Methylglyoxal and glyoxalase I in atherosclerosis

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Abstract

Cardiovascular disease, caused predominantly by atherosclerotic plaque rupture, remains one of the leading causes of death. However, the mechanism of plaque rupture remains largely unknown. Recent studies have linked high metabolic activity in inflamed atherosclerotic plaques to the development of plaque rupture. AGEs (advanced glycation end-products) are known to be formed as a result of high metabolic activity and are higher in rupture-prone than stable plaques. Furthermore, AGEs seem to be more than mere markers of metabolic activity, as recent studies have elucidated that AGEs and their major precursor, MG (methylglyoxal), may have an important role in the progression of atherosclerosis and plaque rupture. MG can be detoxified by Glo1 (glyoxalase I), thereby preventing the accumulation of MG and MG-derived AGEs. In the present review, data concerning MG, Glo1 and AGEs in the context of plaque phenotype are discussed.

Introduction

CVDs (cardiovascular diseases), such as heart attack and stroke, are major causes of mortality worldwide [1]. The disease underlying the vast majority of CVDs is atherosclerosis. An atherosclerotic plaque is formed by accumulation of lipids and inflammatory cells between the medial and intimal layers of arteries. Advanced atherosclerotic plaques consist of a necrotic core containing cholesterol and dead macrophages, flanked by macrophage-rich shoulder regions, and are covered by a fibrous cap containing smooth muscle cells and collagen. Stable plaques are characterized by more collagen deposition, smooth muscle cells, fewer macrophages and a small necrotic core [2]. Plaques at highest risk of rupture are rich in macrophages and have a large necrotic core. In addition, infiltration of leaky intraplaque vessels contribute to intraplaque haemorrhages, increasing the risk of plaque rupture [3]. Ruptured plaques are defined by a disruption of the fibrous cap and a thrombus that is continuous with the necrotic core, which may occlude an artery [3]. The risk of plaque rupture is essentially a balance between inflammatory activity and growth of the necrotic core against thickness of the fibrous cap [4]. It is unknown why, in some plaques, the fibrotic processes dominate, leading to stable plaques, whereas in other plaques inflammation and necrosis take over, leading to thinning of the fibrotic cap.

Previous studies have linked high metabolic activity in atherosclerotic plaques [5] to the development of plaque rupture. One feature of increased metabolic activity is formation of AGEs (advanced glycation end-products) [6]. Indeed, several studies have shown that AGEs accumulate in human atherosclerotic plaques [7–10], and we found recently that higher levels of AGEs in human carotid atherosclerotic plaques are associated with a rupture-prone phenotype [11].

AGEs are a large family of extensively sugar-modified proteins, but, rather than glucose itself, the dicarbonyl MG (methylglyoxal) has been identified as the most reactive AGE precursor [12]. MG has attracted a lot of attention as a key player in vascular dysfunction, particularly due to its capacity to induce the formation of oxidative stress, cell death and endothelial dysfunction [12,13]. Therefore accumulation of MG and MG-derived AGEs may be a major contributing factor to plaque rupture. In the present review, we describe various mechanisms of AGE formation, with a particular focus on MG, and we discuss potential mechanisms for how AGEs may contribute to atherosclerotic disease and how lowering AGEs may have potential to treat CVD.

Formation of AGEs

It is thought that the formation of AGEs is an inevitable consequence of (human) biology, associated with aging [14]. Increased metabolic activity, such as in diabetes, is associated with increased AGE formation [15].

The Maillard reaction

Formation of AGEs was first described by Louis Camille Maillard in 1912 [16]. The Maillard reaction is initiated by the reaction of reducing sugars with protein residues, resulting first in the formation of a Schiff base and subsequently, by structural rearrangements, in Amadori products. Only a small proportion of Amadori products are further, and irreversibly, modified to AGEs. In the body, this entire reaction-chain occurs slowly over a period of months. Therefore AGEs derived from the Maillard reaction are mostly formed on long-lived proteins, such as on collagen [14].

Key words: advanced glycation end-product (AGE), atherosclerosis, glyoxalase I, methylglyoxal, plaque rupture.

Abbreviations: AGE, advanced glycation end-product; CML, N^{ε}-(carboxymethyl)lysine; CVD, cardiovascular disease; Glo, glyoxalase; LDL, low-density lipoprotein; MCP1, monocyte chemoattractant protein 1; MG, methylglyoxal; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine or 5-hydro-5-methylimidazolone; MMP-9, matrix metalloproteinase 9; NF-κB, nuclear factor κB; RAGE, receptor for AGEs; STZ, streptozotocin; THP, tetrahydroimidazolone; TNFα, tumour necrosis factor α.

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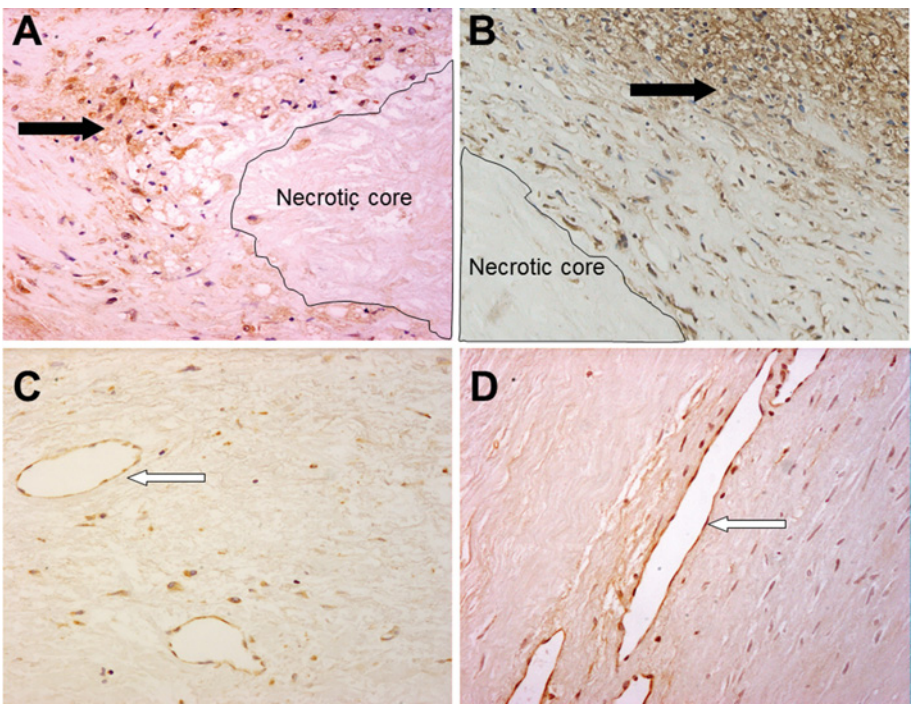
Figure 1 | Immunohistochemical staining of advanced carotid atherosclerotic lesions.
Images showing staining of CML (**A**) and MG-H1 (**B**) in the cytoplasm of macrophages (indicated by black arrows) surrounding the necrotic core (indicated by the black line). In addition, CML (**C**) and MG-H1 (**D**) accumulate in plaque vessels (indicated by white arrows). Images taken at ×200 magnification.



AGE formation via MG and the glyoxalase system

It is now well established that intermediates of glycolysis are much more potent glycating agents than glucose itself [17], suggesting that *in vivo* formation of AGEs intracellularly is more abundant than AGE formation via the slow Maillard reaction on connective tissues. During glycolysis, highly reactive intermediates are formed, including the dicarbonyl compound MG [12]. MG reacts primarily with arginine residues to form the AGEs MG-H1 [N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine or 5-hydro-5-methylimidazolone], argpyrimidine and THP (tetrahydroimidazolone) as well as with lysine residues, to form N^{ε}-(carboxyethyl)lysine.

MG can be detoxified by Glo1 (glyoxalase I) and Glo2 (glyoxalase II) and reduced glutathione (GSH) into D-lactate [18], thereby preventing accumulation of MG and MG-derived AGEs. Overexpression of Glo1, the rate-limiting enzyme in the glyoxalase system, has been shown to reduce MG and AGEs in diabetes [15], and protects against vascular dysfunction [19], retinopathy [20] and nephropathy [19] in diabetic rats.

MG and AGE formation has mainly been studied in the context of hyperglycaemia, although recent work has demonstrated that inflammation and hypoxia are also major determinants of MG formation [11]. For instance, both hypoxia and inflammatory mediators such as TNFα (tumour necrosis factor α) are associated with decreased Glo1 activity in U937 monocytes, which may explain the concomitant increase in MG found under these conditions [11].

AGEs in atherosclerosis

Several studies have described AGE accumulation in human atherosclerotic plaques of the aorta [7], coronary artery [8], carotid artery [9] and femoral artery [10], using antibodies against glycated proteins [7–9] or the well-characterized AGE CML [N^{ε}-(carboxymethyl)lysine] [10,21,22]. Therefore accumulation of AGEs in atherosclerotic lesions seems to be irrespective of the site of plaque development. Specific MG-derived AGEs MG-H1 [11] and THP [23] have also been detected in atherosclerotic plaques. Moreover, we found recently that CML and MG-H1 levels in atherosclerotic plaque, as measured in homogenates of plaques with ultrahigh-performance liquid chromatography–MS/MS, are associated with a rupture-prone phenotype [11], suggesting that AGEs may contribute to events leading to plaque rupture. Furthermore, in line with their high metabolic activity, macrophages are the predominant cells in which CML and MG-H1 accumulate in plaques, particularly surrounding the necrotic core [11] (Figures 1A and 1B). Additionally, staining of these AGEs was also found in plaque vessels (Figures 1C and 1D).

Factors contributing to AGE accumulation in atherosclerosis

Several metabolic factors such as inflammation, hypoxia and oxidative stress promote an increased accumulation of AGEs in atherosclerotic plaques, whereas defensive mechanisms against glycation may be decreased.

AGE formation via glycolysis

In human atherosclerotic plaques, [^{18}F]fluorodeoxyglucose-uptake experiments have shown that the macrophage is the primary cell in which a high degree of glycolysis takes place [5]. It has been demonstrated in cultured macrophages that inflammation is a stronger trigger for glucose uptake than hyperglycaemia [24]. The uptake of glucose under these circumstances may contribute to increased formation of MG. Indeed, we demonstrated recently that the cytokine TNFα and hypoxia strongly increased MG and AGEs in monocytes [11]. These findings are in accordance with strong associations between plaque inflammatory markers and AGE levels, and a striking co-localization between hypoxia and AGEs in advanced atherosclerotic plaques in macrophages [11].

By contrast, hyperglycaemia leads to rapid accumulation of AGE precursors, including MG in endothelium [17]. Furthermore, accumulation of AGEs in endothelial cells has been linked with atherosclerotic lesion initiation in diabetes [25].

Lipid-derived AGE accumulation

In addition to formation of AGEs from glucose and glycolysis-derived precursors, AGEs can also be derived from lipid peroxidation, of which the AGE CML is a well-characterized example [26,45]. Since macrophages take up large quantities of oxidized LDL (low-density lipoprotein) in atherosclerotic lesions, AGEs derived from lipid peroxidation in macrophages may also be of great physiological importance in atherosclerosis. In addition, it has been demonstrated that LDL itself is a target for MG. Modification of LDL by MG decreases its particle size [27], increasing its atherogenicity, and reduces its affinity for the LDL receptor [28], thereby decreasing its clearance from the circulation and promoting trapping of LDL in the vessel wall. Indeed, both CML and MG-H1 levels are associated with lipid content of the plaque [11], indicating that AGEs are (partly) derived from lipid peroxidation and/or AGE-modified LDL particles in atherosclerotic plaques.

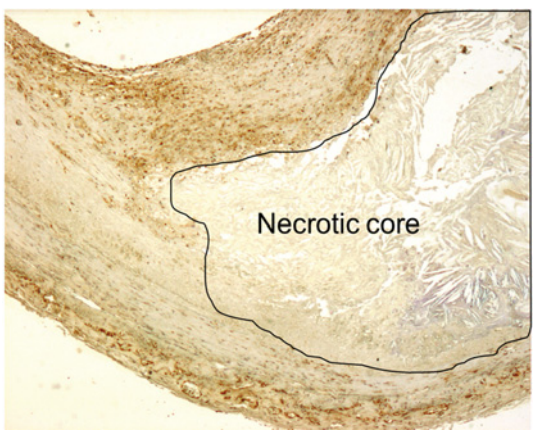
Decreased Glo1 expression in ruptured plaques

Glo1 is abundantly present in the cytosol of virtually all cells in the plaque (Figure 2). We demonstrated recently that mRNA, protein and activity levels of Glo1 are lower in ruptured plaques, which may leave cells in atherosclerotic plaques more susceptible to AGE accumulation. Furthermore, previous experimental studies have shown decreased Glo1 activity under conditions of metabolic stress, such as hyperglycaemia [29], ischaemia [30], hypoxia and inflammation [11]. Given the high metabolic activity in atherosclerotic plaques, down-regulation of Glo1 may lead to increased glycation in the plaque. Previous work has shown that the promoter of Glo1 harbours an NF-κB (nuclear factor κB)-responsive element [31], indicating a link between inflammation and reduced Glo1 expression. In addition, the Glo1 protein is susceptible to post-translational modifications, in particular phosphorylation in response to TNFα, a key mediator of inflammation [32]. Moreover, post-translational modification of Glo1 by oxidized glutathione (GSSG) and nitrosylation strongly inhibits Glo1 activity [33,34]. The presence of such modifications on Glo1 in the plaque remains unclear.

Glo1 is absent from the necrotic core of atherosclerotic plaques (Figure 2). Therefore it is not conceivable that Glo1 offers full protection against extracellular AGE formation, such as AGE formation from lipid oxidation of LDL particles.

Figure 2 | Immunohistochemical staining of an advanced carotid atherosclerotic lesion

Staining shows abundant staining of Glo1 in the cytoplasm of cells throughout the plaque, except for the necrotic core (indicated by the black line). Images taken at ×40 magnification.



Potential mechanisms for how MG and AGEs contribute to plaque rupture

Two major mechanisms by which AGEs damage tissues and contribute to the initiation, progression and rupture of plaques can be proposed: first, intracellular glycation of proteins, leading to impaired cell function, and secondly, binding of AGEs to cellular receptors and subsequent modulation of inflammatory gene expression.

Cytotoxicity of MG

Accumulation of MG in macrophages may contribute to growth of the necrotic core of rupture-prone plaques, by induction of apoptosis. This hypothesis is supported by the fact that AGEs accumulate predominantly in the macrophages surrounding the necrotic core, co-localizing with the apoptosis marker caspase 3 [11]. *In vitro*, MG induces apoptosis of macrophages in an oxidative-stress-dependent mechanism [35].

Furthermore, AGE-positive plaque vessels also stain for caspase 3 [11]. Therefore accumulation of MG and AGEs in plaque vessels may contribute to their dysfunction and leakage, further exacerbating their risk of intraplaque haemorrhage and subsequent plaque rupture. Accordingly, MG impairs proteasome function in endothelial cells [36], and indeed induces apoptosis [37]. Interestingly, AGEs have been shown to promote angiogenesis [38,39], which suggest that accumulation of AGEs in plaques may not only influence the function, but also the amount of plaque vessels. This may be of pathophysiological relevance, as a higher amount of plaque microvessels is strongly associated with a rupture-prone plaque phenotype [3].

Furthermore, it has been demonstrated that intracellular accumulation of MG in endothelial cells causes dysfunction as indicated by expression of adhesion molecules such as VCAM-1 (vascular cell adhesion molecule 1) expression, which can be prevented by Glo1 overexpression [40]. Therefore AGE accumulation in endothelial cells may contribute to atherosclerotic plaque formation by attracting more monocytes to the plaque. Interestingly, El-Osta et al. [41] demonstrated elevated MCP1 (monocyte chemoattractant protein 1) expression and concomitant adverse epigenetic changes in endothelial cells of even normoglycaemic *Glo1*-knockdown mice [41], suggesting that exposure to MG leads to a prolonged risk of atherosclerosis.

Inflammation through interaction with RAGE

RAGE (receptor for AGEs) links the accumulation of AGEs to inflammatory pathways associated with atherosclerosis. Activation of RAGE leads to activation of NF-κB [42] and downstream inflammatory signalling. Furthermore, genetic deletion of RAGE greatly reduces atherosclerosis in $Apoe^{-/-}$ (apolipoprotein E-knockout) mice [43]. In addition, administration of soluble RAGE reduced atherosclerosis in STZ (streptozotocin)-injected diabetic $Apoe^{-/-}$ mice. Moreover, in human plaques, RAGE co-localizes with MMP-9 (matrix metalloproteinase 9), a major collagen-digesting enzyme, and RAGE signalling is implicated in weakening of the fibrous cap of the plaque [44]. CML, which is present in high amounts in atherosclerosis and is considered an important ligand for RAGE [45] is associated with MMP-9, as well as IL-8 (interleukin 8) and MCP1 in plaques [11]. However, many inflammatory cytokines, such as S100b and HMGB1 (high-motility group box 1) [46] are ligands for RAGE, and which of these ligands is the major ligand for RAGE in the plaque is not clear. Interestingly, an interplay between RAGE and the glyoxalase system has been described, as Glo1 overexpression prevents RAGE up-regulation [47].

Targeting AGEs for CVD therapy

Evidence for a causal role for AGEs in atherosclerosis is obtained primarily by the use of AGE inhibiting compounds in $Apoe^{-/-}$ mice.

The best characterized inhibitor of AGE formation is aminoguanidine [48]. Administration of this compound reduced plaque formation in STZ-induced diabetic $Apoe^{-/-}$ mice [49]. In addition to AGE- and MG-lowering properties, aminoguanidine has been described as an antioxidant [50] and inhibitor of nitric oxide formation [51]. Likewise, pyridoxamine (vitamin B_6) is considered an AGE inhibitor, with antioxidant and anti-inflammatory properties [52], that can reduce atherosclerosis in STZ-induced diabetic $Apoe^{-/-}$ mice [53].

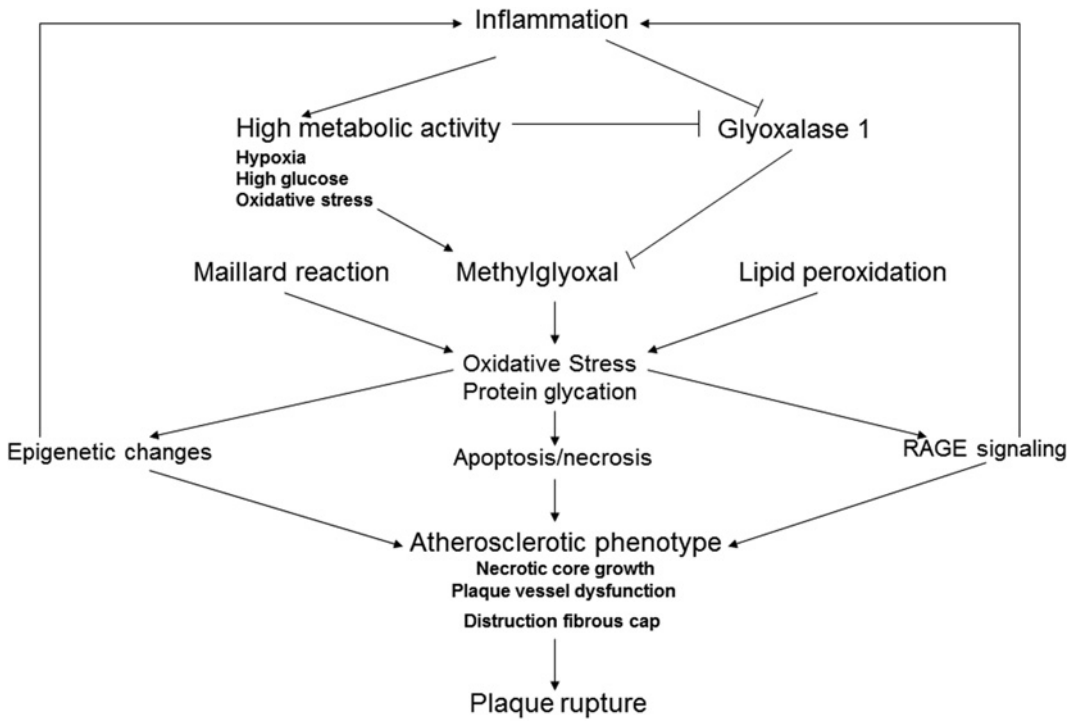
Alagebrium is a compound capable of breaking pre-existing AGE cross-links that has also been shown to lower MG levels directly [54]. Alagebrium has been shown to reduce atherosclerosis in STZ-induced diabetic $Apoe^{-/-}$ mice, although it is unclear whether or not this beneficial effect was attributable to its AGE- or MG-lowering properties [49]. Despite these animal studies, it is currently unknown whether these compounds indeed reduce cardiovascular events in humans [55], as mice do not spontaneously develop plaque rupture. Interestingly, established drugs to treat CVD, such as simvastatin, have also been shown to reduce the accumulation of AGEs in human carotid plaques [9]. Similarly, several glucose-lowering [56] and antihypertensive compounds [57] have been shown to have AGE-lowering properties. Whether beneficial effects of these drugs are partly attributable to AGE lowering, or whether the AGE-lowering effects of these compounds merely reflect their anti-inflammatory, glucose lowering or other actions remains to be determined.

Conclusion

AGEs are higher in rupture-prone plaques and may contribute to the increased risk of plaque rupture. This may be aggravated further by an impaired defence against glycation by a down-regulation of Glo1 and, as a consequence, increased formation of MG. Cytotoxicity and induction of inflammation are potential downstream effects of MG and AGE accumulation. A simplified overview of how this axis may link inflammation to the development of plaque rupture is given in Figure 3.

Many unresolved questions remain regarding precise mechanisms and the extent to which MG and AGEs contribute to progression of atherosclerosis. Importantly, interventions that specifically reduce AGEs without intervening in other athero-reducing mechanisms are still lacking, and it has not been shown that AGE interventions in humans reduce cardiovascular risk. Intervention studies, preferably with more specific inhibitors of MG and/or AGEs, are needed to determine further the extent to which MG and AGEs play a causal role in human atherosclerosis. In addition, interruption of RAGE signalling may prove successful in treating human atherosclerotic disease, as it is in mice. Furthermore, increasing Glo1 expression and/or activity may prove valuable to reduce cardiovascular risk. For example, beneficial effects of the angiotensin receptor blocker candesartan on retinopathy have been attributed to its Glo1-restoring capacities [58]. Furthermore, AGEs measured in the circulation may serve as biomarkers of CVD risk. Unfortunately, little research has focused on which specific circulating AGEs best reflect AGE accumulation

Figure 3 | **Simplified overview of how MG and AGEs may link plaque inflammation and development of plaque rupture.**



in atherosclerotic lesions. The value of specific AGEs for risk prediction for CVD should be determined in large prospective cohort studies.

Taken together, AGEs, MG and Glo1 may contribute to the increased risk of plaque rupture in inflammatory and metabolically active plaques. Developing therapeutic interventions directed at reducing AGE levels, restoring Glo1 activity and/or interrupting in the RAGE axis is a major challenge to combat the development of CVD.

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