

Molecular Basis of Arterial Stiffening: Role of Glycation – A Mini-Review

David R. Sell^a Vincent M. Monnier^{a, b}Departments of ^aPathology and ^bBiochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio, USA

Key Words

Aging · Diabetes · Intervention · Advanced glycation · Oxidation · Collagen · Elastin

Abstract

Arterial stiffening is a progressive, ubiquitous and irreversible aging process that is interwoven with and accelerated by various diseases such as diabetes, atherosclerosis and hypertension. In large arteries, aging is characterized by decreased turnover of collagen and elastin and increased advanced glycation end-products (AGEs) and cross-links. Elastic fibers undergo lysis and disorganization subsequent to their replacement by collagen and other matrix components. These events cause the loss of elasticity and induce stiffening. Conceptual approaches to minimize AGE accumulation in arteries include caloric restriction, exercise, low dietary intake of AGEs, deglycation enzymes, increased clearance of AGEs, antagonists of AGE receptors and pharmaceutical interventions. Much optimism exists in the ability of 'AGE breakers' such as alagebrium (ALT-711) to cleave AGE cross-links and reverse the age-related stiffening of arteries. However, there is little evidence that these agents actually break pre-existing AGE cross-links in vivo. In contrast, many of these anti-AGE agents share in common the ability to che-

late metals, thus acting as inhibitors of metal-catalyzed AGE and protein carbonyl formation. Future work on interventions into the causes of arterial stiffness in aging needs to address more rigorously the relationship between stochastic forms of damage, such a glycation and oxidation, and the changes in elastic fiber structure thought to contribute to loss of arterial elasticity.

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Introduction

Research into the causes and factors regulating arterial stiffness has been the subject of several recent reviews [1]. As such, arterial stiffness is a progressive aging process that is accelerated by many age-associated disorders including hypertension, metabolic syndrome, diabetes, atherosclerosis and renal disease, implying thereby metabolic factors in its pathogenesis [1]. Because of the voluminous amount of literature on this subject, this review focuses on the role of glycation and advanced glycation end-products (AGEs), i.e. the Maillard reaction (fig. 1), in the age-related accelerated stiffening of the arterial extracellular matrix (ECM). As for all age-driven processes, the age component of arterial stiffening is complex and

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Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com© 2012 S. Karger AG, Basel
0304-324X/12/0583-0227\$38.00/0Accessible online at:
www.karger.com/gerDavid R. Sell, PhD
Department of Pathology, Room 5301
Case Western Reserve University School of Medicine, 2103 Cornell Road
Cleveland, OH 44106-7288 (USA)
Tel. +1 216 368 2930, E-Mail david.sell@case.edu

has many facets, but is usually described as intrinsic, ubiquitous, and gradual. Importantly, the degree of arterial stiffening may vary greatly among different individuals of the same age, and even between different tissues of the same person, suggesting a complex 'biological clock' phenomenon that could be under genetic control, but with many environmental interactions involving stochastic processes such as advanced glycation end-product (AGE) formation and oxidative processes [2]. Additionally, there is a distinction between chronological versus biological aging in the mechanistic aging processes of arteries. Undoubtedly, chronological arterial aging is irreversible while biological arterial aging is mutable.

As reviewed by Cattani et al. [3], other factors that may be important in the regulation of arterial stiffness include endothelial and smooth muscle cells (SMCs) which control cell signaling and regulate vascular tone, respectively. Also, a tight relationship exists between adhesion structures and points of attachment between the vascular SMCs and the ECM including adhesion molecules and integrins [3].

Role of Glycation in the Vascular Wall

Glycation and AGE formation is thought to have a profound effect in enhancing age-related arterial stiffening especially in a carbonyl-enriched environment such as diabetes and end-stage renal disease where total carbonyl stress is dramatically increased [2]. AGE formation begins in the classical Maillard reaction pathway by nonenzymatic reaction of reducing sugars with lysyl residues of proteins (fig. 1). In the arterial wall, the principal proteins targeted by this reaction are collagen and elastin. In human abdominal aorta, for example, these proteins account for ~90% of its dry weight, i.e. ~60% collagen and ~30% elastin [4]. As for the reducing sugar, when it is glucose, the reaction is referred to as 'glycation'. However, this term is widely used to refer to other reducing sugars reactions with proteins as well.

The Maillard reaction is initiated by Schiff base formation between the free aldehyde of open-chain glucose with the ϵ -amino group of a lysyl residue which rearranges to an Amadori product, i.e. fructosamine or fructosyl-lysine. This product, in turn, can undergo a further series of complex reactions involving dehydration, condensation, fragmentation and cross-linking to form AGEs. When oxidation is involved, the reaction pathway is termed 'glycoxidation'. Additionally, AGEs can form through pathways involving the oxidation of lipids, thus

termed 'lipoxidation' producing advanced lipoxidation end-products, i.e. ALEs [2]. A conceptual scheme summarizing the major known glycation pathways is shown in figure 1.

Glycation by glucose is relatively rapid and in part reversible. Thus, Amadori product formation is in equilibrium with ambient blood glucose concentration. The degree of Amadori product formed is proportional to glucose concentration and the lysyl residue content of the protein. It is influenced by the protein tertiary structure, i.e. surface residues are more susceptible to glycation versus buried residues. Additionally, the work by Reiser et al. [5] suggests that the primary structure of collagen may be the major determinant for adduct formation by glucose. Interestingly, they found that collagen glycation is not random but occurs at preferential sites which are conserved with age. However, higher-order structure may influence the relative distribution of glucose adducts among the preferred sites [5, 6]. In contrast, AGE formation is nonreversible (cf. cross-link breakers below) whereas levels are proportional to the Amadori product concentration as well as the protein turnover rate.

Reducing sugars such as glucose may degrade when partially attached to primary amines to form reactive α -oxoaldehydes. These reactive dicarbonyl precursors (e.g. glyoxal, methylglyoxal, 3-deoxyglucosone) are potent glycating agents. Glyoxal and methylglyoxal can come from sources other than glucose and may react with proteins to form AGEs directly without going through the classical Maillard reaction pathway just described [7]. Of further complexity and consequence is that they also may form by the degradation of Schiff base adducts, Amadori products, glycolytic intermediates and lipid peroxidation, all of which can form AGEs [7].

As depicted in figure 1, glyoxal may also come from lipid peroxidation, inflammation (oxidation of glycolaldehyde to glyoxal by neutrophils) and inhalation of environmental pollutants such as cigarette smoke (not depicted in fig. 1). Additional sources for methylglyoxal (fig. 1) include fragmentation of glyceraldehyde-3-phosphate intermediate of glycolysis which accumulates during inhibition of GAPDH by hyperglycemia; metabolism of acetone, aminoacetone and threonine; and putative direct ingestion through the diet (not depicted in fig. 1).

Interestingly, the term 'carbonyl stress' was introduced a number of years ago [8] to describe the excessive accumulation of these compounds as derived in full or in part from nonoxidative processes. In contrast, during 'oxidative stress' compounds originate *exclusively* from oxidative reactions [2]. However, other researchers have

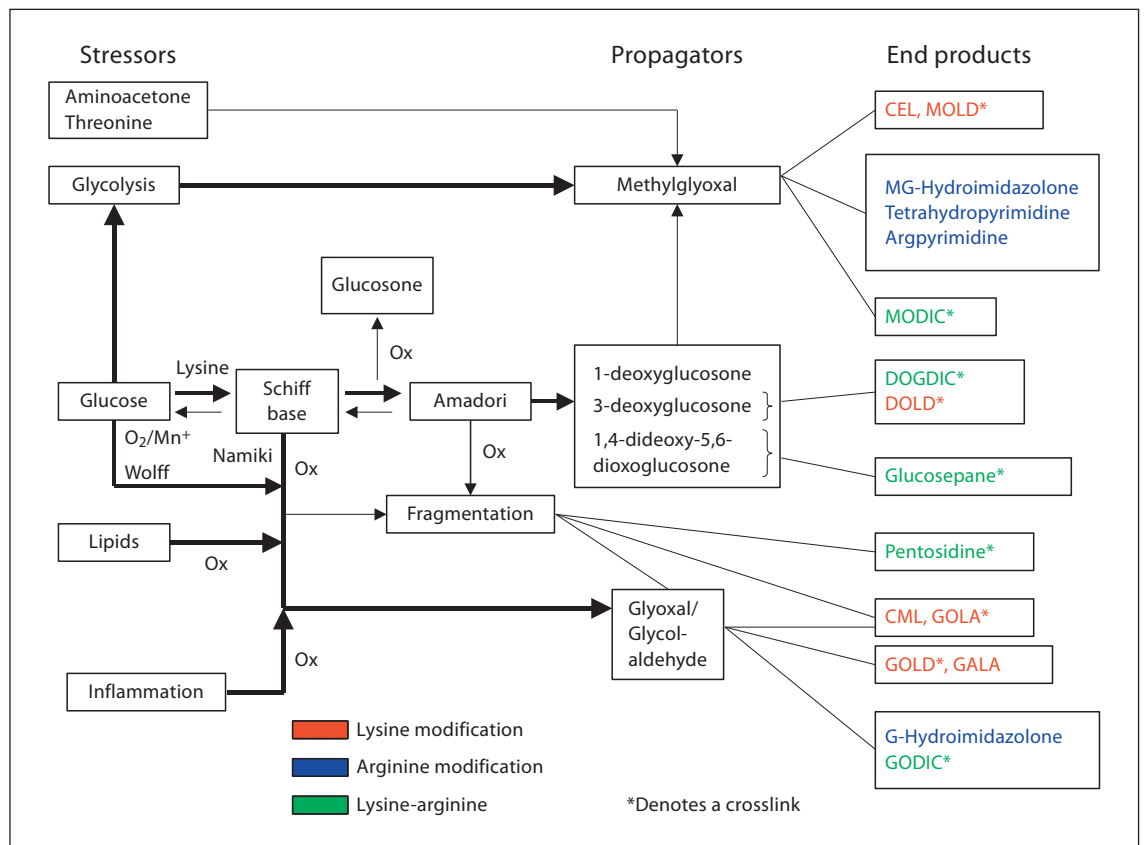


Fig. 1. Conceptual scheme of the Maillard reaction summarizing the major known glycation pathways involved in arterial stiffness. CEL = Carboxyethyl-lysine; CML = carboxymethyl-lysine; DOGDIC = 3-deoxyglucosone-derived imidazolium cross-link; DOLD = 3-deoxyglucosone-lysine dimer; G = glyoxal; GALA = glycolic acid-lysine-amide; GODIC = glyoxal-derived imidazolium cross-link; GOLA = glyoxal-lysine-amide; GOLD = glyoxal-lysine dimer; MG = methylglyoxal; MOLD = methylglyoxal-lysine dimer; MODIC = methylglyoxal-derived imidazolium cross-link.

used this term more broadly; namely, to indicate excessive carbonyl formation in glycooxidative and lipoxidative processes [9].

Importance of Metal-Catalyzed Oxidation in AGE Formation in Arteries

Of further significance is the ability of redox-active transition metals such as Cu^{2+} , Fe^{2+} , Mn^{2+} and Zn^{2+} to catalyze carbonyl formation in proteins. This catalysis may be especially important in arterial tissue due to its known propensity to accumulate metals with age [10] as well as with heavy metal exposure associated with arterial stiffness [11]. Secondly, as discussed below, inhibitors of AGE formation which have been successful at

decreasing arterial stiffness have known ability to chelate metals.

Normally, trace levels of these metals are essential to drive biological reactions; however, when in excess, or in partially sequestered state, they can be cytotoxic as a consequence of their participation in an array of cellular disturbances characterized by oxidative stress and free radical production, i.e. reactive oxygenated species (ROS). Indeed, carbonyl content of proteins has been the most generally used method in the estimation of protein oxidation and damage [12]. Of particular interest for connective tissues is the ability of metals to directly oxidize proteins by a hydroxyl radical-mediated and metal-catalyzed oxidation (MCO) reaction [12] causing deamination of lysine residues to form the carbonyl, α -amino adipic acid- δ -semialdehyde, also known as allsine. This is the same

intermediate in the physiological cross-linking of collagen and elastin by lysyl oxidase [12]. However, other mechanisms have been proposed for allysine formation as well including the oxidative degradation of lysine residues by α -dicarbonyl sugars by MCO-mediated Strecker degradation [12].

Age-Related Morphological Changes in the Vessel Wall Inducing Stiffness

As previously discussed, the arterial wall is composed predominantly by collagen and elastin. Collagen provides the structural framework while elastin provides extensibility, but contributes little to the mechanical and stability of the vessel. However, collagen does have limited elastic properties. Tendon collagen, for example, can be stretched to strains of approximately 10% beyond which irreversible damage occurs. In contrast, elastin elongates and straightens under tension $\leq 200\%$ [13].

Morphologically, aging results in a dramatic increase in stiffness of the arterial wall [14]. There is a loss of functionality attributed to a progressive age-related elastolysis and fragmentation of elastin [14] as well as elastocalcinosi of the medial layer of large arteries [15]. In some large arteries, e.g. aorta, elastin is replaced by less distensible collagen with age which is accelerated by hypertension and atherosclerosis. However, in others, e.g. cerebral arteries, there is no change in elastin content with age, but instead, a structural reorganization of elastin occurs paralleled to its loss of functionality with age [14]. Overall, these events weaken the vessel wall as well as its compliance.

Mechanism for Protein Damage by Glycation in the Arterial Wall

For collagen, glycation induces AGE formation and cross-linking which results in increased stiffening of the vessel wall. It is not yet known whether AGEs and their cross-links accumulate at all major glycation sites or if they also have preferential sites of formation in collagen [5]. However, work with globular model proteins such as ribonuclease showed that glucosepane cross-links could only form at sites where lysine and arginine residues were not further apart than 7 Å [6]. In comparison, elastin has a low level of glycation sites due to its low content of lysine residues versus collagen [16]. Importantly, glycation of elastin still occurs and may have dire consequences for

optimal functioning of the cardiovascular system during aging and diabetes [16]. However, the literature yields no evidence of a link between AGEs and elastin cross-linking in vivo [15]. Consequently, it is believed that AGE formation and cross-linking would be more important in hypertension where elastin synthesis is stimulated, namely tropoelastin, which has a higher proportion of lysine residues [16].

Elastin is prone to the Fenton reaction, whereas carboxymethyl-lysine (CML) modifications of this protein would result in a vicious cycle, as proposed by Saxena et al. [17] consisting of protein aging by glycation and CML formation; binding of redox active metals such as copper (Cu^{2+}) or iron (Fe^{3+}) resulting in a CML-protein metal complex; and induction of lipid peroxidation which itself leads to CML formation by glyoxal. Furthermore, at sites of inflammation, myeloperoxidase can potentially mediate CML formation by oxidizing serine and forming the CML precursor glycolaldehyde. The resultant ROS generated by this cycle would induce elastolysis of the arterial wall in a similar mechanism to that described for actinic elastosis of human skin by photo-enhanced CML formation [18]. In support, glycated elastin and collagen have been shown to have increased affinity for metal ions including copper and iron [19].

Evidence for AGE Formation in Arteries Being Associated with Arterial Stiffening

AGEs levels in aorta are significantly elevated by age, diabetes, renal failure, hypertension and diet [20–22]. These include carboxymethyl-lysine (CML), carboxyethyl-lysine, pentosidine, NFC-1 (nonfluorescent compound), i.e. glyoxal and methylglyoxal hydroimidazolones and pyralline [20–22]. The age-related increase of pentosidine and glyoxal/methylglyoxal hydroimidazolones in aorta correlated directly with its stiffness in both control and diabetic subjects [20]. Several of these AGEs have been associated with aortic collagen either by ELISA measurements in collagenase digests (pyralline) [21] or by co-localization with collagen fibers by immunohistochemical staining (CML) [23]. In another study, pentosidine levels, as determined in isolated elastin from the tunica media of human aorta, were significantly elevated by end-stage renal disease and correlated with calcium deposition [22]. This result was further confirmed immunohistochemically which showed co-localization of calcium deposits with pentosidine and elastin fibers [22].

Impact of AGE Precursors in Arterial Stiffening

Methylglyoxal is the most reactive precursor in the formation of AGEs in aging [24] and diabetes [25]. As shown in figure 1, it is reactive with arginine and lysine residues to form the minor cross-links MOLD and MODIC in association with collagen stiffening. As previously discussed, methylglyoxal can be detoxified by glyoxalase-1 into D-lactate. However, in a study of gene polymorphisms, glyoxalase-1 was not associated with arterial stiffness [26].

Glyoxal, as formed by glycation, lipid peroxidation and inflammation reactions, can form a number of minor collagen cross-links including GOLA, GOLD and GODIC (fig. 1). However, as previously discussed, glyoxal is a precursor for the adduct CML which, in turn, is thought to play an important role in the age-related degradation of elastin by its ability to chelate transitional metals and produce ROS, thus inducing arterial stiffness.

Glucose is a precursor for a number of collagen cross-links including the minor cross-link pentosidine and the major cross-link glucosepane (fig. 1). The latter probably plays a significant role in arterial stiffness.

Cellular Defense Mechanisms against Glycation

As reviewed [27], a number of cellular defense mechanisms have been proposed to lower AGE precursors and AGE formation. These include the presence of endogenous low-molecular agents serving as free radical scavengers/antioxidants (uric acid, bilirubin, reduced glutathione) and a number of enzymatic defenses (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, heme oxygenase and thioredoxin). For glyoxal and methylglyoxal, the glyoxalase-1 and aldoketo reductases (particularly aldose reductase) provide the major defense [24]. Overall, the enzymatic defense against glycation, particularly glyoxalase-1, declines with age and may contribute to the pathobiology of aging [24]. Furthermore, these defense mechanisms may be ineffective and thus irrelevant in the acellular environment of ECM for large elastic arteries.

Role of AGE Receptors in Processes of Arterial Stiffening

Binding of AGEs to receptors on the surface of endothelial cells on the vessel inner wall would elicit a number of negative responses. In this, activation of RAGE (recep-

tor for AGE) would induce cell signaling resulting in oxidative stress, increased expression of cytokines and adhesion molecules, and activation of nuclear factor-kappa B (NF- κ B) [28, 29]. The sum of these events would down-regulate antiglycation defense thus leaving the vascular bed including cells and ECM susceptible to an array of ROS, AGE, and cross-link formation. However, it has recently been argued that the concept of 'AGE receptors' – defined as cell-surface proteins which bind AGE-modified proteins to extract AGE-modified proteins from circulation and induce functional responses – has serious flaws of which the most critical one is the use of supra-physiologic protein modifications to characterize the AGE receptor, thus creating artifacts and invalidating conclusions [30].

Skin Autofluorescence as a Biomarker for Arterial Stiffness

Autofluorescence has commonly been used as a marker and measure of AGE formation and protein cross-linking in vivo. In studies of the vasculature, this has included collagen-link, elastin-link and ECM-link autofluorescence. However, it has frequently been criticized for this purpose primarily due to its nonspecificity since its formation can occur through reactions other than with sugars. Recently, there has been a pragmatic interest in autofluorescence due to the introduction on the commercial market of diagnostic devices (i.e. AGE Reader, DiagnOptics, Groningen, The Netherlands, and SCOUT DS, VeraLight, Inc., Albuquerque, New Mex., USA) capable of noninvasively measure autofluorescence of human forearm skin in screening individuals for diabetes and its complications. In short, and most importantly, skin autofluorescence (as measured by the AGE Reader) was significantly associated with age and arterial stiffness (as measured by pulse wave velocity) in both nondiabetic end-stage renal disease patients and in healthy control subjects, ages approximately 30–80 years [31]. Thus, this promising technology might provide useful information on arterial stiffening and vascular risk. However, the cause and effect relationship is not clear and there are numerous confounding factors such as sex, ethnicity and race on the interpretation of the fluorescent measures. Additionally, the chemical nature of skin autofluorescence as to specific AGE structures is poorly understood [31].

Plasma AGEs as Biomarkers for Vascular Disease and Stiffness

A number of studies have explored the role of AGEs – either as modifications to plasma/serum proteins or free circulating levels – as markers for arterial stiffness and vascular disease such as hypertension, atherosclerosis and stroke. Since this topic has previously been reviewed by Monnier et al. [32], this work represents only an update. In short, circulating levels of AGEs in both diabetic and nondiabetic individuals have been related to aortic stiffness and hypertension [33], arteriosclerosis [34] and atherosclerosis [35]. Although not reviewed here, a secreted isoform of RAGE, i.e. soluble RAGE (sRAGE) believed to be involved in the removal/detoxification of AGEs, increased in plasma of patients with atherosclerosis [36] and stroke [37].

Interventions of Glycation in Arterial Stiffness

Here we review nine conceptual approaches for minimization of AGE accumulation as related to protein damage of collagen and elastin in arteries by the Maillard reaction.

Caloric Restriction

The effect of caloric restriction in reducing AGE formation in arteries has not been studied but assumed to be the same as other tissues. In this, it is conceptualized that by decreasing the glycation burden through caloric restriction, it will minimize or even reverse the formation of Amadori products and prevent AGE accumulation in arteries. AGE levels in skin collagen are efficiently decreased through caloric restriction in rodents and in part in rhesus [38], but not in squirrel monkeys [38]. In diabetes, decreasing glycemia in humans through intensive therapy over 7–10 years has been found efficient at decreasing skin collagen glycation and AGE formation in individuals with type 1 diabetes [39]. However, data are not available in nondiabetic humans, in part because long-term caloric restriction is difficult to achieve in humans. Short-term low-calorie diet caused weight reduction and successfully reduced arterial stiffness and increase vascular compliance in *ob/ob* mice [40] and obese men [41] when given for 4 and 12 weeks, respectively. Because of the short duration of this intervention, it is likely that the reduced arterial stiffness is primarily due to the lowering of vascular resistance in the smaller vessels rather than slowing down the stiffness of the larger ones.

Exercise

Studies in humans [42] and rodents [43] have shown that the age-related increase in arterial stiffness is less pronounced in individuals with regular exercise. It is proposed that the Maillard reaction results in intermolecular cross-links between the triple helices of collagen fibers thus rendering these fibers too stiff for their optimal functioning and consequently of the particular tissue involved [44]. In adult men, there is a significant inverse relationship between autofluorescence in skin by AGE Reader versus muscle strength and power as measured by grip strength and leg extension power [45]. In rodents, long-term exercise decreased plasma levels of protein-link autofluorescence and CML [46] as well as inhibited the age-related cross-linking of collagen in heart muscle as determined by collagen solubility [47]. It is thus hypothesized that exercise and training increases the turnover rate of collagen and hence attenuates the age-related cross-linking by the Maillard reaction in tissues [44]. However, this hypothesis has not been rigorously tested.

Engineering of Deglycating Enzymes

Another conceptual strategy is to target Amadori products by using ‘deglycating’, ‘deglycase’ or ‘Amadoriase’ enzymes, thus effectively removing the Amadori product and preventing AGE formation. Presently this approach is more hypothetical than practical and is geared toward the treatment of diabetic complications. Thus, considerable more research is needed. In short, three groups of deglycating enzymes have been discovered so far [48]: (1) FAD-containing fructosyl-amine oxidases, (2) ATP-dependent fructosamine 3-kinase (FN3K) and FN3K-related protein (FN3K-RP), and (3) ATP-dependent fructoselysine 6-kinase and glucoselysine-6-phosphate deglycase. The FN3K and FN3K-RP enzymes have the ability to deglycate proteins intracellularly, such as glycated hemoglobin. Since they are ATP-dependent enzymes, they are not suitable to prevent the consequences of excessive glycation of ECM proteins. In contrast, the FAD enzymes recognize only low-molecular-weight substrates such as glycated amino acids and small peptides, i.e. they have no activity against glycated proteins [49]. Thus, complex protein engineering would be required to adapt these enzymes into molecular tools for probing the impact of the Maillard reaction in aging and age-related diseases [48].

Decrease Intake of Exogenous AGEs

Exogenous AGEs are believed to be absorbed into the circulation from reactions between sugars and proteins

in foods. Circulating AGEs have been correlated with vascular compliance in humans and may therefore also induce vascular stiffening [33]. Other studies have shown that exogenous sources of AGEs such as tobacco smoke and the diet play an important role in elevating circulating and tissue-deposited AGE levels [9]. The importance of diet-derived AGEs has been confirmed by a number of experimental studies with humans and rodents showing an association between increase AGE intake and the development and progression of diabetes and nondiabetes-related pathologies [9]. It has been hypothesized that these diet-derived AGEs have the same ability to cross-link proteins and induce oxidative stress and inflammation as their endogenous sources [9]. However, a considerable amount of controversy exists as to what the exact contribution of these exogenous sources means to the total body burden of AGE levels in tissues and what their significance means to overall health. Thus, further investigations are needed [50]. As to caloric restriction, a diet consisting of low AGE intake may be difficult to maintain over the long term in humans and thus the practicality of achieving such a diet, especially in healthy individuals, is in question.

Clearance of AGEs

Impaired renal clearance of AGE precursors and free AGEs contributes to increase plasma circulating AGEs and AGE residues of proteins in the tissues during uremia. Both hemodialysis and peritoneal dialysis were able to remove free AGEs in the plasma, but the efficiency varied among the dialysis method as well as the specific AGE measured: CML, CEL, G-hydroimidazolone, MG-hydroimidazolone, 3-deoxyglucosone, pentosidine (fig. 1) [51]. Additionally, it has been postulated that certain AGE-receptors such as AGE-R1 (p60), AGE-R2 (p90) and AGE-R3 (galectin-3) could conceivably modulate the clearance of AGEs [28, 52]. However, this has never been proven and, as previously discussed, the concept of AGE receptors has flaws.

Antagonists of AGE Receptors

A soluble form of RAGE (sRAGE) is known to block AGEs from binding to RAGE on cells and inducing pathological responses. In short, treatment of diabetic mice with sRAGE causes the suppression of atherosclerosis (streptozotocin diabetic Apo E^{-/-} mouse model) [28] and renal failure (C57BLKS/J *db/db* mouse model) [53]. Although not yet tested for its therapeutic value, levels of circulating endogenous secretory RAGE (esRAGE) are significantly lower in diabetic individuals

and have been associated with arterial stiffness, but not age [29].

Turnover Stimulation/Stem Cell Treatment

It has been proposed that arterial stiffening could be corrected by increasing the turnover of collagen and elastin by treatment with lineage cells from fibroblasts and mesenchyme [54], or by using endothelial progenitor cells to treat patients with hypertension [55]. However, these proposals are highly speculative and currently there are no known methodologies for such treatments.

Blockage of Endogenous AGEs

Since the introduction of aminoguanidine (pimage-dine) as a 'super nucleophile' to block the Maillard reaction in vivo, a large number of studies with this and other agents have been found effective in reducing AGE accumulation in tissues in experimental diabetes [56]. These include ACE inhibitors, ALT-711 (alagebrium), aspirin, AT1 antagonists, pyridoxamine, benfotiamine, carnosine, metformin, OPB-9195, pyridoxamine, sRAGE, thiamine, thiazolidinediones. Additionally, the anti-dementia drug, tenilsetam, and a number of polyphenolic compounds including curcumin, resveratrol and green tea extract have also been found effective.

The beneficial effect of aminoguanidine in reducing arterial stiffness in diabetes is well documented. In rodent models, it prevented the detrimental changes of cardiac structure and function [57], improved cardiac compliance, and decreased vascular hypertrophy [58]. It also attenuated diabetes-induced AGE formation and cross-linking of collagen in the arterial wall [59]. Similar beneficial effects have been observed in a rodent model of hypertension [60]. In aging studies, aminoguanidine was able to prevent the age-related increase in cardiac hypertrophy and arterial stiffening [61] as assessed by heart weight, impedance and elastic modulus of the aorta, carotid distensibility and thoracoabdominal pulse wave velocity. However, it is unlikely that any of these findings in rodents could be extended to humans because of the reported side effects of aminoguanidine associated with its chronic administration during a human trial which included vasculitis and abnormalities in liver function [62].

Despite their diverse chemical structures and a variety of different mechanisms of action, one intriguing observation concerning all of these compounds is that many of them have chelating properties [63], and that chelators themselves have beneficial properties against diabetic complications [64]. In diabetes, there is a strong association between transition metals and diabetic complica-

tions [64]. However, in normal aging, the role of redox active metals in explaining the age-related increase in stiffness and cross-linking is questionable. In a study from this laboratory, aminoguanidine was found to have only a very minimal effect in inhibiting the age-related increase in tail collagen cross-linking in normal rats [65]. Also, it had no effect at retarding the age-related increases of pentosidine and CML in insoluble collagen from skin and tendon [65]. Thus, these results raise important questions on the mechanism of AGE formation and cross-linking of collagen during normal aging as opposed to that found in diabetes.

Breakers of Glycation Cross-Links

Chemists have known for a long time that the carbon-carbon bond of α -diketones can be cleaved by certain thiazolium salts [56]. Thus, the concept of 'AGE cross-link breakers' was formulated based upon the assumption that these compounds can 'break' dicarbonyl bridges thought to form during the Maillard reaction and play a role in the cross-linking process [66]. Working on this assumption, PTB (N-phenacylthiazolium bromide) was initially synthesized as an agent to selectively cleave these putative bridges, i.e. the Amadori dione cross-link [66]. However, because of the unstable nature of PTB in physiological buffers, i.e. PTB undergoes rapid hydrolysis forming a cyclic hemithioacetal [67], analogs were tested of which the stable ALT-711 was identified, i.e. alagebrium (phenyl-4,5-dimethylthiazolium bromide) [66], also referred to as PMT [68]. In vitro, PTB can rapidly cleave the model diketone phenylpropanedione with release of benzoic acid [66]. It was also demonstrated that treatment of diabetic rat red blood cells (RBC) with PTB or alagebrium [66] caused the breaking of the RBC-IgG cross-link and the rapid release of IgG from the RBC's surface. Most importantly, however, when either PTB or alagebrium were incubated with tail collagen from diabetic rats *ex vivo*, they restored the fragmentation pattern on cyanogen bromide digestion to that observed in healthy control rats suggesting that these agents were able to cleave the diabetes-induced and AGE-mediated cross-links of collagen.

However, the concept of 'AGE breakers' defined as agents that effectively cleave pre-existing AGE structures and cross-links in vivo is controversial. First, the α -dicarbonyl structure mechanistically proposed as the Amadori dione cross-link [69] has not been found in vivo, nor in vitro [70]. Thus, its involvement, as well as its actual existence in Maillard processes in vivo, has been seriously questioned [70]. Secondly, attempts to repeat some of

the AGE breaker experiments just described have failed. In these studies, although PTB and alagebrium were able to cleave model compounds, they both failed to break AGE cross-links (as determined by collagen solubility) in *ex vivo* samples of skin and tail collagen from diabetic rats [68]. Similarly, Mentink et al. [71] failed to detect any cleavage of cross-links (as determined by differential scanning calorimetry) by alagebrium when added to incubations of rat tail tendons or skin collagen which were highly modified with AGEs and cross-links by incubating with 100 mM glucose or ribose for 43 days. Thirdly, the stability of AGE breakers in solution has been questioned [7, 63, 67, 71]. Thus, it has been concluded that the term 'AGE breaker' is a misnomer [56] and mechanistically a flawed concept [48]. There is no evidence to date that any AGE 'breaker' can cleave any crosslinks of the advanced Maillard reaction.

Alternatively, there is overwhelming evidence that both PTB and alagebrium are inhibitors of AGE formation [48, 56]. The most likely mechanism for their in vivo action is their properties in metal chelation [48, 56, 63, 72]. Thus, especially in the diabetic milieu, this means inhibiting the oxidative degradation of lysine residues by α -dicarbonyl sugars, such as methylglyoxal, or their fragments by MCO-mediated Strecker degradation as proposed by Suyama and colleagues [73]. However, other mechanisms have been proposed as well including their antioxidant activities [71], their ability to inhibit autooxidation of ascorbate [63] and glucose [71], and their ability to promote the metabolism of triosephosphates thus avoiding the generation of the AGE precursor methylglyoxal [74].

By far the most studied and most advanced anti-AGE agent in clinical development is alagebrium. Although not reviewed here, in animal models including rodents, dogs and monkeys, alagebrium was effective in reducing stiffness in the large arteries and myocardium while enhancing cardiac output, functioning and performance. In short, alagebrium improved cardiovascular function in animal models of aging, diabetes, atherosclerosis and hypertension. However, in human clinical studies, the results have been mixed depending upon the age, severity and extent of heart failure in patients treated. Overall, clinical trials using alagebrium to target systolic hypertension have not supported benefits. Recently the BENEFICIAL trial has been conducted in The Netherlands to examine the effects of alagebrium on exercise capacity and cardiac function in patients with systolic heart failure. Unfortunately, these results also do not support benefits [75].

Conclusions

Human aging, especially in the large arteries, is characterized by decreased turnover of ECM (mainly elastin and collagen) paralleled with increased AGE formation and cross-linking, though few studies have been carried out with human tissues per se. The elastic layers which bear most of the pulsatile pressure stresses undergo fracture, fragmentation, splitting and disorganization subsequent to their gradual replacement by collagen fibers and proteoglycans [1]. Collectively, these events cause the loss of elasticity and induce stiffening and incomppliance in arteries. The treatment and reversal of arterial stiffness has been met with both great optimism and pessimism. In retrospect, over the past 20 years, the field has exploded with a variety of newly proposed drug agents and therapies to inhibit AGE formation especially in the treat-

ment of diabetes [56, 72]. Much reliance exists on the ability of 'AGE breakers' to cleave AGE cross-links in ECM, thus reversing the age-related stiffness in arteries and even the aging process. However, as reviewed here, there is presently little evidence that these agents actually break pre-existing AGE cross-links in vivo. Conversely, pharmacological treatment of aortic stiffness appears impossible [1]. This assessment is due to the age-related morphological changes in elastic fibers which appear impossible to correct. Drugs currently used to treat arterial stiffness, such as nitroglycerine, which have a dominant effect on the muscular arteries, have no effect on the aorta [1]. Finally, while the consequences of age-related stiffening of arteries are easily understood and well documented, it remains to be seen whether decreasing oxidative and carbonyl stress would indeed prevent stiffening and result in lower morbidity and mortality.

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