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Original article

Comparison of N^{ε}-(carboxymethyl)lysine, an oxidation-dependent AGE, by metal ions

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Abstract

Advanced glycation end products (AGEs) are formed by the Maillard reaction, a non-enzymatic reaction that occurs during the browning of food. AGE formation is promoted by heating and oxidation by metal ions. However, there are no reports comparing the form of AGE contents from each reducing sugar depending on the type of metal ion. Therefore, we used an immunochemical method to evaluate the effects of Cu and Fe on the formation of N^{ε}-(carboxymethyl)lysine (CML), an oxidation-dependent AGEs formed from glucose, fructose, and ribose, which are representative reducing sugars in the body. Consequently, the CML increased with Fe in glucose, Cu in fructose, and Fe in ribose. The Amadori rearrangement products, prepared by reacting each of the above three types of reducing sugars with bovine serum albumin under anaerobic conditions, were subjected to the Fenton reaction with hydrogen peroxide and various metal ions. The amount of CML produced was significantly increased in glucose with Fe and Cu, and in ribose with Cu compared to the control, however in fructose, no change was observed for any of the metal ions. This indicates that the metal ions that affect CML formation differ depending on the reducing sugar used. In conclusion, the amount of CML formed by metal ions is greater from the reaction of reducing sugars with proteins than from the Amadori rearrangement products; therefore, it is thought that CML is mainly produced via dicarbonyl compounds produced by the oxidation of reducing sugars.

***KEY WORDS*:** advanced glycation end products (AGEs), glycation, ELISA, N^{ε}-(carboxymethyl) lysine (CML), metal ions

Introduction

The Maillard reaction is a reaction in which reducing sugars and free amino acids or amino residues of proteins react non-enzymatically with heating or aging of food to produce browning. In this reaction, the carbonyl groups such as glucose react non-enzymatically with the amino acid residues of proteins. The carbonyl and amino groups dehydrate and condense to form Amadori rearrangement products via Schiff bases, which are subsequently transformed into advanced glycation end-products (AGEs) through oxidation and dehydration reactions. In addition to this pathway, AGEs are formed by dicarbonyl compounds (*e.g.*, glyoxal) produced by the autoxidation of reducing sugars, or by the decomposition of Amadori rearrangement products [1)]. Factors that affect the progress of this reaction include the type of sugars or amino acids, temperature, and metals. It is known that metal ions promote oxidation reactions and increase formation of AGEs [2)].

Because this reaction is a non-enzymatic chemical reaction, it is promoted by the concentration of the substrate, and as the temperature increases, the AGE content formed varies depending on the method of cooking [3)]. However, this reaction proceeds gradually at physiological temperatures. Therefore, AGEs are also produced from reducing sugars, proteins, and nucleic acids in living organisms. However, AGEs formed *in vivo* at a constant temperature of approximately 37 °C include low-molecular-weight N^{ε}-(carboxymethyl)lysine (CML), rather than polymers such as melanoidin produced in food. It has also been reported that the formation of endogenous AGEs in living organisms causes the denaturation of skeletal muscle proteins and the expression of chaperone-like functions in crystallin [4, 5)]. CML produced from Lys residues is generated by the oxidation of Amadori rearrangement products [6, 7)]. Therefore, endogenous AGEs are strongly affected by temperature and oxidation.

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Reactive oxygen species (ROS) are highly reactive in the body and are involved in oxidation reactions, however, their hail-life is short. ROS produced by white blood cells are essential for killing bacteria during infection. On the other hand, the generated ROS are removed by many reactive oxygen scavengers such as superoxide dismutase (SOD) and catalase, as well as low molecular weight antioxidants. These ROS include superoxide produced from oxygen, and hydroxyl radicals produced by hydrogen peroxide produced from superoxide in the presence of transition metals. Hydroxyl radicals, in particular, have a high reactivity and a strong effect on metabolism and cell damage, such as the formation of short-chain aldehydes derived from fatty acids such as acrolein [8], lipid peroxidation, and DNA damage [9]. In diabetes, chronic hyperglycemic conditions lead to increased oxidative stress [10]. It is also known that ROS are generated from Amadori rearrangement products generated in the initial reaction of the Maillard reaction [11]. Therefore, chronic hyperglycemic conditions and reactive oxygen species produced from Amadori rearrangement products further promote AGEs formation, which may lead to the progression of diseases, including diabetes and diabetic complications.

Copper and iron are present in the blood and tissues as trace elements in the body [12], however there have been no papers evaluating whether the form of AGE contents differs depending on the type of metal ion. Therefore, we hypothesized that CML formation from reducing sugars is differentially regulated by various metal ions. Immunological techniques were used to evaluate the amount of CML produced from each reducing sugar using each metal ion. The effects of metal ions on CML formation were compared in (1) the initial reaction between sugars and protein, and (2) the intermediate Amadori rearrangement product.

Materials and Methods

Sample Preparation

For the initial reaction between sugar and protein, 2 mg/mL bovine serum albumin (BSA: Sigma, Germany) was mixed with glucose, fructose (Kanto Chemical, Tokyo, Japan), or ribose (Fujifilm Wako Pure Chemical, Osaka, Japan) in phosphate buffered saline (PBS) to prepare solutions with 5 mM and 30 mM, which correspond to the blood glucose levels of healthy subjects and diabetic patients. Copper sulfate (Fujifilm Wako Pure Chemical), anhydrous iron(II) chloride (Fujifilm Wako Pure Chemical), and diethylenetriamine-N,N,N',N,N''-pentaacetic acid (DTPA; Dojindo Laboratories, Kumamoto, Japan) were prepared in distilled water. Copper sulfate (final concentration: 0.4 mM), anhydrous iron chloride (final concentration: 0.4 mM), and DTPA (final concentration: 1 mM) were added to each sugar solution. These were incubated at 37 °C for 0, 3, and 7 days, stored at −20 °C, and CML was measured by enzyme-linked immunosorbent assay (ELISA). Because high concentrations of reducing sugars have antioxidant properties [13], to prepare Amadori rearrangement products using chemical synthesis (2), 2mg/mL BSA was mixed with glucose, fructose, or ribose, and the sugar concentration was adjusted to 1,600 mM in phosphate buffer. DTPA (final concentration 1 mM) was added, incubated at 37 °C for 7 days, and then dialyzed using a dialysis membrane to recover glycated BSA. The protein concentration was then measured, and hydrogen peroxide (final concentration 0.1 mM) was added. Copper sulfate (final concentration 0.4 mM), anhydrous iron(II) chloride (final concentration 0.4 mM), and DTPA (final concentration 0.1 mM) were added to the mixture. These were incubated at 37 °C for 1 h, and CML was measured by ELISA.

ELISA

The incubated samples were diluted with PBS to 1 μg/mL BSA and added to a 96-well plate (Clear Flat-Bottom Immuno Nonsterile 96-Well Plates, Thermo, USA) and CML was measured according to the standard method [14]. The plate was coated with antigen and blocked with 0.5 % gelatin (Sigma, Germany). For the primary antibody, 100 μL of monoclonal anti-CML antibody (0.5 μg/mL) was added to the plate and left at room temperature for 1 h. Then, 100 μL of horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody (KPL, USA) was added to the plate and incubated at room temperature for 1h, after which color development was performed using OPD (Fujifilm Wako Pure Chemical Industries). After color development, the reaction was stopped with 2 M sulfuric acid and the absorbance was measured at 492 nm using a TECAN infinite M PLEX.

Statistical analysis method

Statistical analysis was performed using R-4.2.1. for Windows. The Bonferroni analysis was used to test for statistical significance.

Results

Comparison of CML formation by type of reducing sugar and type of metal ion

The results of ELISA using anti-CML antibodies showed that CML formation increased in an incubation time-dependent manner with 5 mM and 30 mM glucose. Furthermore, the amount of CML was higher with the addition of Fe compared to that in the control (***Fig. 1-a, b***). The levels of generated CML also increased with the addition of Cu compared to the control. Furthermore, CML formation was more significant with Fe than with Cu at a high glucose concentration of 30 mM.

CML formation increased in a time-dependent manner at fructose concentrations of 5 and 30 mM. The levels of generated CML were higher with the addition of Cu than that of the control (***Fig. 1-c, d***). In contrast, no change was observed with the addition of Fe compared with the control. Furthermore, CML formation was more significant with Cu than with Fe at fructose concentrations of both 5 and 30 mM. CML formation tended to increase in an incubation time-dependent manner with 5 mM and 30 mM ribose, a five-monosaccharide. The amount of CML was higher with the addition of Fe than with the control (***Fig. 1-e, f***).

For each reducing sugar, the addition of DTPA, a metal ion chelator, inhibited CML formation.

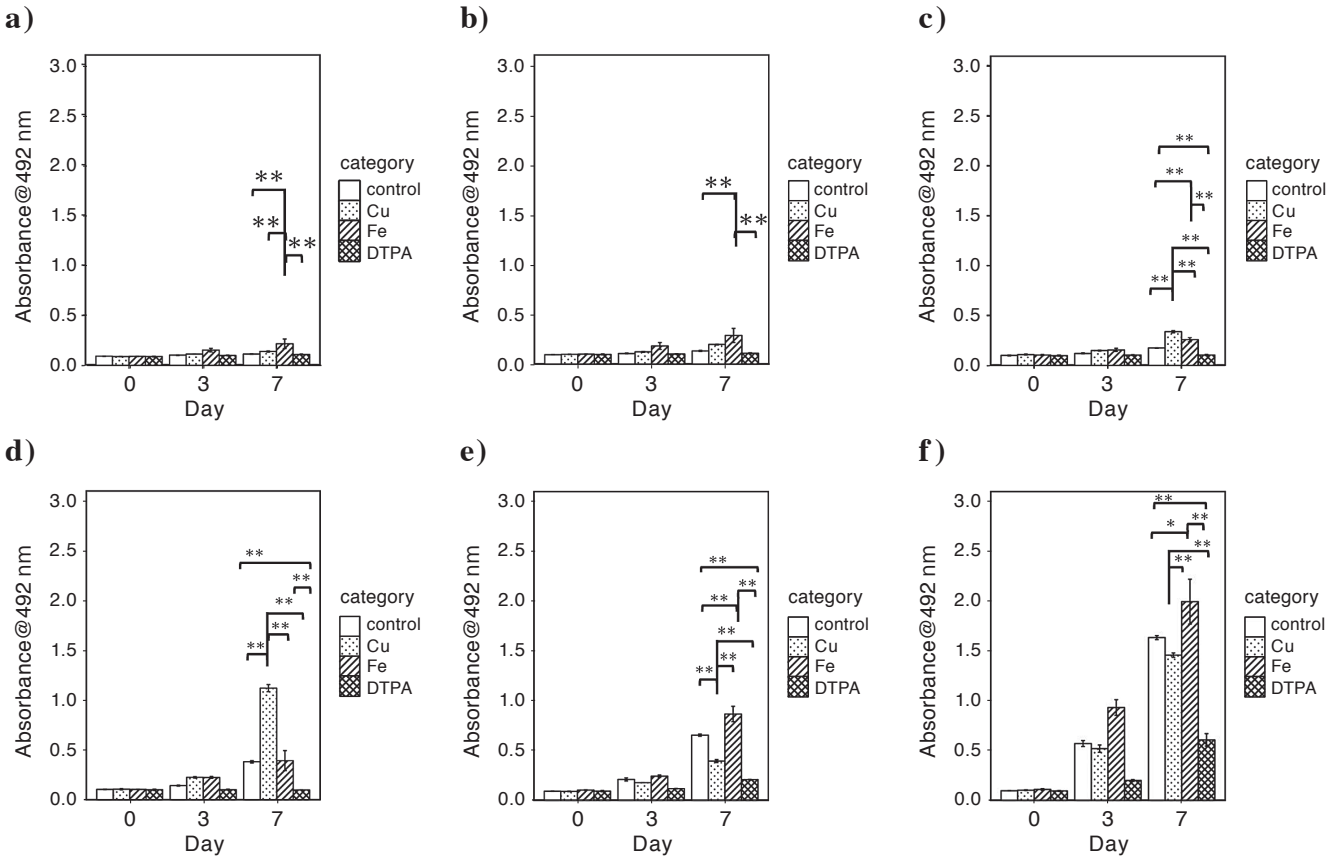

Fig. 1. **Comparison of generated CML levels from glucose, fructose or ribose -BSA with each metal ion.**

a) CML levels of 5 mM glucose. None: control (no metal), Circle: Cu, Stripe: Fe, Crosshatch: DTPA.
b) CML levels of 30 mM glucose. None: control (no metal), Circle: Cu, Stripe: Fe, Crosshatch: DTPA.
c) CML levels of 5 mM fructose. None: control (no metal), Circle: Cu, Stripe: Fe, Crosshatch: DTPA.
d) CML levels of 30 mM fructose. None: control (no metal), Circle: Cu, Stripe: Fe, Crosshatch: DTPA.
e) CML levels of 5 mM ribose. None: control (no metal), Circle: Cu, Stripe: Fe, Crosshatch: DTPA.
f) CML levels of 30 mM ribose. None: control (no metal), Circle: Cu, Stripe: Fe, Crosshatch: DTPA.

Bar indicates standard deviation. * $p < 0.05$, ** $p < 0.01$ by Bonferroni's multiple test, n = 3, performed on each sample at day 7. CML, N^{ε}-(carboxymethyl)lysine; BSA, bovine serum albumin; DTPA, diethylenetriamine-N,N,N',N,N"-pentaacetic acid.

Comparison of CML formation by glycated BSA and type of metal ion

When metal ions and hydrogen peroxide were added to glycated BSA to examine the changes in CML formation, the results showed that in glucose-derived glycated BSA, CML formation increased even in Cu, and the highest CML levels were formed in Fe. In fructose-derived glycated BSA, the levels of generated CML did not change regardless of the metal ion added. In ribose-derived glycated BSA, CML formation was higher in Cu than in Fe (***Fig. 2***).

Discussion

Minerals, such as copper, iron, calcium, and magnesium are present in the body. Among them, copper and iron are trace elements. Copper is important for angiogenesis and the synthesis of neurotransmitters, and iron is important for immunity and energy metabolism.

The addition of Fe and Cu promotes oxidation reactions [6, 15, 16]. On day 7, compared to the control without metal addition, the levels of generated CML were increased in some cases by the addition of metals. Among them, the amount of CML was highest in glucose, a 6-monosaccharide, with Fe, in fructose, with Cu, and in ribose, a 5-monosaccharide, with Fe, indicating that the metals that affect CML formation differ depending on the type of reducing sugar. Fe and Cu increase in the kidneys and liver of streptozotocin-induced diabetic rats[17]. Therefore, since CML formation was promoted more at high concentrations of sugar, it is highly likely that the presence of Cu(I) and Fe(II) in proteins promote CML formation in a hyperglycemic state.

CML can be formed from Amadori rearrangement products or dicarbonyl compounds such as glyoxal (***Fig. 3***)[1, 7]. In this study, we used the Fenton reaction with hydrogen peroxide to evaluate whether the increased CML was generated

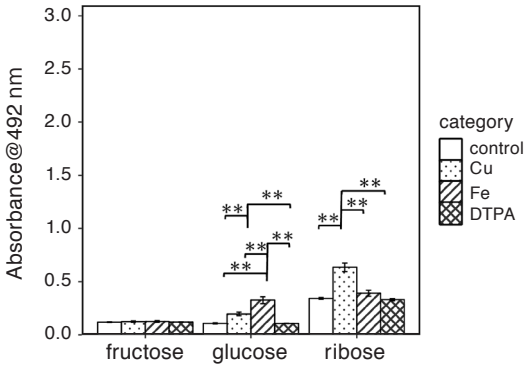

Fig. 2. **Comparison of generated CML levels from each reducing sugar derived Amadori-products with each metal ion.**

Pink: control (no metal), Circle: Cu, Stripe: Fe, Crosshatch: DTPA. Bar indicates standard deviation. **p < 0.01 by Bonferroni's multiple test, n = 3. CML, N^{ε}-(carboxymethyl)lysine; DTPA, diethylenetriamine-N,N,N',N,N"-pentaacetic acid.

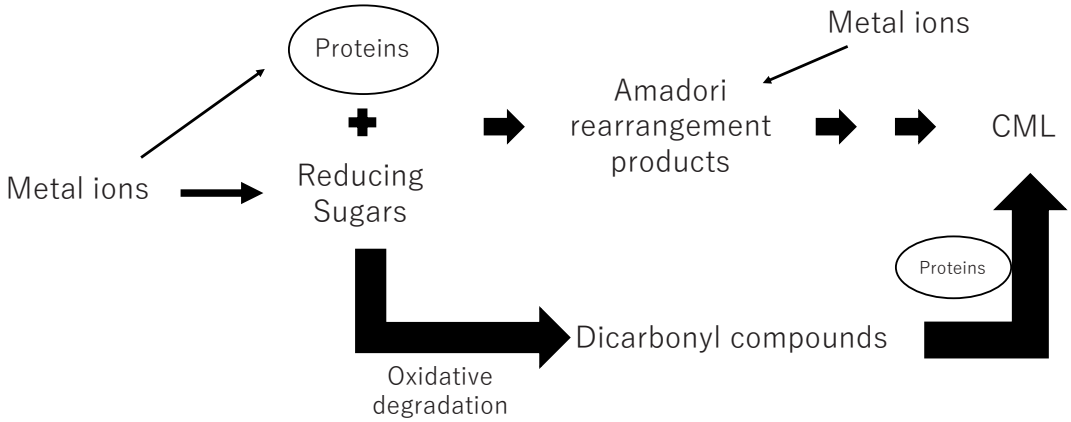

Fig. 3. **The process of CML formation.**

Most CML levels were generated by dicarbonyl compounds rather than by Amadori rearrangement products influenced by metal ions. CML, N^{ε}-(carboxymethyl)lysine.

from Amadori rearrangement products by oxidation with the addition of metal ions[6]. In fructose-derived glycated BSA, no change in CML contents was observed for any metal (***Fig. 2***). This may be because glucose-lysine, which is generated by the Heyns rearrangement from the reaction of fructose with protein, has an oxidation-independent structure[18], so CML formation may not have progressed even with the addition of metal ions. Therefore, it is possible that the main pathway for CML formation from fructose in the presence of metal ions is dicarbonyl compounds[19]. Furthermore, Fe and Cu promoted CML formation in glucose-derived glycated BSA. In the case of ribose-derived glycated BSA, Cu produced high levels of CML (***Fig. 2***), whereas in the reaction in which BSA + ribose + metal ions were incubated simultaneously, Fe produced high levels of CML, but not Cu (***Fig. 1-e, f***). Metal ions form complexes with proteins[20]. The reason why CML did not increase in the present case of BSA + ribose, or glucose + Cu may be that the formation of a complex with Cu(I) and BSA, which affected the formation of CML from glucose or ribose via dicarbonyl compounds. In the case of fructose, the dicarbonyl compounds produced by Cu(I) before the formation of a complex with Cu(I) and BSA may have promoted CML formation.

Therefore, the enhanced CML formation caused by the addition of metals to the reaction system of glucose, fructose, or ribose with protein may not be due to Heyns rearrangement, but may be due to the promotion of the formation of dicarbonyl compounds such as glyoxal through the autoxidation of these reducing sugars.

Several trace elements, such as iron and copper, exhibit catalytic activity in living organisms and enhance the activities of enzyme components and enzymes. Enzymes containing iron include catalase and peroxidase, while enzymes containing molybdenum and iron include xanthine oxidase. They also play a role in storage and transportation; a typical example is hemoglobin, which transports oxygen, an iron-containing protein. Iron and copper are involved in the hydroxyl radicals generated from superoxide and hydrogen peroxide[16], and iron and copper ions *in vivo* are bound to proteins such as metallothionein, hemoglobin, and Cu, Zn-SOD, and are therefore unlikely to exist as free metal ions. However, the binding between these metal ions and proteins is weak. The concentration of trace metals in living organisms is regulated by the balance between excretion and reabsorption via transporters[21]. Therefore, when the extracellular metal ion concentration increases, the transporters present on the

cell membrane surface decrease in order to regulate it [22]. Changes in the extracellular metal ion concentration due to metabolic abnormalities may induce oxidation reactions and affect protein denaturation. When Cu, Zn-SOD is glycated, a Fenton reaction occurs between Cu and the Cu contained in the SOD, progressing the cleavage of the protein [23]. It has also been reported that mutations in Cu, Zn-SOD cause excessive accumulation of Cu in neural tissues, and CML accumulation is enhanced, simultaneously [24]. Furthermore, because Cu damages DNA, metal ions may react with glucose and fructose as a protective effect to prevent damage caused by metal ions, and CML formation may progress.

It has been reported that the epsilon amino group of lysine forms a complex with iron ions, and is converted to a carbonyl [25]. Although the generated carbonyl group may undergo a condensation reaction with other amino residues, the conversion of the carbonyl group to a carboxymethyl group has not been reported. In contrast, this study describes CML formation from Amadori rearrangement products and the pathway of CML formation via dicarbonyl compounds generated by reducing sugars that do not react with proteins in the presence of metal ions, which is thought to be different from protein carbonylation by metal ions (***Fig. 3***) [26].

By adding metal ions, CML levels were generated in a shorter time. Typically, it takes several weeks to several months to form AGEs contents by reacting reducing sugars with proteins or nucleic acids. Therefore, to prepare AGEs levels in a short period of time and screen inhibitors, the method of adding metal ions may be effective. However, the metal-chelating ability of the inhibitor must also be considered. It has been reported that aminoguanidine and flavonoids, which are AGE inhibitors, also inhibit AGE formation by chelating ability. The involvement of metals always needs to be considered in AGE research.

Conflict of interest declaration

All authors declare that they have no conflict of interest.

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