Oxidative Stress Biomarker and Its Application to Health Maintenance
Guest Editor: Yasukazu Yoshida

Detection of AGEs as markers for carbohydrate metabolism and protein denaturation

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Approximately 100 years have passed since the Maillard reaction was first reported in the field of food chemistry as a condensation reaction between reducing sugars and amino acids. This reaction is thought to progress slowly primarily from glucose with proteins *in vivo*. An early-stage product, called the "Amadori product", is converted into advanced glycation end products. Those accumulate in the body in accordance with age, with such accumulation being enhanced by lifestyle-related diseases that result in the denaturation of proteins. Recent studies have demonstrated that intermediate carbonyls are generated by several pathways, and rapidly generate many glycation products. However, accurate quantification of glycation products *in vivo* is difficult due to instability and differences in physicochemical properties. In this connection, little is known about the relationship between the structure of glycation products and pathology. Furthermore, the interaction between proteins modified by glycation and receptors for advanced glycation end products is also known to induce the production of several inflammatory cytokines. Therefore, those inhibitors have been developed over the world to prevent lifestyle-related diseases. In this review, we describe the process of protein denaturation induced by glycation and discuss the possibility of using the process as a marker of age-related diseases.

Key Words: AGEs, glycation, post-translational modification, diabetic complications, aging

Research on advanced glycation end products (AGEs), which are produced by a browning reaction that occurs between reducing sugars and the amino residues of proteins and free amino acids (Fig. 1), was first reported by Louis Camille Maillard in 1912. Therefore, this reaction is called the Maillard reaction, rather than glycation, in the field of food science, and the end products of this reaction are also called melanoidins. The Maillard reaction also proceeds *in vivo* by several reactions, including oxidation and condensation between reducing sugars and proteins more gradually than that observed in food processing, resulting in the induction of denaturation of proteins (Fig. 2). In other words, although carbohydrates are indispensable for ATP production, an excess of these molecules arises in irreversible functional disorders of proteins in patients with disordered metabolism. In fact,

the level of hemoglobin A1c (HbA1c), an early-stage product of the Maillard reaction, is used worldwide as a clinical marker of glycemic control in patients with diabetes, as it reflects the blood glucose level over the previous 1–2 months. However, because the stability of and methods used to detect each AGE structure differ, the clinical application of AGE analyses has not fully progressed.

Immunochemical Detection of AGEs

The quantification of AGEs *in vivo* was originally performed using the fluorescent characteristics of AGEs. Monnier *et al.*[1] reported that the accumulation of fluorescent AGEs increases in the dura mater of the brain in an age-dependent manner (Fig. 1). Subsequently, an anti-AGE antibody was developed as a more specific tool for detecting AGEs,[2] and the involvement of AGEs in various diseases has been reported. However, since the epitope structures of anti-AGEs antibodies were not identified until the 1990s,[3] little is known about the relationship between the structure and pathology of AGEs. We previously demonstrated that 6D12, which has been reported to be a monoclonal anti-AGE antibody in the 1990s,[3] recognizes both N^ε-(carboxymethyl)lysine (CML) and N^ε-(carboxyethyl)lysine (CEL) (Fig. 3).[4] CML is generated by the oxidative cleavage of Amadori products by hydroxyl radicals[5] and peroxynitrite,[6] thus suggesting that CML is an important biological marker of oxidative stress *in vivo* (Fig. 4). In contrast, CEL is generated from methylglyoxal via the Embden-Meyerhof pathway.[7]

Since AGEs are modified amino acids with molecular weights of less than 500 Da, preparation of structure-specific anti-AGE antibody is difficult. Although 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is the most conventional coupling reagent for small molecules and produces peptide bond between carrier protein and hapten, EDC-conjugated hapten-carrier adducts often fail to produce immune responses against small molecule haptens. Therefore, CML, a major antigenic AGE structure, was conjugated to human serum albumin (HSA) with three different cross-linkers, EDC, bis(sulfosuccinimidyl)suberate

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doi: 10.3164/jcbn.13-112
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J. Clin. Biochem. Nutr. | July 2014 | vol. 55 | no. 1 | 1–6
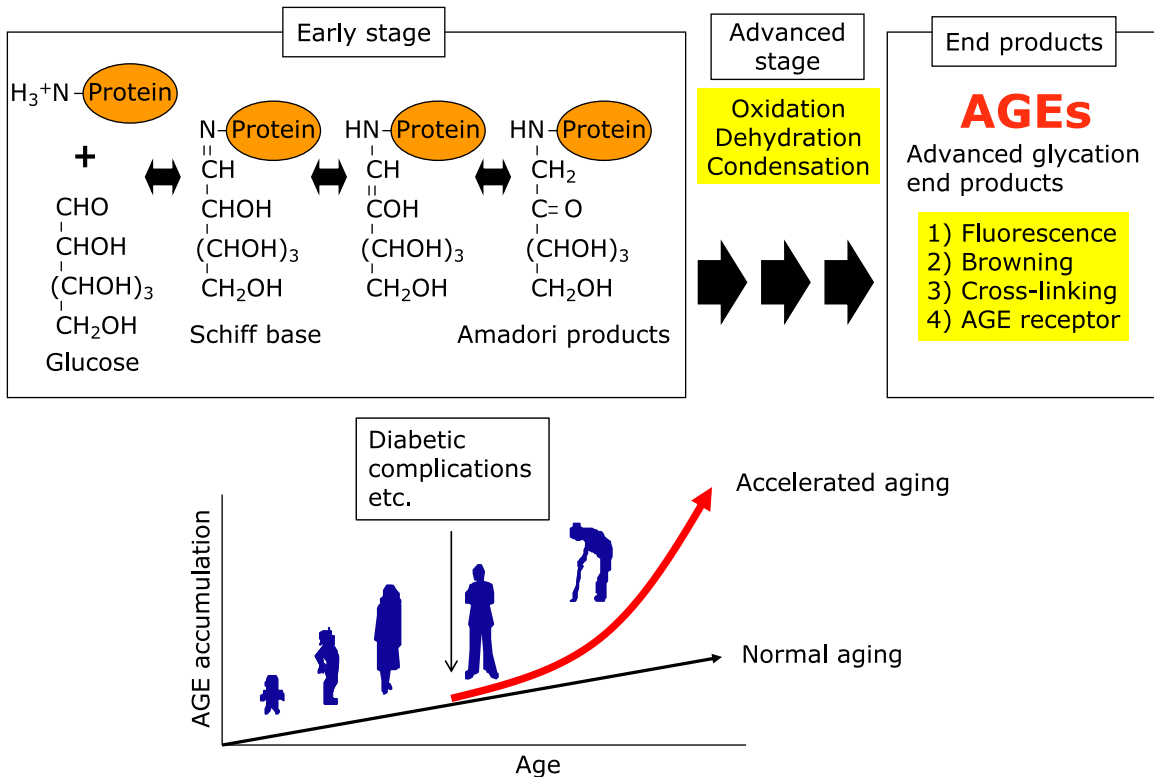
Fig. 1. Maillard Reaction. Reducing sugars such as glucose and ribose react with amino residues of proteins and free amino acid, and reaction that occurs between reducing sugars and generate AGEs through formation of Schiff base and Amadori products. AGEs are characterized by a yellow-brown color, an autofluorescence, intra- and intermolecular cross-linkings. AGEs are recognized by several AGEs receptor such as receptor for AGE (RAGE), and AGEs-RAGE interaction is reported to activate cell signaling pathways. AGEs accumulate in the body in accordance with age, with such accumulation being enhanced by lifestyle-related diseases such as diabetic complications that result in the denaturation of proteins.
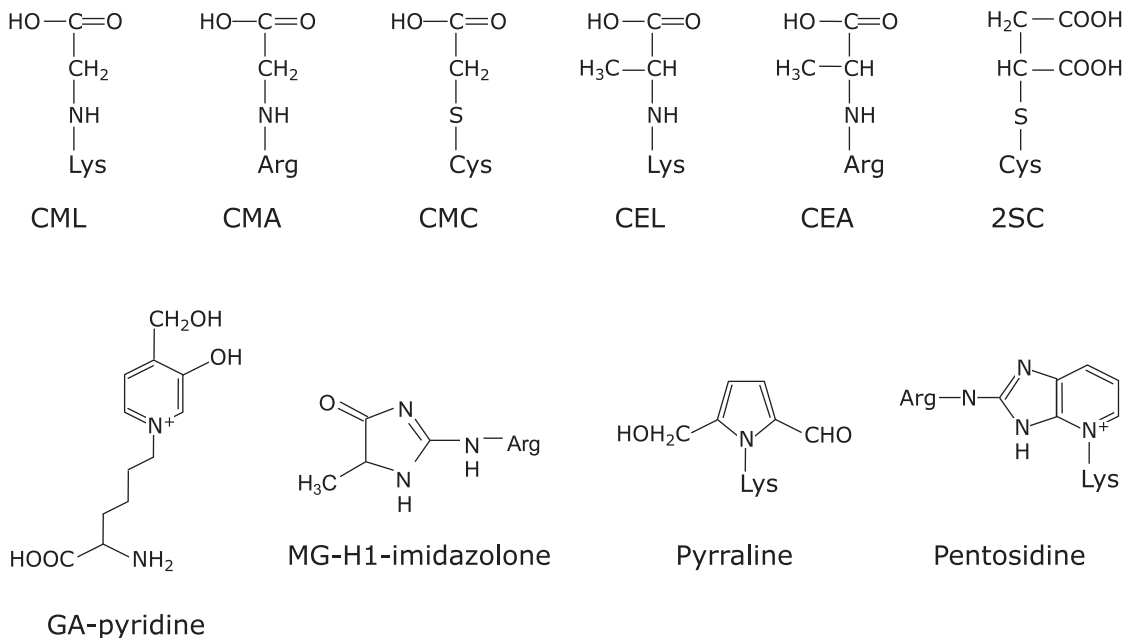
Fig. 2. Possible pathway for AGEs formation and those biological impacts to protein modification. The Maillard reaction proceeds *in vivo* between reducing sugars and proteins, resulting in the induction of denaturation of proteins. Intermediate aldehydes such as glyoxal, methylglyoxal, glucosone and glycolaldehyde rapidly modify proteins *in vivo*.
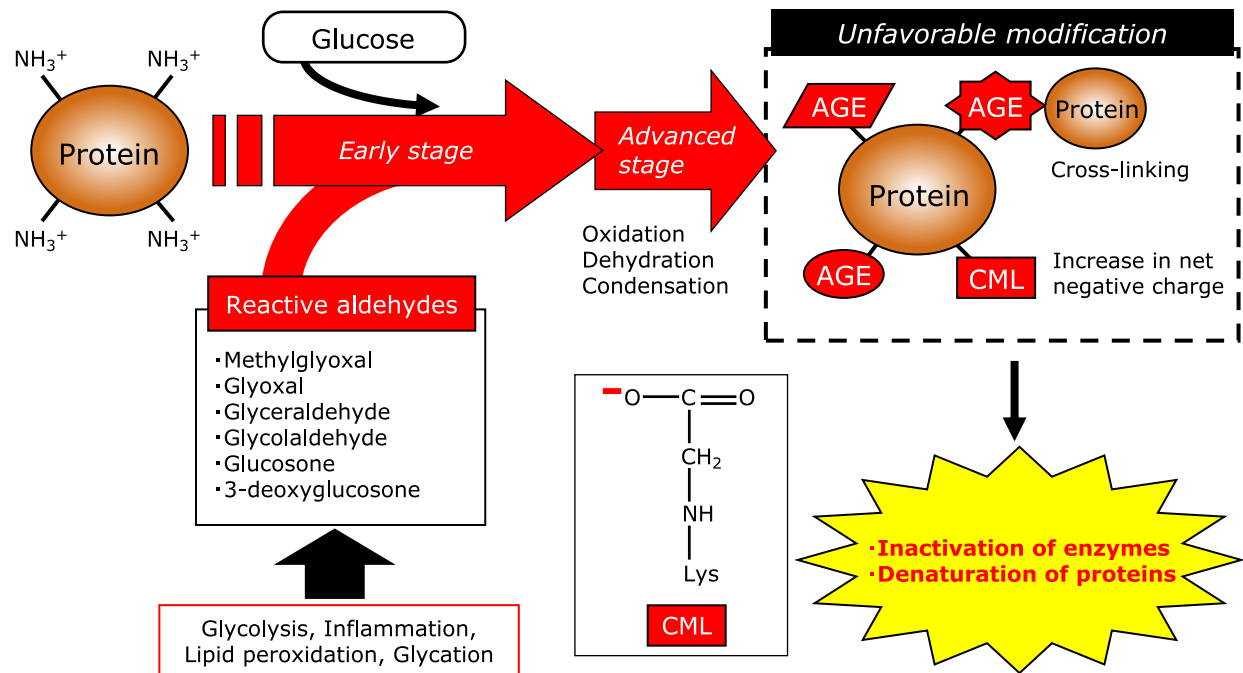
Fig. 3. Reported AGEs structures. AGEs are generated not only from glucose but also from intermediate carbonyls via glycolysis, lipid peroxidation and inflammatory response. Typical AGEs structures were shown among AGE structures reported to date.

(BS3) and glutaraldehyde, and their efficacy in the production of antibodies was compared. Although all three CML-conjugated HSAs were strongly recognized by anti-CML antibody, only CML-conjugated HSA prepared by glutaraldehyde cross-linking produced an antibody against CML.[8] Similarly, antibodies against CEL, 2SC and CMC were also obtained by conjugation to carrier proteins using glutaraldehyde, indicating that glutaraldehyde is a promising cross-linker for production of antibody against small molecules.

We previously identified new AGE structure derived from glycolaldehyde (GA) in human atherosclerotic lesions. GA is formed from serine by action of myeloperoxidase and reacts with proteins to form several products. Prominent among them is CML. Because CML is formed from several pathways as described above, we attempted to identify unique structures characteristic of the reaction of GA with protein. To this end, monoclonal antibodies (GA5 and 1A12) and polyclonal antibody (non-CML-GA) specific for GA-modified proteins were prepared. These antibodies specifically reacted with GA- and hypochlorous acid-modified BSA, but not with BSA modified by other aldehydes, indicating that the epitope of these antibodies could be a specific marker for GA-modified protein. By HPLC purification, GA5-reactive compound was isolated and its chemical structure was characterized as 3-hydroxy-4-hydroxymethyl-1-(5-amino-5-carboxypentyl) pyridinium cation. This compound named as GA-pyridine (Fig. 3) was also recognized by both 1A12 and non-CML-GA, demonstrating that GA-pyridine is an important antigenic structure in GA-modified proteins.[9] 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. These results suggest that GA-mediated protein modification may contribute to atherogenesis.[9] Taken together, anti-AGEs antibodies that epitope structures were identified are useful for evaluating the biological significance of AGEs (Fig. 4).

Difficulty of Measuring AGEs in Physiological Samples

Although the quantification of AGEs using instrumental analyses is superior to that of immunochemical analyses, anti-AGE antibodies are a convenient tool for estimating the content and examining the histological localization of AGEs. However, the immunochemical measurement of AGEs in physiological samples is associated with potential artifacts due to pretreatment techniques, such as heating and alkaline treatment. For instance, the pentosidine level in physiological samples is used as a sensitive marker for the early diagnosis of renal failure. In the quantitative measurements of pentosidine reported to date, a rapid enzyme-linked immunosorbent assay (ELISA) has been widely used to estimate the plasma/serum pentosidine levels in a number of clinical samples, because high performance liquid chromatography (HPLC) methods require multiple preparation steps before the analysis. However, the currently used clinical analysis of the plasma/serum pentosidine level by ELISA requires incubation of the plasma/serum at 100°C for 15 min to inactivate the protease,[10] which is required before the anti-pentosidine antibody can bind to the pentosidine. The pentosidine content, measured by HPLC, in the serum increased by heating in a temperature- and time-dependent manner.[11] Same tendency was also observed in CML formation. Thus, CML was generated from glycated HSA by heat treatment (above 80°C), and increased in a time-dependent manner.[12] These results demonstrated that AGEs could be generated artificially through the heating process.

Inhibitors for AGEs Formation

Recent studies have demonstrated that AGEs are generated not only from glucose but also from aldehydes such as glyoxal, methylglyoxal,[13] glucosone[6] and glycolaldehyde,[9] and those aldehydes rapidly modify proteins (Fig. 2). For instance, Mclellan *et al.*[14] demonstrated that plasma methylglyoxal concentrations in insulin-dependent diabetic patients were 7-times higher than those of healthy individuals. These reports indicate that the modification
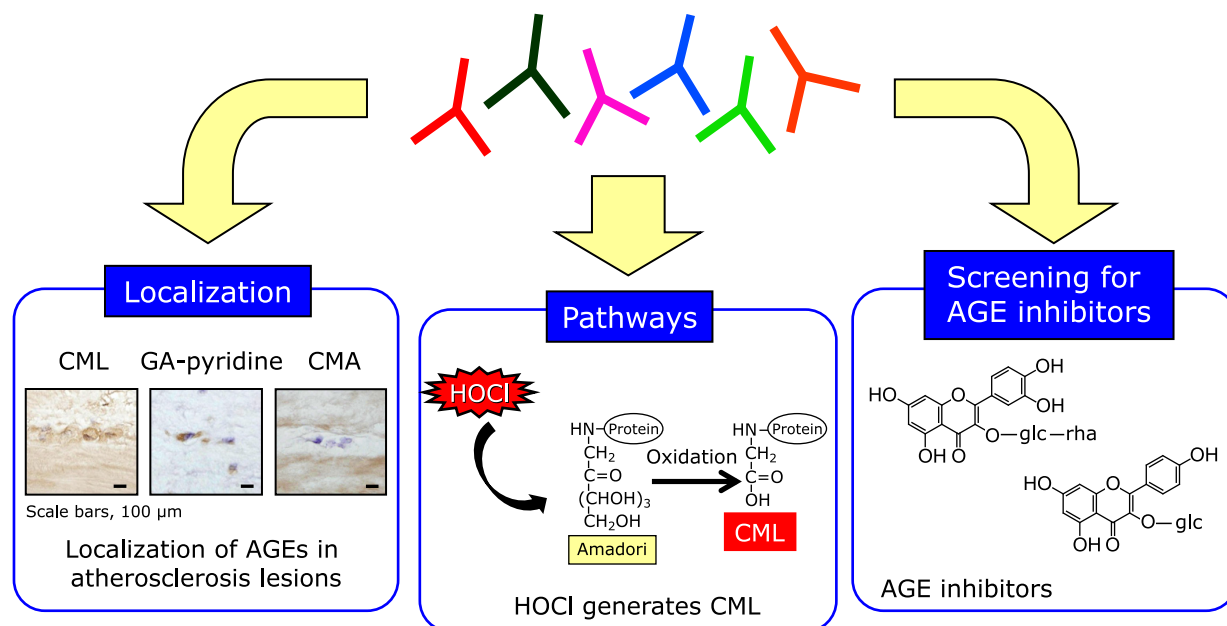
Fig. 4. Usefulness of antibody library against AGEs structures. Monoclonal anti-AGEs antibodies that epitope structures were identified are useful for evaluating the biological significance of AGEs such as localization, pathway for AGE formation and screening of AGE inhibitors.

of proteins with aldehydes *in vivo* may contribute to the development of organ disorders such as diabetic nephropathy[15] and retinopathy[16] (Fig. 2). Therefore, AGE inhibitors have been developed over the world to prevent lifestyle-related diseases such as diabetic complications and atherosclerosis. Aminoguanidine is the first AGE inhibitor with an amino residue which traps the aldehyde group of reducing sugars.[17] Thiamine and its derivative, benfotiamine, are known to decrease methylglyoxal level *in vivo* and inhibit the development of incipient nephropathy[18] and retinopathy[19] in streptozotocin-induced diabetic rats. Pyridoxamine has been shown to significantly inhibit the progress of nephropathy[20] and retinopathy,[21] although the serum glucose concentration was not changed in a rat model of streptozotocin-induced diabetes.

It is known that preventive medicine is the most important approach to preventing the development of lifestyle-related diseases such as atherosclerosis and diabetic complications. We believe that the daily intake of AGEs inhibitors in natural products can play a beneficial role in preventing the pathogenesis of lifestyle-related diseases. Therefore, natural compounds were screened as potential inhibitors of AGEs formation (Fig. 4). We previously reported that *Astragalus radix* samples for the natural herb-derived compound inhibits formation of CML and pentosidine from the reaction of bovine serum albumin and ribose. Our findings confirmed that astragaloside significantly inhibits both of these AGEs,[22] although the inhibitory effect of the compound has not yet been confirmed *in vivo*. Furthermore, we also reported that high concentration (>1 mM) of catechol compounds such as epicatechin, gallic acid and 4-MC exhibits enhancing effects of CML formation by producing hydrogen peroxide, 0.01 mM of these compounds inhibits CML formation due to their high antioxidative activity.[23] This study provides the evidence that natural compounds containing catechol residues enhance CML formation and that high dose of flavonoid supplementation should be conducted with care to prevent any unfavorable aspects of antioxidants.

Discovery of New Post-translational Modification

Glycation research has recently attracted attention with respect to the study of lifestyle-related diseases, including metabolic syndrome, abnormalities in carbohydrate and/or lipid metabolism. In addition, although quantifying the amount of AGEs *in vivo* is difficult due to instability and differences in physicochemical properties, measuring the levels of various AGE structures *in vivo* is now possible due to advances in instrumental analysis techniques, such as gas or liquid chromatography tandem mass spectrometry (LC-MS/MS). As a result, the involvement of AGEs and their pathology have been evaluated at the molecular level.

We previously demonstrated that S-(2-Succinyl)cysteine (2SC) is formed by a reaction between the thiol group of proteins and fumarate, a Krebs cycle intermediate. The level of 2SC significantly increases during the maturation of 3T3-L1 fibroblasts to adipocytes. For example, the fumarate concentration increases >5-fold during adipogenesis in medium containing 30 mM glucose, producing a >10-fold increase in 2SC-proteins in adipocytes compared with undifferentiated fibroblasts grown in the same high-glucose medium[24] (Fig. 5). Furthermore, several proteins, including adiponectin and heat shock proteins, have been identified using matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry.[25] Although AGE structures, such as CML and pentosidine, are commonly measured in biological samples due to their stability and autofluorescent properties, 2SC is a prominent post-translational modification observed during the maturation of adipocytes (Fig. 5), demonstrating that the specific detection of each AGE component, including 2SC, is necessary in order to clarify the relationship between post-translational modifications and disease. 2SC is detected in human plasma and urine by gas chromatography mass spectrometry,[26] whereas the relationship between diseases and 2SC levels in plasma or urine has not been reported yet. We speculate that the increase in fumarate and 2SC is the result of mitochondrial stress in the adipocyte during adipogenesis and that 2SC may be a useful biomarker of mitochondrial stress in obesity, insulin resistance, and diabetes.
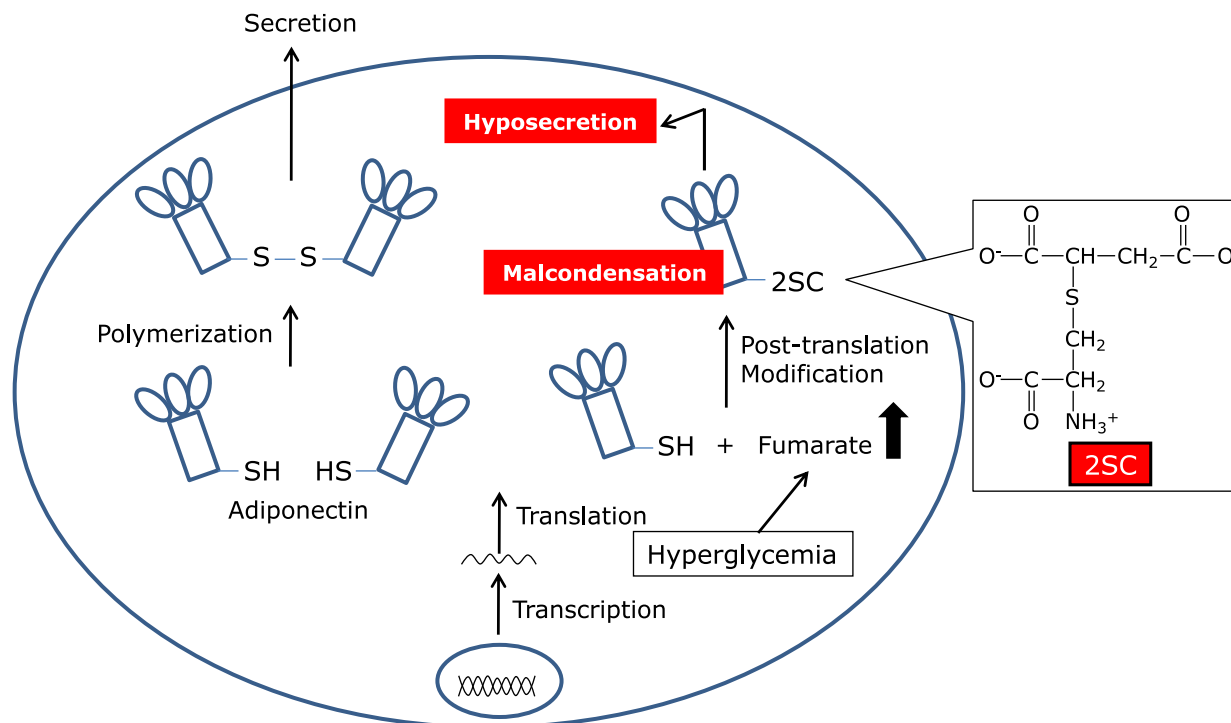
Fig. 5. Succination of adiponectin proteins by fumarate affects the secretion and condensation in adipocytes. 2SC is formed by a reaction between the thiol group of proteins and fumarate, a Krebs cycle intermediate. 2SC-proteins such as adiponectin increased in adipocytes under hyperglycemic condition, resulting in the hyposecretion and malcondensation of adiponectin.

Question about AGE Receptors

The interaction between proteins modified by AGEs and AGE receptors, such as scavenger receptor(s) on macrophages and smooth muscle cells, is known to induce the production of several cytokines, including plasminogen activator and transforming growth factor-beta.[27] Although highly-modified AGE-bovine serum albumin (high-AGE-BSA) was significantly recognized by human monocyte-derived macrophages and Chinese hamster ovary cells which overexpress such scavenger receptors as CD36, SR-BI (scavenger receptor class B type-I), and LOX-1 (Lectin-like Ox-LDL receptor-1), the mildly-modified-AGE-BSA (mild-AGE-BSA) did not show any ligand activity to these cells. Furthermore, when ^{111}In-labeled high- or mild-AGE-BSA were injected into the tail vein of mice, the high-AGE-BSA was rapidly cleared from the circulation whereas the clearance rate of the mild-AGE-BSA was very slow, similar to the native BSA.[28] Thornalley *et al.*[29] demonstrated that end-stage renal disease is associated with a significant increase in the molecular mass of HSA (+255 Da, relative to the control subjects) and ~3% of lysine residues were modified. However, our study using a MALDI-TOFMS analysis demonstrated that the molecular mass of the mild-AGE-BSA was 658 Da larger than the native BSA, indi-cating that our experimentally prepared mild-AGE-BSA is already more profoundly modified than physiological human serum albumin under (patho)physiological conditions. This study demonstrates the evidence that the ligand activity of the AGE-proteins to the scavenger receptors and its pharmacokinetic properties depend on their rate of modification by AGEs, and we should carefully prepare the AGE-proteins *in vitro* to clarify the physiological significance of the interaction between the AGE-receptors and AGE-proteins.

Acknowledgments

Conflict of Interest

No potential conflicts of interest were disclosed.

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