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ACCUMULATION OF ADVANCED GLYCATION END PRODUCTS IN AGE-DEPENDENT DISORDERS

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ABSTRACT. INCUBATION OF PROTEINS with reducing sugars such as glucose and fructose lead, through formation of Schiff bases and Amadori products, to the generation of advanced glycation end-products (AGE) of the Maillard reaction. Recent studies have demonstrated that accumulation of AGE in tissue proteins increases with the pathogenesis of diabetic complications and atherosclerosis, strongly suggests an association of AGE for the development of age-related disorders. Since this reaction progresses non-enzymatically almost all of the proteins in accordance with reducing sugar concentration and the half-life of each protein, it is different from enzymatic post-translational modification such as phosphorylation and acetylation of some specific proteins. Although the formation of Amadori product, relatively stable intermediates of the reaction, is reversible, the formation of AGE is irreversible reaction, indicating that AGE-formation may result in the irreversible denature and inactivation of proteins in vivo. Although the formation of AGE was believed to proceed on extracellular lesion from the relatively inert aldehyde such as glucose, many recent publications have strongly demonstrated that AGE is also formed on intracellular space from more reactive intermediates in carbohydrate metabolism. Furthermore, several AGE inhibitors such as pyridoxamine and thiamine have shown promise in model systems for inhibiting both the Maillard reaction and the development of diabetic complications. This review describes the proposed pathways for the formation of AGE during the Maillard reaction and role of the reaction in the pathogenesis of age-related diseases.

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INTRODUCTION

Advanced glycation end-products (AGEs) are formed via nonenzymatic glycation of proteins with reducing sugars such as glucose, glucose 6-phosphate, fructose and ribose, and are composed of heterogeneous structures characterized by a yellow-brown color, an autofluorescence, intra- and intermolecular cross-linkings (FIG. 1). AGE is also recognized by AGE receptor expressed on cell surface membrane. Several AGE-specific structures were identified, which include pyrroline [1], pentosidine [2], cross-lines [3], N^ε-(carboxymethyl)-lysine (CML) [4], N^ε-(carboxyethyl)lysine (CEL) [5], GA-pyridine [6], glyoxal-lysine dimer (GOLD) and methylglyoxal-lysine dimer (MOLD) [7], imidazolone [8], and vesperlysines A, B and C [9] (FIG. 2). Among these products, several *in vitro* experiments demonstrated that CML [10] is a major antigenic AGE structure *in vivo*. CML is generated through three pathways *in vitro*; by oxidative cleavage of Amadori products, cleavage of Schiff base through Namiki pathway and autoxidation of glucose through glyoxal. Among these, oxidative cleavage of Amadori products into CML is considered a major pathway *in vivo* because this pathway mostly progresses under physiological phosphate concentrations.

The AGE research originally began in the field of food chemistry and has been enormously expanded to *in vivo* study since 1990's because of the development of polyclonal and monoclonal antibodies against AGE-modified proteins. These immunochemical approaches have greatly contributed to understanding the biological significance of AGE in the pathogenesis of age-enhanced diseases [11]. For instance, AGE modification is found to be involved in the normal aging [12], as well as in the pathogenesis of several age-enhanced diseases such as diabetic nephropathy [13], atherosclerosis [14], diabetic retinopathy [15], hemodialysis-associated amyloidosis [16], chronic renal failure [17], and Alzheimer's disease [18]. Furthermore, AGE accumulated in hippocampal neurons, neurofibrillary tangles, peripheral nerves, atherosclerotic lesions in rats aortas, atherosclerotic coronary arteries, murine amyloid, peritoneum in patients on continuous ambulatory peritoneal dialysis [19], human skin elastin in actinic elastosis

[20], cardiac tissues of renal transplant patients [21], pulmonary fibrosis [22]. Therefore, AGE is now widely accepted as one of important post-translational modifications of proteins that contribute to the aging and pathological process (FIG. 1).

IMMUNOCHEMICAL DETECTION OF AGE

Immunological strategies have been used to demonstrate the presence of AGE in several human and experimental animal tissues and enhanced AGE accumulation has been actually demonstrated in several pathological tissues. Therefore, accurate measurement of AGE content is fundamental to studies on aging process and the pathogenesis of age-dependent diseases. We previously demonstrated that CML is formed from Amadori product artificially by alkaline treatment [23]. Thus, incubation of glycated human serum albumin (HSA) in 0.1 N sodium hydroxide led to the formation of CML whereas reduced glycated HSA or non-glycated HSA did not generate CML. These results strongly demonstrated that alkaline treatment might result in false-positive or high background in CML analysis. We applied this procedure to measure the content of Amadori product in human serum. Human sera (N = 224) were incubated with 0.1 N sodium hydroxide for 16 h at 37°C to convert Amadori product into CML and formed CML was determined by anti-CML antibody (6D12). CML level in alkaline-treated human sera were correlated well with glycated albumin value ($r^2 = 0.912$) which was determined by HPLC. Likewise, alkaline-treated glycated-bovine serum albumin also significantly increased the reactivity with 6D12 in accordance with Amadori content, demonstrating that Amadori compounds on HSA and BSA were measured as CML by alkaline-treatment [23].

Amadori product is also converted into CML by heating process. In immunochemistry, the heat-induced epitope retrieval technique is extensively used with formalin-fixed paraffin-embedded tissue sections. However, we demonstrated that CML was generated by heating, directly from oxidative cleavage of Amadori products or via several reactive aldehydes, might serve as a false-positive results observed in the immunohistochemical study with the anti-CML antibody after antigen retrieval

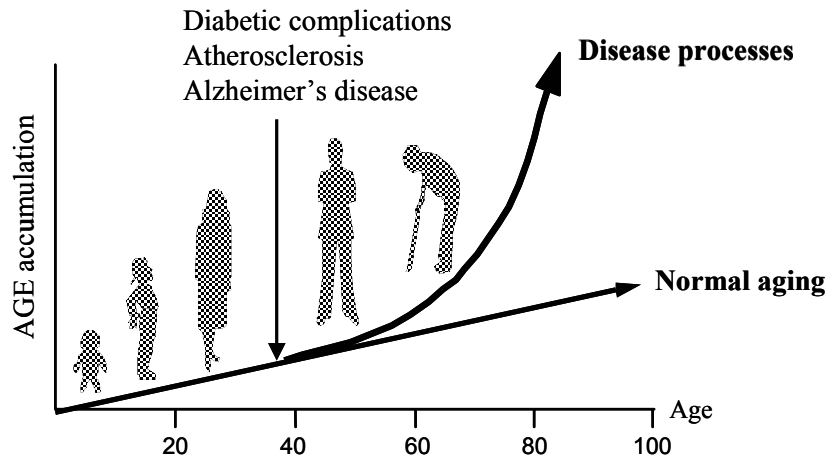
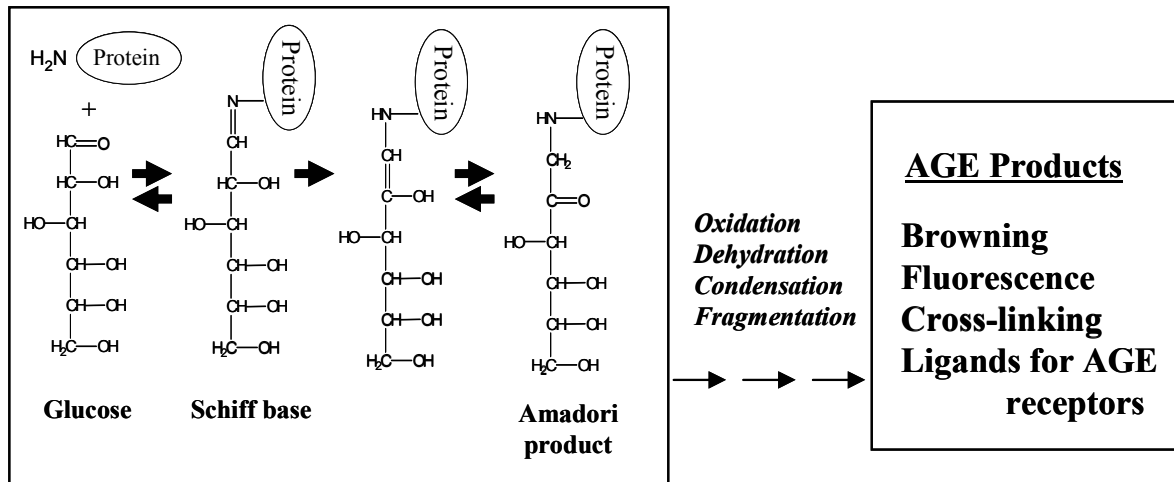


FIGURE 1. THE MAILLARD REACTION. Accumulation of AGEs is enhanced during the pathogenesis of age-related diseases such as diabetic complications, atherosclerosis and Alzheimer's disease.

by heating [24]. Therefore, it is necessary to take into consideration the possibility of artificial AGE formation during sample preparation to prevent false-positive and undesirable high background.

ROLE OF REACTIVE OXYGEN SPECIES (ROS) IN AGE FORMATION

ROS plays an important role not only in the formation of several ROS-dependent AGEs such as CML and pentosidine but also in the formation of cross-linking of proteins [25]. As described above,

CML is formed in three pathways in vitro. Since CML formation is inhibited by antioxidants and antioxidative conditions, the involvement of ROS in CML formation has been suggested. However, little is known about which ROS is responsible for this process. To solve the issue, the effect of ROS such as superoxide anion radical ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\text{OH}\cdot$) on the formation of CML was measured. Since the Fe^{2+} -induced CML formation was enhanced by the addition of H_2O_2 , it seems reasonable to expect that $\text{OH}\cdot$ generated by Fenton reaction between Fe^{2+} and H_2O_2 , derived endogenously from Amadori

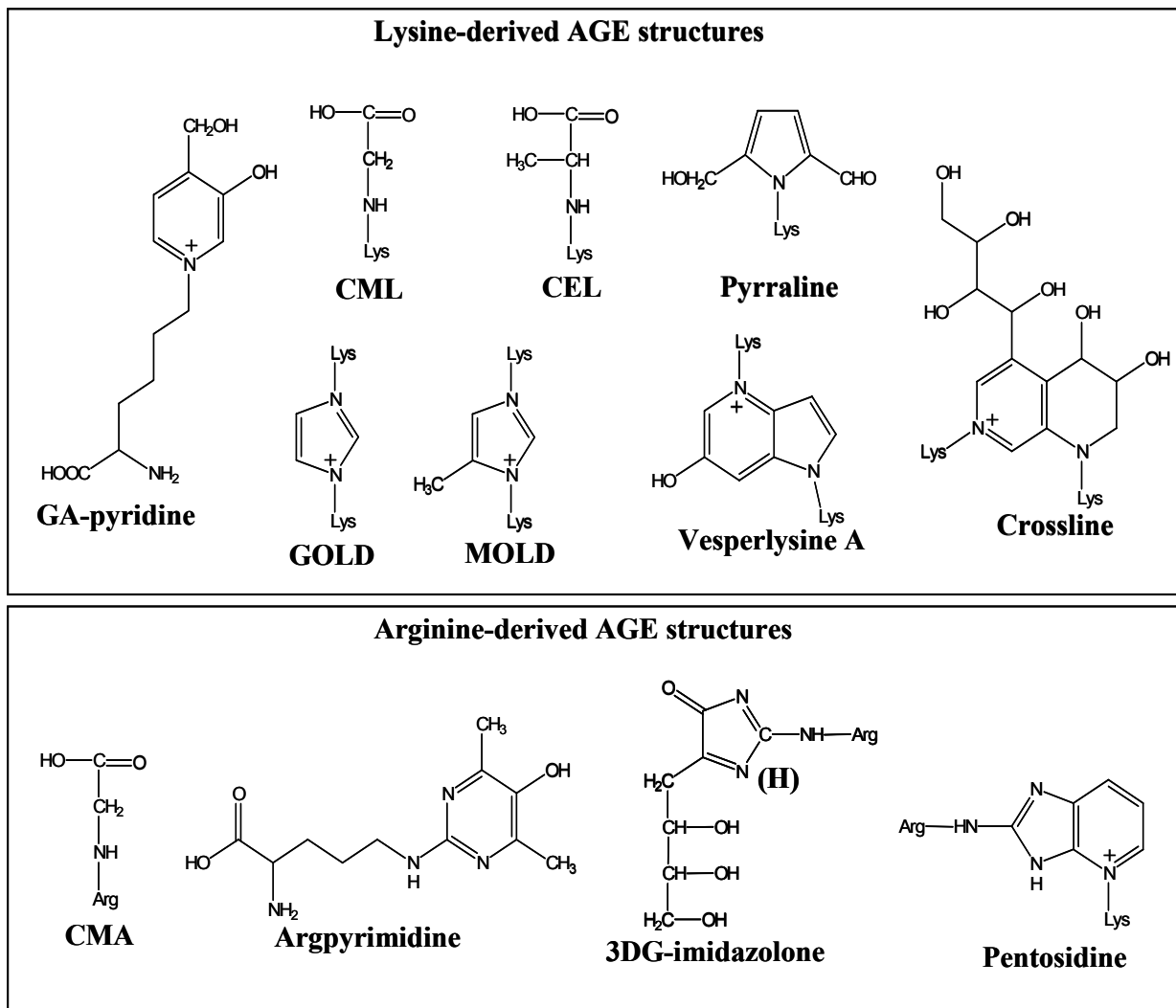


FIGURE 2: STRUCTURES OF AGEs.

products, might play an important role in the formation of CML from Amadori product on glycated proteins (FIG. 3) [26]. Furthermore, to clarify a direct role of $\text{OH}\cdot$ in the Fe^{2+} -induced CML formation from Amadori product, effects of several radical scavengers on CML formation was also examined. Results clearly showed that Fe^{2+} -induced CML formation was significantly inhibited by the addition of catalase, whereas superoxide dismutase had no effect on it [26]. These results demonstrated that $\text{OH}\cdot$ generated by Fenton reaction between Fe^{2+} and Amadori product-derived endogenous H_2O_2 plays an important role in oxidative cleavage of Amadori compounds into CML.

CML is also generated from oxidative cleavage of Amadori product by peroxynitrite (ONOO^-). Nitric oxide (NO) is known to play a role in endothelium-derived relaxing factor, and exhibits several physiological functions such as inhibition of neutrophil adhesion, platelet aggregation and regulation of vascular elasticity. Furthermore, NO is extremely reactive to $\text{O}_2^{\cdot-}$, generating ONOO^- (rate constant of ONOO^- formation is $7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$), which functions as an oxidant to proteins, vitamins and DNA (FIG. 3) [27]. For instance, inactivation of Mn-superoxide dismutase by ONOO^- is thought to be involved in the rejection mechanism in kidney transplantation [28]. The half-life of nitrate protein

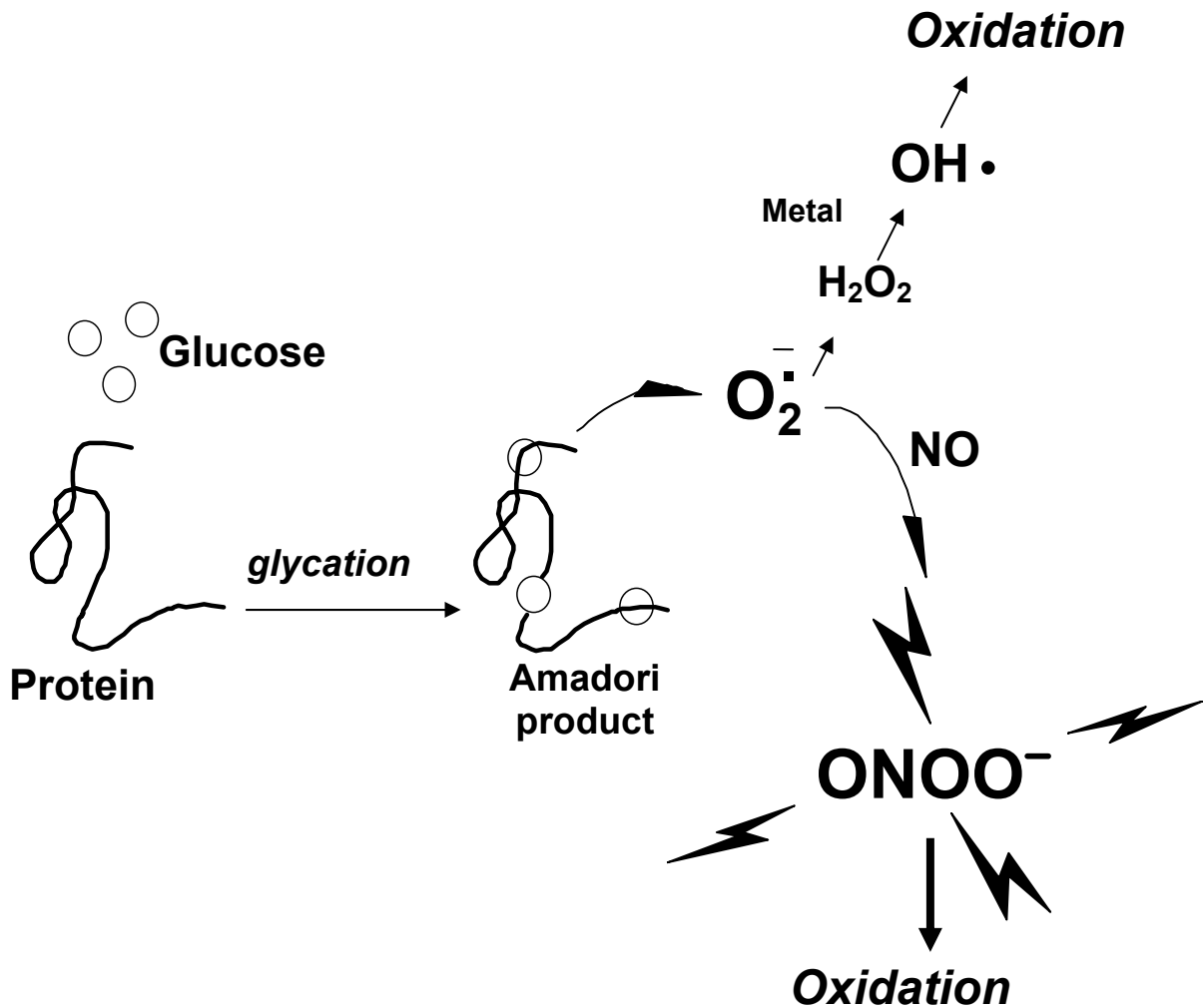


FIGURE 3: GENERATION OF REACTIVE OXYGEN SPECIES FROM THE MAILLARD REACTION. Although CML is generated from oxidative cleavage of Amadori product, Amadori product is also known to generate $O_2^{\cdot-}$, leading to the production of $OH\cdot$ and $ONOO^-$.

by $ONOO^-$ is shorter than that of the native protein. NO and $O_2^{\cdot-}$, both of which can be generated from aortic endothelial cells under hyperglycemia, can disturb the functions of those cells and are known to induce vascular disorders. Furthermore, $O_2^{\cdot-}$ generated from glycated low-density lipoprotein (LDL) reacts with NO , and decreases the NO -induced modulation of cellular cyclic GMP levels. Thus, these reports indicate that the generation of $ONOO^-$ as well as $O_2^{\cdot-}$ is enhanced by hyperglycemia. $ONOO^-$ is known to exhibit

considerably greater toxicity than the $OH\cdot$ generated extracellularly because $ONOO^-$ is formed by a diffusion-limited reaction between $O_2^{\cdot-}$ and NO , with a half-life of 1.9 second at pH 7.4 that permits diffusion over several cell diameters. We also demonstrated that $ONOO^-$ induces CML formation not only from Amadori product but also from the $ONOO^-$ -treated glucose pathway in which glucosone and glyoxal play an important role [29]. Since the rate constant of $ONOO^-$ formation is 1,000-fold higher than that of the $OH\cdot$ radical and

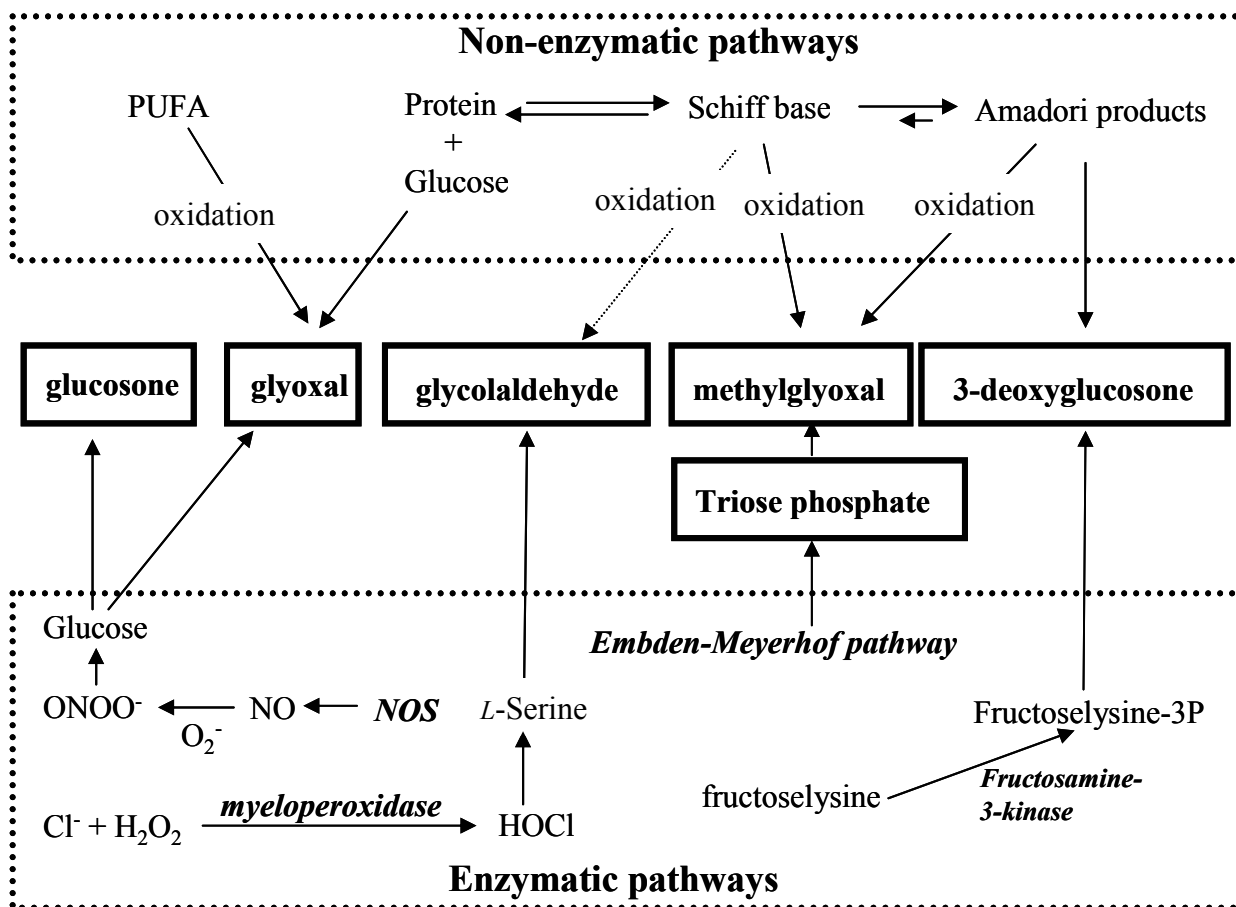


FIGURE 4: GENERATION OF INTERMEDIATE ALDEHYDES. Reactive aldehydes are generated during the Maillard reaction (non-enzymatic pathway) and carbohydrate metabolism (enzymatic pathways), both of which enhance AGE formation. PUFA, polyunsaturated fatty acid.

the half-life of ONOO⁻ is 10⁹-times longer than that of OH[•] radical under physiological condition, it is likely that the formation of CML *in vivo* is mediated by ONOO⁻ rather than OH[•] in pathological conditions. Thus, ONOO⁻ is highly reactive and potentially a major oxidant and source of CML *in vivo* even in the absence of free metal ions.

IDENTIFICATION OF NOVEL AGE STRUCTURE *IN VIVO*

As described above, monoclonal and polyclonal antibodies are widely used to demonstrate the presence of AGE-modified protein *in vivo*. Monoclonal antibody is also applied to identify

novel biological marker in pathological lesions. Several reactive aldehydes such as 3-deoxyglucosone, glyoxal and methylglyoxal are formed from Maillard reaction and metabolic pathway *in vivo* and are considered to be precursors of AGEs (FIG. 4). In a parallel pathway involving both enzymatic and non-enzymatic reactions during inflammation, leukocytes are activated to secrete myeloperoxidase that mediates the formation of hypochlorous acid (HOCl) from H₂O₂ and chloride. A reactive aldehyde such as glycolaldehyde (GA), which is formed by reaction of HOCl with serine, reacts to form chemical modifications in protein. GA is known to react with protein amino residues to give brown-colored cross-linked structures. A recent

study demonstrated that the reaction of myeloperoxidase-derived GA with RNase led to CML, indicating that CML is formed in GA-modified protein [30]. However, since CML is also formed during the Maillard reaction by oxidative cleavage of Amadori product, CML is not a specific marker for myeloperoxidase-induced protein modification *in vivo*. Therefore, to obtain a specific marker for myeloperoxidase-induced protein modification *in vivo*, it is required to identify a GA-protein adduct(s) other than CML. Demonstration of such a specific marker in atherosclerotic lesions would establish a role for the myeloperoxidase system in chemical modification of proteins during atherogenesis. To this end, monoclonal antibodies (GA5 and 1A12) as well as polyclonal (non-CML-GA) antibodies that are specific for GA-modified proteins have been prepared [6]. These antibodies specifically reacted with GA-modified or hypochlorous acid-modified BSA, but not with BSA modified by other aldehydes such as glucose, glyoxal, methylglyoxal and 3-deoxyglucosone, indicating that the epitope structure of these antibodies could be specific for GA-modified proteins. Following successive HPLC purification procedures, the GA5-reactive compound was isolated and its chemical structure was found to be 3-hydroxy-4-hydroxymethyl-1-(5-amino-5-carboxypentyl) pyridinium cation. This compound named as GA-pyridine could be recognized both by 1A12 and non-CML-GA, indicating that GA-pyridine is an important antigenic structure in GA-modified proteins. Immunohistochemical studies with GA5 demonstrated the accumulation of GA-pyridine in the cytoplasm of foam cells and extracellularly in the central region of atheroma in human atherosclerotic lesions [6]. These results suggest that myeloperoxidase-mediated protein modification via GA may contribute to atherogenesis.

AGE RECEPTOR

Cellular interactions with AGE-proteins are known to induce several biological responses, not only endocytic uptake and degradation, but also induction of cytokines and growth factors, which are likely linked to the development of diabetic vascular complications. These responses are thought to be

mediated by AGE-receptors which include a RAGE (receptor for AGE) [31], SR-A (scavenger receptor type A) [32], CD36 [33] LOX-1 (lectin-like oxidized LDL receptor-1) [34], SR-BI (scavenger receptor class B type I) [35] and megalin [36]. Our recent studies using peritoneal macrophages obtained from SR-A-knockout mice [32] showed that SR-A plays a major role, particularly in the endocytic degradation of AGE-proteins by macrophages and macrophage-derived cells. However, since scavenger receptor families recognize only extensively modified AGE protein [37], which are unlikely to be found *in vivo*, it is critical to identify the AGE structure(s) responsible for recognition by the scavenger receptors.

CONCLUSION

Recent studies have suggested that several aldehydes, such as GA, glyoxal, methylglyoxal, glyceraldehydes-3-phosphate, 3-deoxyglucosone and glucosone are generated from the Maillard reaction (non-enzymatic pathway) and metabolic pathways (enzymatic pathways) (FIG. 4), serve as important intermediates for the formation of AGE structures. AGE inhibitors such as pyridoxamine and thiamine have developed based on in a series of experiments identifying formation pathway of AGE *in vivo*. Pyridoxamine, which traps intermediates in the Maillard reaction and lipid peroxidation reaction, significantly inhibits the development of retinopathy and neuropathy in the streptozotocin (STZ)-induced diabetic rat [38]. Furthermore, thiamine and its derivative, benfotiamine, also inhibit the development of incipient nephropathy in STZ-rat by increasing activity of transketolase, which convert glyceraldehydes-3-phosphate into ribose-5-phosphate, in renal glomeruli [39]. Taken together, these results suggest that treatment with AGE inhibitors may be a potential strategy for the prevention of clinical diabetic complications.

We also demonstrate that accumulation of CML in diffuse intimal thickening and atherosclerotic plaque in patients with end-stage renal disease significantly correlates with the duration of hemodialysis, but not with the duration of diabetes [40]. The result suggests that accumulation of AGE is enhanced not only under hyperglycemia but also successive inflammatory response. Further studies

will be important for understanding the pathophysiological role of AGE and developing potential drugs to prevent age-related disorders.

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ABBREVIATIONS USED:

AGE: advanced glycation end-products
CEL: N^ε-(carboxyethyl)lysine
GOLD: glyoxal-lysine dimer
MOLD: methylglyoxal-lysine dimer
CML: N^ε-(carboxymethyl)lysine
HSA: human serum albumin
BSA: bovine serum albumin
ROS: reactive oxygen species
O₂^{•-}: superoxide anion radical
H₂O₂: hydrogen peroxide
OH•: hydroxyl radical
ONOO⁻: peroxynitrite
NO: Nitric oxide
HOCl: hypochlorous acid

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