Chapter 9

Impact of Advanced Glycation End Products on Endothelial Function and Their Potential Link to Atherosclerosis

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Additional information is available at the end of the chapter

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Abstract

The role of advanced glycation end products (AGEs) in cardiovascular diseases is a matter of interest in the last years and the strong association between the action of AGEs on their receptor (RAGE) and atherosclerosis has attracted increased attention. The aim of this chapter is to review the results of our laboratory and others on the molecular mechanisms triggered by AGEs in the endothelium that could participate in the atherosclerotic process. These mechanisms and molecular pathways could be the source of new therapeutic targets against atherosclerosis or vascular disease. Oxidative stress in endothelium induced by AGEs triggers molecular signaling pathways that produce an inflammatory response or even endothelial dysfunction. Adhesion molecules expression at the membranes of endothelial cells as a consequence of this response or induced by other mechanisms involving AGEs mediates the adhesion of leukocytes to endothelium. This adhesion is a key step in the atherogenesis process and the possible involvement of AGE-RAGE axis in this process should be considered as a potential therapeutic target. Finally, potential pharmacological modulation of AGE-RAGE axis activity at the endothelium is suggested, but the specific pharmacological tools available nowadays are missing; respectively, drugs used for the treatment of cardiovascular and metabolic diseases could be helpful for AGE-RAGE axis modulation, thus also affecting endothelial (dys)function.

Keywords: advanced glycation end products, atherosclerosis, endothelial dysfunction, oxidative stress, receptor for advanced glycation end products, vascular adhesion molecules, vascular inflammation

1. Introduction

Cardiovascular diseases resulting from atherosclerosis have become the most important cause of mortality and morbidity in the general population [1]. Although atherosclerosis develops as a consequence of multiple risk factors such as hypertension, dyslipidemia, diabetes, aging and smoking, the common pathway for its development is endothelial dysfunction and vascular inflammation [2]. In the last two decades, the role of advanced glycation end products (AGEs) in the development of endothelial dysfunction has gained increasing interest [3–5], initially as a possible molecular mechanism of diabetic cardiovascular complications [3], and, in the last years, as an independent risk factor of vascular injury [6].

AGEs are products of non-enzymatic molecular modifications of proteins and lipids that affect the structure and function of the target molecule. They are produced endogenously by spontaneous reactions, but pathophysiological conditions may accelerate their formation and they also contribute to disease by different mechanisms.

AGEs comprise a heterogeneous group: the most studied are pentosidine and Nε-carboxymethyllysine (CML) and quantitatively, the most important in the tissues are the hydroimidazolones like CML [7]. AGEs are formed by a combination of glycation, oxidation, and/or carbonylation reactions both in the extra- and in the intracellular space. Other processes involving lipid peroxidations in the cell membranes lead to the formation of advanced lipid end products, as for example, malondialdehyde [8]. The classical mechanism of AGE formation is the slow Maillard reaction between glucose or reducing sugars and proteins [9]. The interaction between the carbonyl groups of reducing sugars and amino groups of proteins results in the formation of a Schiff base within a few hours. Intramolecular rearrangement of the Schiff base results in more stable Amadori products [9]. An example of these types of products is glycated hemoglobin or glycated albumin, the former is widely used in clinical practice for diagnosis and follow-up of diabetes mellitus and the last could be regarded as a smart alternative to modified hemoglobin for the same purposes, with less dependence on hematological diseases and intracellular conditions. Finally, the process of oxidation of the Amadori products leads to reactive carbonyl compounds and subsequently to the formation of AGEs within weeks to months. AGEs can also be formed intracellularly. Glucose is altered into reactive carbonyl compounds during glycolysis pathway, of which the best-known is methylglyoxal. The chemical reaction between these carbonyl compounds and proteins can result in AGEs [10].

Absorption of exogenous AGEs also contributes to their accumulation in tissues. Tobacco smoke contains highly reactive glycation products which rapidly form AGEs *in vitro* and *in vivo* and therefore, increase the serum AGEs levels in smokers compared to non-smokers [11]. The content of AGEs in food depends on the temperature at which food products are prepared, with oven frying being the most severe inducer [12]. Approximately 10% of the ingested AGEs are absorbed from the gastrointestinal tract into the blood [13]. The final level of AGEs accumulation depends on their clearance and the metabolic mechanisms by the kidney and liver, respectively. Increased level of AGEs can be found in patients with either renal [13] or liver failure [14].

The role of AGEs in cardiovascular diseases is a matter of interest in the last years [15], and the strong association between the axis of action of AGEs and their receptor (RAGE) and atherosclerosis or cardiovascular ischemic disease [3, 16, 17] has attracted increased attention.

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The aim of this chapter is to review the results of our laboratory and others on the molecular mechanisms triggered by AGEs in the endothelium that could participate in the atherosclerotic process. These mechanisms and molecular pathways could help in the development of new therapeutic targets against atherosclerosis or vascular disease.

2. Molecular mechanisms triggered by AGEs in the endothelium

It is generally accepted that AGEs target cells by three main mechanisms. First, proteins modified by AGEs have altered biological function, either enzymatic activity, binding properties or structural conformation. Second, extracellular matrix components modified by AGEs interact abnormally with other matrix components and with matrix receptors, such as integrins. This includes also the formation of new links or the alteration of those previously existing, between proteins, which may alter the physical properties of extracellular matrix and cell environment. Third, plasma proteins modified by AGEs bind to cell surface receptors, of which the receptor for AGEs (RAGE) is acknowledged to be the most important, activating intracellular signaling pathways and various cellular responses.

Binding of AGEs to RAGE is responsible for the generation of reactive oxygen species (ROS) and the activation of transcription factors such as nuclear factor-κB (NF-κB), with subsequent changes in the expression of many genes involved in vascular inflammation and endothelial dysfunction [18–20]. Besides from the involvement of AGEs-RAGE axis, the precursors of AGEs, like Amadori products or early glycated products also have a role in the global response of non-enzymatic glycation of proteins, so we will also discuss their effects on endothelial cells.

2.1. AGEs-induced ROS production in the endothelium

One of the first and best studied actions of AGEs on endothelial cells is the induction of ROS. The suggested mechanisms for this action are several and range from the activation of ROS-producing enzymes to the reduction of ROS-neutralizing enzymes. In the first group of enzymes or enzyme complexes are nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [21] and mitochondria [22], whereas in the second, there are endothelial nitric oxide (NO) synthase (eNOS) [23], superoxide dismutase (SOD) and glutathione peroxidase [24, 25]. The molecular mechanisms of these actions have been related to the activation of NF-κB via RAGE [26, 27].

ROS production in endothelial cells has important consequences on endothelial activation. In brain microvascular endothelial cells, AGEs-induced ROS production enhances vascular endothelial growth factor (VEGF) expression, which mediates an increase in cell permeability [28], and platelet tissue factor up-regulation [29]. Other mechanisms of AGEs on endothelial cells promoting endothelial activation or dysfunction are the generation of asymmetric dimethylarginine (ADMA, a metabolic by-product of natural protein modification processes in the cytoplasm of cells, that acts as a competitive inhibitor of NOS) [30], or impaired calcium signaling [31].

It is important to note that the effects of AGEs' precursors (i.e. Amadori products or glycated proteins) on endothelial cells, differ from the effects of AGEs themselves. Several works have focused on this issue (see, for a review, [32]). Amadori products modify eNOS activity and gene expression, promoting apoptosis of endothelial cells [33, 34]. A recent study

performed by our group has highlighted the important molecular and functional differences between early glycated human serum albumin (gHSA) and advanced glycated albumin (AGE-HSA), obtained commercially or by glucose incubation during 4 weeks at 37°C in aseptic conditions, respectively [35]. The respective control molecules of these treatments were unmodified commercial HSA and HSA incubated for the same time than AGE-HSA, but without glucose (Ct-HSA). Molecular characterization of the early and advanced glycation products formed on each modified albumin (gHSA and AGE-HSA) were studied by matrix assisted laser desorption/ionization—time of flight (MALDI-TOF)-mass spectrometry. Once characterized, the effects on ROS production of human umbilical vein endothelial cells (HUVECs) under the stimuli of gHSA or AGE-HSA were compared [35]. Low concentrations of gHSA enhanced long-lasting ROS production in HUVECs, whereas AGE-HSA induced extracellular ROS production after short time of incubation and at lower concentrations than gHSA. Extracellular ROS production of HUVEC was measured by the cytochrome C reduction method, whereas intracellular ROS production of HUVEC was measured by 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (cDCF-DA; Sigma-Aldrich), an intracellular dye for that purpose [36].

Treatment of HUVECs with gHSA (25–100 μg/mL) for different times (4–12 h) induced significant increments of extracellular ROS production with respect to treatment with the same concentration of un-modified albumin (HSA, used as control) [36]. The maximal response (i.e. the quantity of ROS) was obtained with 25 μg/mL gHSA after 4 h of treatment (**Figure 1a**). The effects of AGE-HSA were studied under the same conditions. AGE-HSA increased the extracellular ROS production at lower concentrations (12 μg/mL) and after shorter time of exposure than gHSA (2 h). Another important difference is that, at long incubation periods, the ROS-inducing effects of gHSA were maintained, whereas no significant increases on ROS production were observed with AGE-HSA at 4–8 h (**Figure 1b**).

Similar experiments were designed to measure the intracellular ROS production by using cDCF-DA after 4 h of treatment the HUVECs with gHSA or AGE-HSA (12–50 μg/mL).

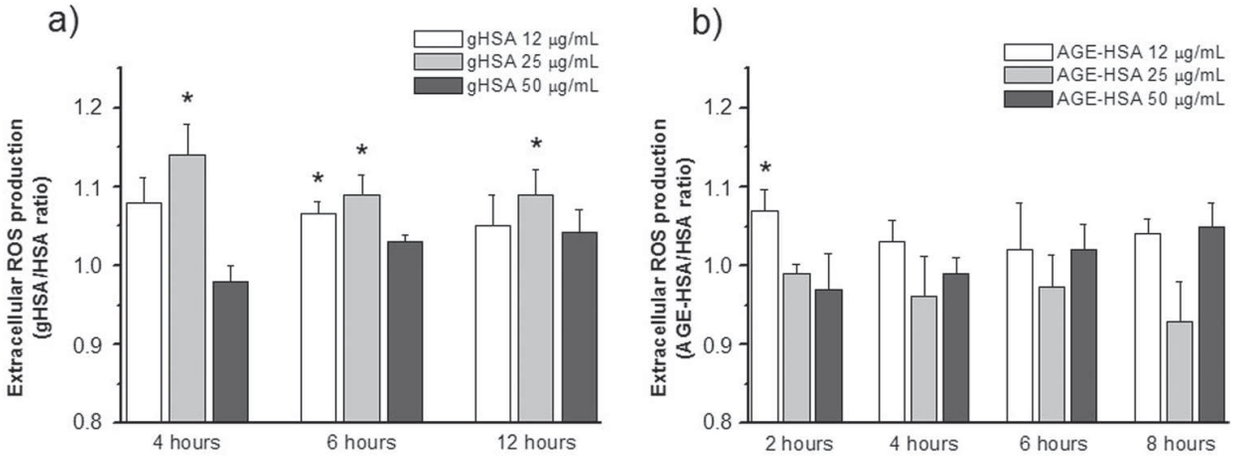

Figure 1. Extracellular ROS production in HUVECs after treatment with different concentrations of (a) gHSA (12–50 mg/mL) or (b) AGE-HSA (12–50 mg/mL), for periods of time indicated on each graph. Columns represent the ratio of ROS produced by treatment/HSA and are expressed as mean ± S.E.M. (vertical bars) of at least three independent experiments. *$p < 0.05$ with respect to the control values (Student's t test).

Interestingly, at 25 μg/mL, gHSA significantly enhanced the intracellular ROS production, whereas AGE-HSA only showed a trend to slightly increase it (**Figure 2**).

Therefore, differences in the induction of ROS production were observed between gHSA (a low glycated product) and AGE-HSA (a high glycated product). Although the effects of AGE-HSA are accepted to be mediated by RAGE, the receptor that mediates the effects of gHSA has not been revealed yet [37], since, the effects of gHSA are not mediated by RAGE [38].

2.2. Expression of adhesion molecules mediating leukocyte adhesion to endothelium

RAGE-ligands interaction induces a series of signal transduction cascades and lead to the activation of transcription factor NF-κB as well as increased expression of cytokines, chemokines, and adhesion molecules [39]. Expression of inducible adhesion molecules is a final common pathway in the development of vascular inflammation and pathology, rendering the vasculature a selective target for circulating peripheral blood cells [27, 40].

A number of studies have demonstrated induction of vascular cell adhesion molecule-1 (VCAM-1) expression in a RAGE-dependent manner when endothelial cells are exposed to AGEs [18]. Moreover, engagement of RAGE by AGEs results in enhanced expression of other adhesion molecules, such as E-selectin and intercellular cell adhesion molecule-1 (ICAM-1) [40–42]. High expression of adhesion molecules in endothelial cells may promote adhesive interactions of circulating monocytes with the endothelial surface, resulting, eventually, in transendothelial migration [43].

We confirmed that AGE-HSA up-regulated ICAM-1 and VCAM-1 expression more than gHSA, in terms of mRNA quantitative changes, measured by total messenger RNA retrotranscription and quantitative real-time polymerase chain reaction (qPCR) [35]. Even while

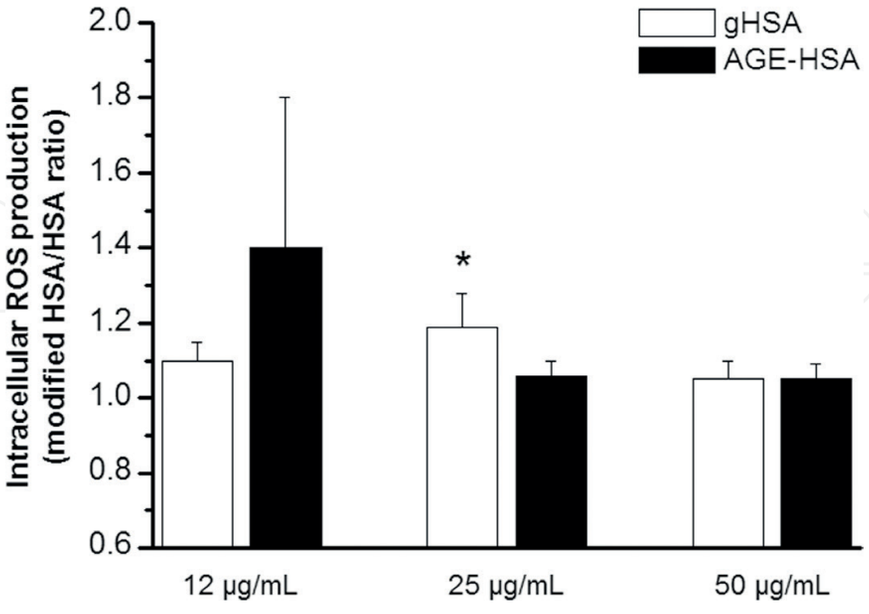

Figure 2. Intracellular ROS production in HUVECs after 4 h of treatment with different concentrations of gHSA (white columns) and AGE-HSA (black columns), as indicated in the x-axis. Results are shown as the ratio modified HSA/control HSA, expressed as mean, in columns, ± S.E.M. (vertical bars) of at least four independent experiments. Comparisons were made between each ratio level and the unit (*$p < 0.05$; Student's t test).

the effects of gHSA seemed to be limited to 4 h- treatment, AGE-HSA up-regulated VCAM-1 and ICAM-1 expression for longer periods of time (from 2 to 6 h). Differences on the active concentrations of both glycation products were also observed: whereas gHSA was only active at 25 μg/mL, AGE-HSA was also effective at 12 and 100 μg/mL (**Figure 3**).

To further confirm the increase in the expression of these adhesion molecules, protein levels of VCAM-1 and ICAM-1 were analyzed by western blot analysis after the treatment of HUVECs with two relevant concentrations of gHSA and AGE-HSA: 25 and 100 μg/mL, in comparison with the same concentrations of unmodified HSA and Ct-HSA, respectively. There was a significant elevation of VCAM-1 and ICAM-1 levels caused by the effect of both AGE-HSA concentrations tested. On the other hand, only the concentration of 25 μg/mL gHSA (but not 100 μg/mL) enhanced the ICAM-1 protein levels (**Figure 4**).

The functional translation of VCAM-1 and ICAM-1 up-regulation was analyzed by the adhesion of peripheral blood mononuclear cells (PBMCs) to HUVEC monolayers after treatment with both types of modified albumins for 4 h (**Figure 5**). After these treatments, the adhesion of calcein-AM-stained PBMCs to HUVEC monolayers after 1 h of incubation and washing of non-adhered

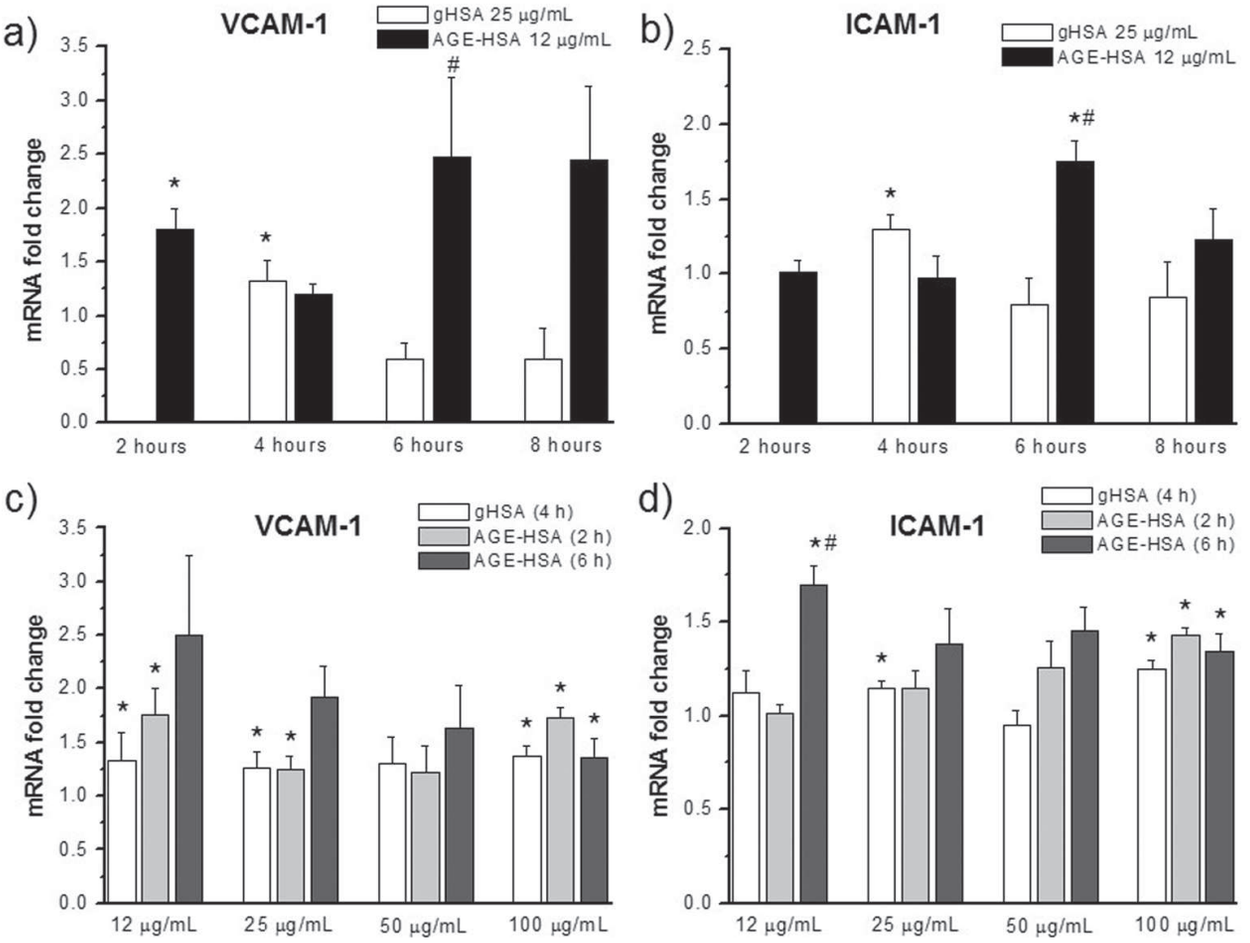

Figure 3. The expression levels of mRNA of VCAM-1 (a and c) and ICAM-1 (b and d) after treatment with gHSA or AGE-HSA at the concentrations and times indicated on HUVEC cultures. Results are shown as the ratio treatment/respective control, expressed as mean (columns) ± S.E.M. (vertical bars) of at least four independent experiments. Comparisons were made between each ratio level and the unit (*$p < 0.05$; Student's t test) and between AGE-HSA and gHSA treated experiments ate the same time of incubation (a and b) or concentration (c and d; #$p < 0.05$; Student's t test).

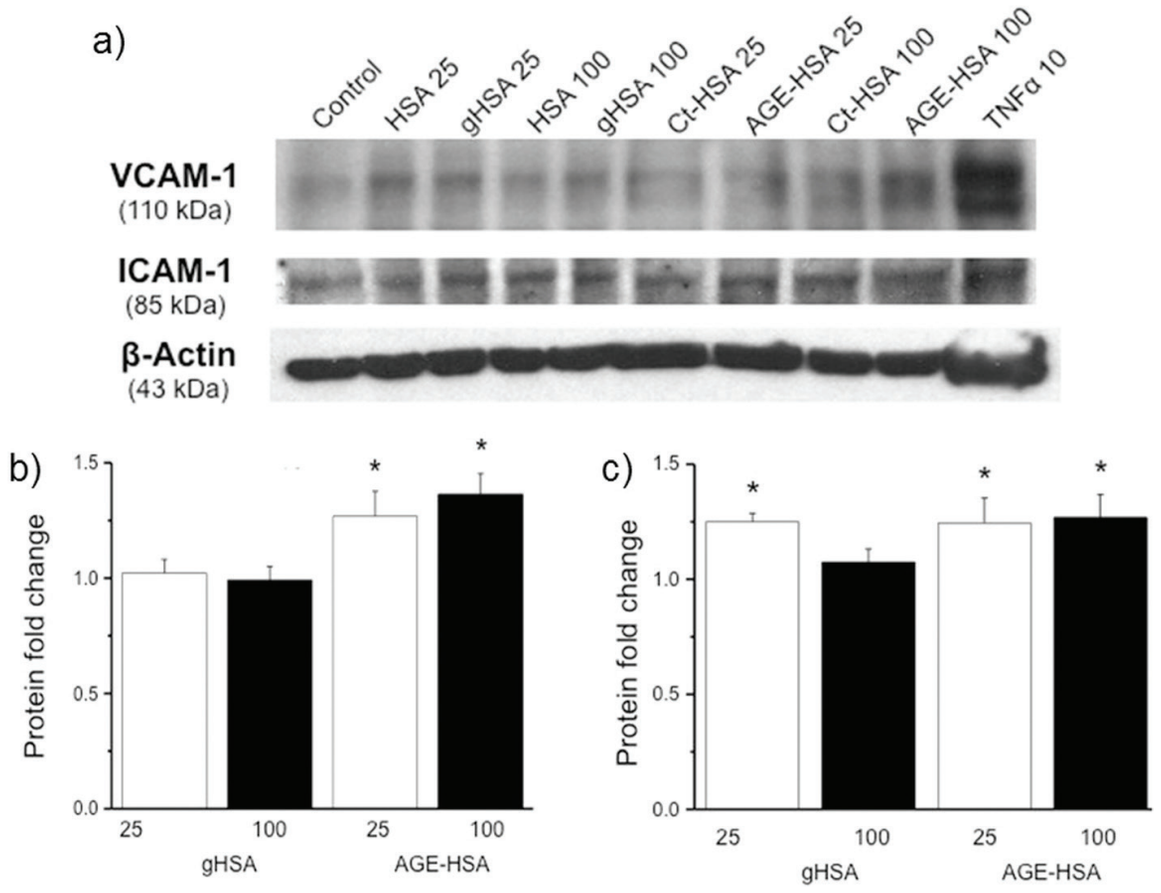

Figure 4. The expression levels of VCAM-1 and ICAM-1 measured by western blot in HUVEC protein extracts obtained after 4 h of treatment with gHSA or AGE-HSA (25 or 100 μg/mL, as indicated). TNF-α (10 ng/mL) was used as a positive inducer control. (a) Representative blots for VCAM-1, ICAM-1 and β-actin. Columns represent the fold change of protein expression for (b) VCAM-1 and (c) ICAM-1 calculated by optical densitometry with respect to β-actin and expressed as mean values (columns) ± S.E.M. (vertical bars) of at least three independent experiments. *$p < 0.05$ with respect to unmodified HSA or Ct-HSA for gHSA and AGE-HSA, respectively (Student's *t* test).

PBMCs was quantified by fluorescence. In these conditions, gHSA (25 μg/mL) induced no significant effect in PBMCs adhesion in comparison with the control HSA. However, AGE-HSA (25 μg/mL) induced a significant increase in the adhesion of PBMC to HUVEC monolayers.

The effects of gHSA and AGE-HSA on PBMCs transmigration through HUVEC monolayers were studied in comparison to the ICAM-1 and VCAM-1 changes of expression. For these experiments HUVEC with transfected green fluorescent protein were grown until confluence onto transwells with 5 μm of pore size (Millipore). After treatment with AGE-HSA (25 and 100 μg/mL) for 4 h PBMCs were layered over the HUVECs and incubated at 37°C. TNF-α (10 ng/mL) was used as a positive control because it induces endothelial cell activation and promotes PBMCs transmigration through the endothelial monolayer. The number of transmigrated PBMCs were estimated by quantification of nuclei acids content with CyQUANT® GR dye (Molecular probes, Invitrogen) at the end of the experiment. Unless for the case of TNF-α, no changes were observed for any of the stimuli after 3 h of treatment. However, after 24 h of HUVEC incubation with 25 μg/mL AGE-HSA, a significant increase in the migration of PBMCs was observed as compared to control (**Figure 6**). On the contrary, higher concentration of AGE-HSA (100 μg/mL), showed no effect in the transmigration of PBMCs. The positive control with TNF-α increased the migration of PBMCs even more than after 3 h (**Figure 6**).

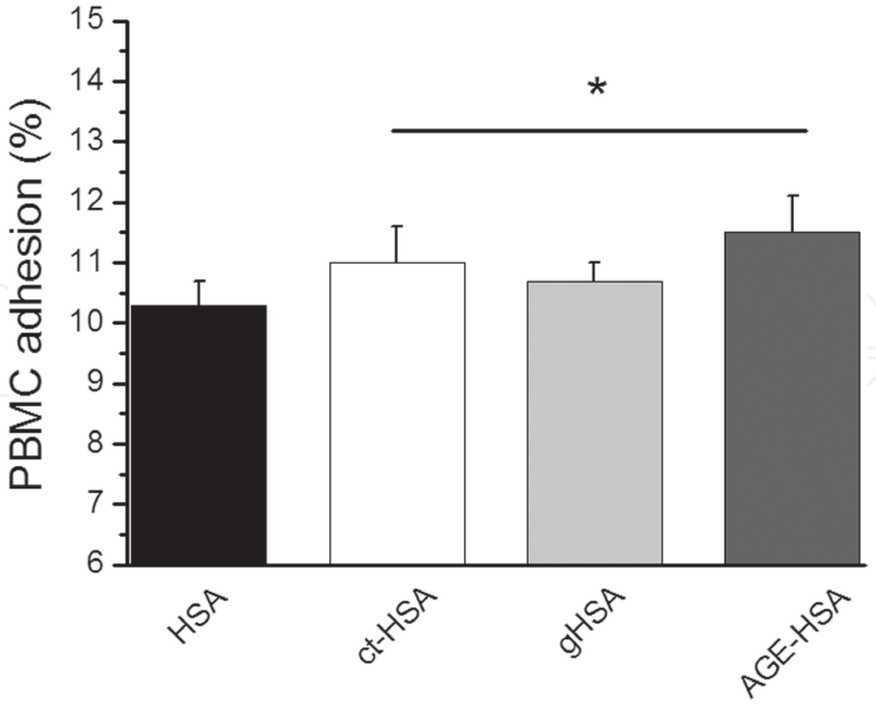

Figure 5. Quantification of the adhesion of PBMCs to a HUVEC monolayer, after treatment of HUVECs during 4 h with gHSA (25 μg/mL) or AGE-HSA (25 μg/mL), compared with HSA (25 μg/mL) or ct-HSA (25 μg/mL), respectively. The graph represents the mean percentage of adhesion (columns) ± S.E.M. (vertical bars) of at least three independent experiments. *$p < 0.05$ between the columns indicated (ANOVA followed by Tukey's test).

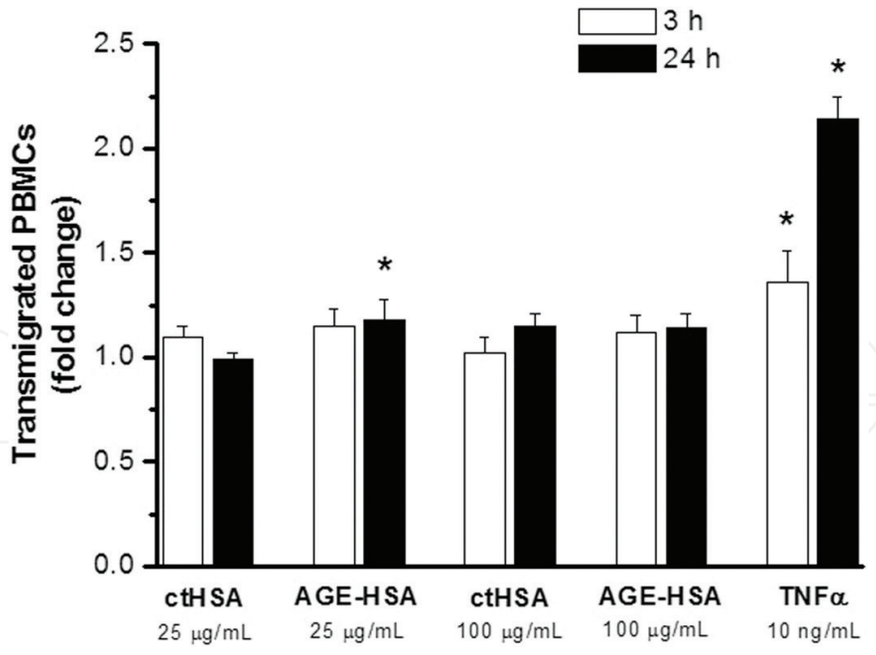

Figure 6. Transmigration of PBMCs through HUVEC monolayers after 3 h (white columns) or 24 h (black columns). Columns represent the mean (columns) ± S.E.M. (in vertical bars) of the increase of PBMCs transmigration after treatment compared to untreated control. *$p < 0.05$ with respect to untreated control (Student's t test).

Given the results obtained in the adhesion molecules expression in HUVECs, another approach was performed repeating the study with *in vivo* glycated albumin obtained from healthy volunteers and from cardiovascular patients, which donated their blood after signing informed consent.

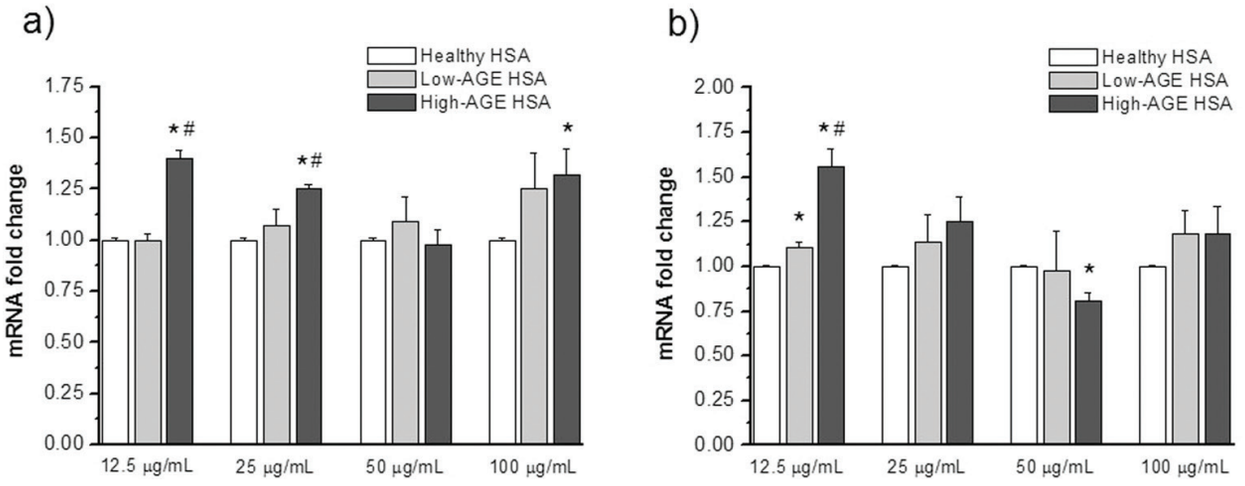

Figure 7. The expression of mRNA for ICAM-1 (a) and VCAM-1 (b) after the treatment of HUVECs with HSA isolated from healthy volunteers (healthy HSA) or with low-AGE HSA and high-AGE HSA from cardiovascular patients for 4 h. Columns represent the fold increase of mRNA expression for each gene and are expressed as mean values (columns) ± S.E.M. (vertical bars) of at least three independent experiments. *$p < 0.05$ with respect to healthy HSA. #$p < 0.05$ with respect to low-AGE HSA (Student's *t* test).

The whole study and protocols were approved by the Ethics Committee for Human Studies at Galicia (Spanish region) in accordance to the 1975 Declaration of Helsinki. Particularly, we analyzed the effect of HSAs categorized in healthy or nonglycated (from healthy volunteers), low-AGE or high-AGE (from cardiovascular patients), according to their content in AGE adducts. Glycation level was estimated by the molecular weight increment of isolated HSAs, due to the incorporation of different glycation products to the molecule. This was measured by mass spectrometry with a 4800 MALDI-TOF/TOF analyzer (Applied Biosystems). On this basis, attending to the increase of HSA molecular weight with respect to non-modified HSA, three stocks of HSA were prepared and categorized as healthy-nonglycated HSA, low-, and high-AGE HSA (molecular weights of 66,481, 66,665 and 66,778 Da for healthy, low-, and high-AGE HSA, respectively). HUVECs were incubated with a range of concentrations of these types of HSAs (12–200 μg/mL) for 4 h. In these conditions, the treatment with high-AGE HSA significantly increased the mRNA expression of ICAM-1 at concentrations of 12.5 and 25 μg/mL with respect to healthy-nonglycated HSA (**Figure 7a**; $p < 0.001$). An increase was also observed at 100 μg/mL concentration with respect to healthy HSA ($p = 0.046$). Moreover, high-AGE HSA was able to induce a significant increase with respect to low-AGE HSA at 12.5 and 25 μg/mL (**Figure 7a**; $p < 0.05$).

In the case of VCAM-1 expression, high-AGE HSA only induced an increase in the mRNA expression at 12.5 μg/mL with respect to healthy HSA and low-AGE HSA (**Figure 7b**; $p < 0.05$). At this concentration, low-AGE HSA also induced an increase in the expression of VCAM-1 with respect to healthy HSA (**Figure 7b**; $p < 0.05$). Finally, at a concentration of 50 μg/mL, high-AGE HAS induced a reduction in the expression of VCAM-1 with respect to healthy HSA ($p < 0.05$). This reduction in the expression of VCAM-1 was only transient as the mRNA levels recovered again at higher concentrations. Altogether, these results suggest that *in vivo* glycation of albumin could have a pro-inflammatory effect in endothelial cells, which would trigger chronic endothelial dysfunction.

PBMCs adhesion to HUVECs was also studied with *in vivo* glycated albumins at 12.5, 25 and 100 μg/mL. HUVECs were treated with these concentrations for 24 h. After that, HUVECs

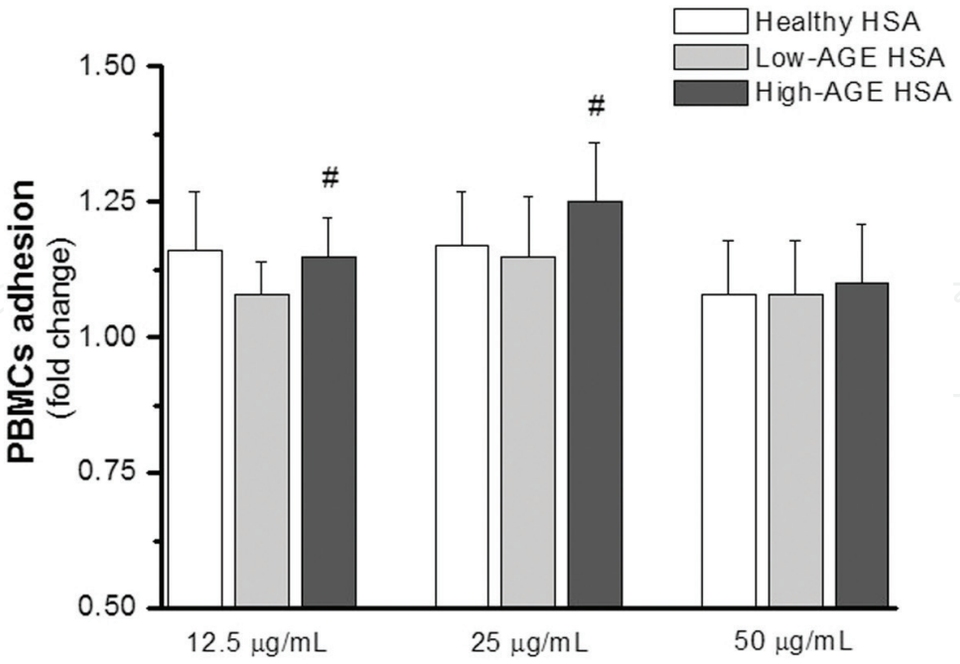

Figure 8. PBMCs adhesion to HUVEC monolayers treated with albumin from healthy volunteers (healthy HSA) or low-AGE HSA and high-AGE HSA from cardiovascular patients for 24 h. Columns represent the fold change of percentage of PBMCs adhered with respect to commercial HSA, expressed as mean values (columns) ± S.E.M. (vertical bars) of at least three independent experiments. #$p < 0.05$ with respect to low-AGE HSA (Student's *t* test).

were incubated with PBMCs for 1 h. A slight but significant increase in PBMCs adhesion (measured as explained above) was observed with high-AGE HSA with respect to low-AGE HSA at 12.5 μg/mL ($p < 0.05$), but not with respect to healthy HSA (**Figure 8**; $p < 0.05$). A trend toward an increase in PBMCs adhesion was also observed after treatment with high-AGE HSA with respect to low-AGE HSA at 25 μg/mL ($p = 0.06$). This suggests that *in vivo* glycated albumin needs more time to induce PBMCs adhesion than highly *in vitro* glycated albumin (AGE-HSA).

3. Potential implications for pharmacological modulation of AGE-RAGE axis activity

In an attempt to counteract the inflammatory effects of AGE-HSA, we selected three RAGE inhibitors: a soluble form of RAGE (sRAGE; R&D systems), used at 0.25, 0.5 and 1 ng/mL; a monoclonal antibody against RAGE (anti-RAGE; R&D systems), used at 5, 10 and 20 μg/mL; and the RAGE antagonist FPS-ZM1 (Calbiochem, Merck Millipore), used at 125, 250, 500 and 1000 nM. HUVECs were pre-treated with different concentrations of these inhibitors and 50 min later treated with 25 μg/mL AGE-HSA. The inhibitory effect of these agents on the expression of VCAM-1 and ICAM-1 in HUVECs was studied.

However, contrary to what we expected, blockade of RAGE by using sRAGE, anti-RAGE antibody and FPS-ZM1 was not sufficient to counteract the AGE-induced VCAM-1 and ICAM-1 up-regulation at any of the concentrations tested under our experimental conditions. Our results may suggest that on endothelium, other RAGE-independent mechanisms may also

be acting to increase adhesion molecule expression and induce inflammation. Other possible explanation for these results is that the pharmacological tools actually available to block RAGE activity are not able to block the effects of AGEs at the endothelial level. However, the results obtained on *in vivo* models of disease are promising, as we comment below.

To investigate the effects of RAGE blockade in pathological conditions, many studies have used soluble forms of RAGE or anti-RAGE antibodies, which can antagonize RAGE-ligand interaction to competitively inhibit the activation of RAGE signaling [39, 44, 45]. Evidence from these studies has shown that RAGE blockade protected against various disease challenges. Soluble RAGE, which competes with cellular RAGE for ligand binding, has been able to reduce inflammatory responses in several models tested. Streptozotocin-induced diabetic apoE−/− mice treated with once daily injections of murine sRAGE showed suppressed acceleration of atherosclerotic lesions in a dose-dependent manner [46]. In parallel with decreased atherosclerotic lesion area and the complexity of the atheroma plaque composition, the levels of tissue factor, VCAM-1, AGEs, and nuclear translocation of NF-kB were decreased in the aortas of sRAGE-treated mice [42, 46]. In other work, sRAGE-treated mice displayed significant stabilization of the lesion area at the aortic root. Compared with diabetic mice receiving albumin (placebo), those receiving sRAGE had significantly diminished activity of monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase-2 (COX-2), VCAM-1 and matrix metalloprotease 9 (MMP-9) within aortic tissue [47]. Similarly, administration of sRAGE resulted in a highly significant decrease in atherosclerotic lesion area in parallel with decreased vascular expression of pro-inflammatory RAGE ligand S100/calgranulins and VCAM-1 and MMPs [48]. Moreover, sRAGE-treated non-diabetic mice displayed significantly decreased atherosclerosis and vascular inflammation [47, 48].

Further studies using anti-RAGE IgG fragments to block ligand binding to RAGE have confirmed these results, especially at the highest dose (up to 10 μg/mL) tested [49]. Exposure of HUVECs to AGE-bovine serum albumin induced expression of VCAM-1 and increased adhesiveness of the monolayer for T lymphoblast of the Molt-4 cell line, which was inhibited by addition of anti-RAGE IgG or sRAGE [40]. Activation of signaling pathway on endothelial cells by advanced oxidation products resulted in overexpression of VCAM-1 and ICAM-1 at both, gene and protein levels, something that was prevented by blocking RAGE with either anti-RAGE IgG or excess sRAGE [27]. Administration of anti-RAGE IgG or sRAGE strongly blocked the increase in vascular permeability in diabetic rats injected with human diabetic red blood cells [50]. Mice treated with sRAGE or anti-RAGE F(ab')2 fragments displayed significantly lower intima/media ratio (a marker of negative vascular remodeling after injury) compared to vehicle-treated animal models of femoral artery injury [51]. However, despite the fact that both, sRAGE and anti-RAGE IgG were able to reduce inflammatory responses in all models tested so far [42, 46, 50, 52], no significant decrease in ICAM-1 and VCAM-1 expression was observed after pre-treatment with soluble RAGE or anti-RAGE antibody, under our experimental conditions.

A recently developed high-affinity RAGE-specific inhibitor: FPS-ZM1 (N-benzyl-4-chloro-N-cyclohexylbenzamide; Calbiochem, Merck Millipore) [53] was also studied. This inhibitor was developed to interact with the ligand-binding domain of the receptor and block RAGE signaling. In our *in vitro* experimental conditions this approach was also unable to inhibit AGE-induced VCAM-1 and ICAM-1 up-regulation.

It is worth mentioning that, most of the above-mentioned works did not elucidate the precise AGE(s) that trigger signal transduction mechanisms upon interacting with RAGE. Kislinger et al. [54] studied the effect of CML-adducts and showed that CML-mediated VCAM-1 expression on HUVECs was also suppressed in the presence of excess sRAGE or anti-RAGE IgG. Nevertheless, they suggest that the findings presented in their work do not rule out other specific AGE products of glycation or oxidation, such as pentosidine, pyralline, methylglyoxal, and imidazolone [55–57], which are present in our modified albumins. Additionally, they also specified that their findings do not rule out either the presence of other receptors or cellular interaction sites for CML adducts, being possible that other receptors for AGE [58–60] may also engage CML- and AGE-modified adducts. These situations might explain why no reduction in the up-regulation of adhesion molecules is observed after pre-treatment with sRAGE and anti-RAGE antibody under our experimental conditions.

Additionally, Amadori-modified albumin stimulates adhesion of monocytes to endothelial cells through enhanced transcription of the cell surface adhesion molecules E-selectin, VCAM-1 and ICAM-1 [61], implicating an initial endothelial cell activation occurring at atherosclerosis-prone vascular sites [62, 63]. However, Amadori products do not compete with AGE-albumin for binding to AGE receptors such as RAGE [64]. Aortic endothelial cells express specific receptors for Amadori-modified albumin [37, 65]. Although less information is available for the receptor for Amadori products and signaling through Amadori-modified albumin receptors remains obscure, calnexin [66] and nucleophosmin [67, 68] have been reported to be the fructosyl-lysine specific binding proteins [66–68]. Binding of Amadori-modified albumin to calnexin-like receptors may participate in degradation and/or activation of signal transduction processes involved in mediating the biologic activities of Amadori-modified albumin [66]. The E-selectin expression induced by Amadori-modified albumin was 10 or 20 times higher than that induced with three types of AGEs-HSAs and was not suppressed by anti-RAGE antibody [69]. This would explain why RAGE antagonism would not counteract the increase in adhesion molecules expression.

In agreement with this hypothesis, Esposito et al. [70] found that anti-RAGE antibody completely prevented leukocyte adhesion to endothelial cells grown for 8 weeks in high-glucose-containing media, but it did not reduce the adhesion at 24 h. These results demonstrate that AGEs are important mediators of high-glucose-induced endothelial dysfunction after long-term exposure, whereas the same changes in acute exposure occur with the action of mediators other than AGEs. As the formation of Amadori products is highly probable after 24 h incubation in high glucose medium, but not the formation of AGEs, the effects on the inflammation parameters observed by Esposito et al. [70], and not prevented by anti-RAGE antibodies, might be due to the effect of the early glycated products, and not AGEs.

Besides from directly blocking RAGE, alternative pharmacological approaches might turn out to be more promising. Namely, it has been shown that both RAGE and sRAGE can be regulated by currently available pharmacological agents [71]. Other drugs currently in use for diabetic complications have been shown to have an effect on AGE accumulation. These include the antihypertensive angiotensin-converting enzyme inhibitor (ACEI) ramipril [72] and the glucose-lowering drug metformin [73], which both reduce AGE. Forbes et al. [74] demonstrated that compared with placebo, the ACEI perindopril increased human plasma sRAGE levels and reduced plasma AGE concentrations, suggesting an additional mechanistic effect of ACE inhibition in the treatment and prevention of vascular disease. The inhibition of ACE in

rats increased the renal expression of sRAGE and decreased the expression of renal full-length RAGE protein [74]. These investigators also showed that plasma sRAGE levels were significantly increased by inhibition of ACE in both diabetic rats and human subjects with type 1 diabetes [74]. Olmesartan, an angiotensin II type 1 receptor blocker, inhibited the AGE-evoked ROS generation and reduced the expression levels of monocyte chemoattractant protein 1 and ICAM-1 in endothelial cells, subsequently blocking T-cell adhesion to endothelial cells [75].

Other potential agents that may affect circulating sRAGE include the thiazolidinediones [76, 77] and statins [78–80], both of which are known to modulate AGE-RAGE axis. Marx et al. [76] investigated the effects of the two thiazolidinediones available, rosiglitazone and pioglitazone, on RAGE expression in HUVECs. Exposure of HUVECs to thiazolidinedione resulted in a similar reduction in RAGE mRNA expression, via inhibition of NF-κB activation, and in RAGE cell surface expression, demonstrating how these drugs may influence RAGE expression and its deleterious inflammatory activity in subjects with DM [76]. Blockade of the interaction of S100A12 (an endogenous ligand of RAGE) with RAGE by statins at an early stage may prevent inflammation in atherosclerosis and counteract the harmful effects mediated by C reactive protein [81].

Finally, recent results testing new potential drugs have been reported. Curcumin, a polyphenolic natural compound is able to trap methylglyoxal, an important precursor of AGEs [82]. Added on endothelial cell cultures curcumin reduced the intracellular ROS levels and improved cell viability compared with the treatment of methylglyoxal alone. There was also a significant reduction in the expression levels of ICAM-1 [82]. Liquiritin, the 4′-O-glucoside of the flavanone liquiritigenin, reduced AGEs-induced apoptosis and ROS generation in HUVECs and also significantly increased AGEs-reduced SOD activity [83]. It even downregulated the RAGE protein expression and significantly blocked NF-κB activation [83].

4. Conclusions

Oxidative stress induction by AGEs at endothelium triggers molecular signaling pathways that produce an inflammatory response or even endothelial dysfunction. Adhesion molecules expression at the membrane surface of endothelial cells as a consequence of this response or induced by AGEs by other mechanisms mediates the adhesion of leukocytes to endothelium. This adhesion is a key step in the atherogenesis process and the possible involvement of AGE-RAGE axis in it should be considered as potential therapeutic target. Finally, possible pharmacological modulation of AGE-RAGE axis activity at the endothelium is suggested, but specific pharmacological tools available nowadays are not efficient enough; momentarily, drugs used for cardiovascular and metabolic problems could be helpful in modulating the AGE-RAGE axis.

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