in pathologies involving the Ca^{2+}-binding S100 proteins and their association with the multifunctional receptor for advanced glycation end products (RAGE).

In this paper arguments against the opinion that dietary AGEs may be a risk to human health are discussed, with special focus on their interactions with RAGE. First, I would like to explain how these projects developed: after entering the cell, Ca^{2+} is reversibly complexed to specific Ca^{2+}-binding proteins that fulfill multiple functions, including Ca^{2+}-buffering and transport, activation of enzymes, regulation of contraction, secretion, and proliferation. A large family of Ca^{2+}-binding proteins is characterized by a common structural motif, the EF-hand [3, 4]. These proteins decipher the information carried by Ca^{2+} and pass it onto various targets regulating biological functions.

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Abbreviations: AGE, glycation end products**; RAGE,** Receptor for advanced glycation end products

The largest group within this EF-hand Ca^{2+}-binding protein family are the S100 proteins. Their name originally came from two proteins detected in the 100% ammonium sulfate supernatant of a bovine brain extract [5]. More than 20 members are now known, and they have attracted a broad interest in recent years because of their association with human diseases as well as their use in clinical diagnosis. S100 proteins are small (10 – 12 kDa) acidic proteins that contain two distinct EF-hands: a classical one (as in parvalbumin) and an additional S100-specific EF-hand with a Ca^{2+}-binding loop of 14 instead of 12 amino acids [6]. Recently, a uniform nomenclature was introduced in agreement with the European Calcium Society and approved by the The Human Genome Organization (HUGO) nomenclature committee with the recommendation to strictly adhere to this new nomenclature [7].

A unique feature of S100 proteins is that they are secreted from cells upon cell stimulation, exerting cytokine- and chemokine-like extracellular activities [8]. Some of their biological activities are mediated by specific receptors located on the cell surface, including RAGE [9, 10].

RAGE, a member of the Ig-like cell surface receptor superfamily, is expressed at low levels in many adult cell types (Brett *et al.* [11]). The up-regulation of RAGE has been observed in a large number of pathophysiological processes, such as immune/inflammatory disorders and abnormalities associated with diabetes [12]. RAGE can be activated by several classes of ligands (Fig. 1). The first

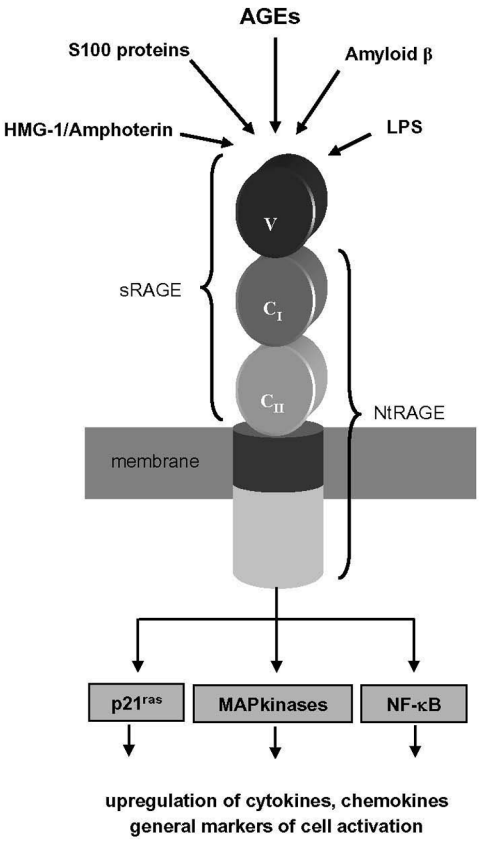

Figure 1. The RAGE signaling network. The RAGE ligands (AGEs, S100 proteins, Amyloid-β, LPS, and Amphoterin/HMGB1) bind to full-length RAGE or N-truncated RAGE (NtRAGE) and activate different signaling pathways, p21ras and mitogen-activated protein kinases (MAPK). Depending on the pathways recruited, RAGE–ligand signal leads to the activation of transcription factors like NF-κB or Sp1 and to cellular responses. In contrast, binding of the RAGE ligands to sRAGE abolishes the activation of RAGE localized on cell surfaces and protects cells against cellular activation [8, 12].

class of ligands is advanced glycation end products (AGE). AGEs are nonenzymatically glycated proteins found in pathologic conditions such as diabetes, renal failure, or cardiac dysfunctions. The second class of ligands includes amphoterin (HMGB-1), β-amyloid peptides, and several members of the S100 protein family.

Engagement of RAGE triggers the activation of various cellular pathways involving MAP kinases and the transcription factor NF-κB, resulting in an increased expression of inflammatory mediators [10]. Activation of RAGE enhances the expression of the receptor itself and initiates a positive feedback loop, resulting in sustained RAGE up-regulation, which has been suggested to be the starting point of chronic cellular activation and tissue damage. Several studies have succeeded in blocking the RAGE/ligand interactions using blocking antibodies or a soluble decoy receptor (sRAGE), resulting in the suppression of atherosclerosis and improved wound healing in diabetic animal models [12].

2 Ligand–RAGE interactions

The binding of AGEs and S100 proteins to the extracellular domain of RAGE (sRAGE) was studied by Biacore surface plasmon resonance [13–15]. Binding studies were performed using recombinant sRAGE fused at the C-terminus to the glutathione-S-transferase (GST) protein. Immobilization of the fusion protein using an antibody against GST resulted in unidirectional orientation on the sensor chip, mimicking the orientation of RAGE on the cell surface.

Overall, our investigations show that the S100–RAGE interactions are very complex. The few selected dimeric S100 proteins bind with different affinities to distinct RAGE domains and activate separate cellular pathways (Leclerc *et al.*, submitted).

When analyzing the 3-D structure of human S100B [15], we discovered different high molecular weight forms of this protein. The structural analysis revealed the octameric architecture of the human Ca^{2+}-loaded S100B with four homodimeric units arranged as two tetramers. The structural data, as well as the binding and the *in silico* docking studies with sRAGE imply that tetrameric S100B triggers RAGE activation by receptor dimerization [14, 16].

From these and other studies we can estimate the high complexity of S100–RAGE interactions:

(i) S100 proteins (probably as multimers) bind with different affinities to different binding sites on RAGE.
(ii) Specific S100 proteins activate different downstream pathways in a dose-dependent manner.
(iii) Glycosylation of RAGE may affect ligand binding but our preliminary studies with S100 proteins did not reveal any difference in binding to unglycosylated (expressed in *Escherichia coli*) or glycosylated (expressed in *Pichia pastoris*) RAGE [14].
(iv) There are different RAGE isoforms to be tested for interaction with ligands.
(v) RAGE ligands and AGEs also bind/activate a number of other receptors as recently shown for HMGB1 interactions with TLR4 [17].

A limited study was initiated to analyze the binding of selected AGEs to the sRAGE (expressed in *E. coli*, not glycosylated) using the Biacore technology.

We have generated modified β-lactoglobulins using lactose and different dicarbonyl compounds that resulted in proteins containing either simple modifications (Amadori products or CML) or complex high-molecular weight complexes (Buetler *et al.*, submitted). These preparations were then analyzed for RAGE binding by the Biacore method. Our results show that only the crosslinked, high-molecular AGE complexes were able to interact with GST–sRAGE. Once these complex AGEs were digested using proteolytic enzymes, the RAGE binding capacity was lost (publication in preparation). Very similar results were also obtained when different glycated HASs were tested in the same model. These results support the view that – as already

shown for S100B [14] – preferentially complex, cross-linked forms of modified AGEs are able to interact with sRAGE and that digested AGEs lose their ability to bind to RAGE.

3 Arguments against the motion

AGEs are generated in the late stages of the Maillard reaction in food and biological systems. A vast number of AGEs are formed by the reaction of reducing sugars or degradation products of carbohydrates and lipids with proteins [18]. High concentrations of AGEs in food have been reported, in association with RAGE, to be toxic and are considered risk factors for diabetes and renal disorders. For example, a potential toxic mechanism is *via* their interaction with and activation of RAGE.

However, there are a number of arguments that make it unlikely that dietary AGEs, by binding to RAGE, are a major health risk.

(i) The Maillard reaction products include low molecular compounds (such as free AGEs, dicarbonyls, aldehydes, ketones, and others) and high molecular weight modified proteins. The latter ones (according to our unpublished results) are candidates that could interact with RAGE, the low molecular compounds may also be toxic but most likely exert their effects independent of RAGE.

(ii) The protein-bound AGEs are digested in the gastrointestinal tract and only free and peptide-bound AGEs can be taken up by the body. These AGEs do not seem to be able to bind to RAGE, indicating that dietary AGEs may not be able to activate RAGE after ingestion and digestion.

Recent studies indicate that food intake results in an increase in serum AGEs and dicarbonyls within a short time [19, 20]. One could argue that these might represent secondary AGEs formed intracellularly as a consequence of postprandial inflammatory reactions because these AGEs have been shown to be ligands of RAGE and to effectively modulate RAGE-dependent gene expression and vascular dysfunction.

However, these two publications do not prove that AGE breakdown products are absorbed from the diet causing deleterious effects. In Stirban *et al.* [19] a test meal was fried for 20 min. One can assume that such a harsh treatment would destroy all natural antioxidants and vitamins and in addition would generate significant amounts of lipid peroxidation products. As a consequence, the observed effects may not be attributed solely to AGEs generated simultaneously.

Schiekofer *et al.* [20] fed casein glycated by sorbitol or glucose to volunteers. The use of the different sugars resulted in significantly higher CML and pentosidine contents in the glucose-treated casein. However, the observed effects on NF-κB activation were not different between the two AGE diets and the authors therefore concluded that the observed postprandial NF-κB activation was independent of the AGE content. In fact, in diabetic patients, the same authors describe that NF-κB activation is linked to glucose control. Thus, the observed postprandial NF-κB activation may be due to the postprandial hyperglycaemia rather than AGEs or inflammation.

It cannot be excluded, however, that dicarbonyls present in food could be absorbed and lead to *in vivo* glycation, although this has not been experimentally tested so far.

(iii) RAGE expression in bovine tissues are ranked as follows: skeletal muscle > lung > heart > liver > kidney > as determined by Western blot analysis [11]. Lohwasser *et al.* [21] also described high RAGE expression in skin.

When considering RAGE expression and AGE accumulation in tissues, it is interesting to note that the tissues with the highest RAGE expression (skin, skeletal muscle, lung) are rarely mentioned in connection with diseases. If AGEs were actually responsible for diseases, we would all have severe muscle, lung, and skin problems, especially with ageing when tissue AGEs accumulate.

(iv) In addition to RAGE, AGEs also bind to a number of other proteins/receptors. These include scavenger receptors A and B, CD36, AGE receptors I, II, and III, oxidized LDL receptor 1, and integrins (Mac-1) [22]. Our preliminary investigation of mRNA expression levels of genes reported to be AGE receptors in several cell lines (including a lung epithelial cell line), showed that RAGE expression contributed to less than 1% of mRNAs encoding other AGE receptor (unpublished data). While mRNA expression cannot directly be translated into protein expression this nevertheless suggests that, among the AGE-binding receptors, RAGE is probably a minor player.

(v) AGE modifications in endogenous proteins represent a small fraction of proteins. The concentration of the modified proteins may not be high enough to lead to substantial binding to RAGE. Also, so far, only highly glycated proteins have been shown to interact with and activate RAGE. However, the endogenously formed AGEs contain only very few modifications *per* protein unit and it is not known whether these minor modifications are able to trigger RAGE activation. Our preliminary data suggest that minor protein modifications are insufficient to induce RAGE binding.

(vi) RAGE has a truncated secretory isoform termed esRAGE (endogenous secretory RAGE, a native RAGE splice variant found in the circulation). This variant contains a 16aa secretory tail added to the extracellular RAGE (sRAGE) domain. This RAGE variant is thought to partially reduce the AGE-mediated damage by acting as a decoy receptor [23].

(vii) A number of other ligands bind to RAGE (Fig. 1). AGEs would have to compete/replace those ligands to be able to chronically activate RAGE. The preliminary Biacore experiments indicate that the AGEs tested have binding affinities that are comparable to some S100 proteins tested. This implies that the local concentrations at the receptor site determine which ligand will bind to and acti-

vate RAGE. However, under inflammatory conditions it is likely that the concentration of S100 proteins, released from activated cells, is considerably higher than the circulating AGE concentration. Some publications also show that under controlled conditions, AGEs will not activate RAGE even though they bind to the receptor [24, 25]. This provides the possibility that AGEs could be antagonists of RAGE activation by the endogenous ligands. This could explain the finding that hemodialysis patients with high circulating AGE levels have a lower mortality rate than those with low circulating AGE levels [26].

(viii) Most AGEs cannot cross the BB barrier and will, therefore, not be able to active RAGE expressed in the brain.

These arguments make it very unlikely that RAGE is a major target for the vast majority of dietary AGEs.

To further prove or disapprove this view, the following points need to be addressed:

(i) Applying the Biacore technology, the binding/affinity of a large number of AGEs to sRAGE and esRAGE should be tested as well as their competition with the natural ligands.

(ii) The binding of AGEs and the AGE – RAGE signalling pathways need to be further investigated in RAGE expressing cells.

(iii) Wild-type and RAGE$^{-/-}$ mice should be fed with dietary AGEs and the effect of these compounds on inflammation, oxidative stress, renal damage, *etc.* should be analyzed to discriminate between RAGE-mediated and RAGE-independent effects.

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4 References

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