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Hydroxyl Radical Mediates N^{ϵ}-(Carboxymethyl)lysine Formation from Amadori Product

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Recent studies demonstrated N^{ϵ}-(carboxymethyl) lysine (CML) in several tissue proteins. Incubation of proteins with glucose leads through a Schiff base to Amadori products. Oxidative cleavage of Amadori products is considered as a major route to CML formation *in vivo,* whereas it is not known which reactive oxygen species (ROS) is involved. The present study is undertaken to identify such a ROS. We prepared heavily glycated human serum albumin (HSA) which contained a high level of Amadori products, but an undetectable level of CML. Incubation of glycated HSA with $FeCl_2$, but not with H_2O_2, led to CML formation which was enhanced by H_2O_2, but inhibited by catalase or mannitol, whereas superoxide dismutase had no effect. Similar data were obtained by experiments using Boc-fructose-lysine as a model Amadori compound. These data indicate that hydroxyl radical generated by the reaction of Fe^{2+} with H_2O_2 mediates CML formation from Amadori compounds.

Long-term incubation of proteins with glucose leads, through formation of early products such as a Schiff base and Amadori product, to advanced glycation end products (AGEs), compounds that have unique properties, such as fluorescence, browning and cross-linking (1). Among AGE-structures so far reported, pentosidine and crosslines are fluorescent and cross-linking compounds whereas pyrraline and N^{ϵ}-(carboxymethyl)lysine (CML) are non-fluorescent and non-cross-linking ones. We have prepared a monoclonal anti-AGE antibody (6D12) (2) and immunological studies using this antibody have successfully demonstrated the presence of AGEs in several human tissues, suggesting a potential link of AGEs to aging (3-5) and age-enhanced disease states such as diabetic complications (6, 7), atherosclerosis (8-10), dialysis-related amyloidosis (11, 12). Our recent study has disclosed that this antibody recognizes a CML-protein adduct as an epitope (13), strongly suggesting an importance of CML among AGE-structures *in vivo.*

CML is formed in three pathways *in vitro,* by oxidative cleavage of Amadori adducts (14, 15) or Schiff bases (16) or by modification with glyoxal generated directly through autoxidation of glucose (17). However, CML formation from Amadori products is thought to represent a major pathway *in vivo* (18). Since CML formation is inhibited by antioxidants and antioxidative conditions, the involvement of oxidative mechanism in this process has been suggested (19, 20). However, it is not known which reactive oxygen species is responsible for this process. To solve the issue, the present study was undertaken to compare reactive oxygen species such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) in their contribution to CML formation from Amadori product.

MATERIALS AND METHODS

Chemicals. Human serum albumin (HSA, fraction V) and hypoxanthine were purchased from Sigma (St. Louis, MO). Xanthine oxidase was purchased from Boehringer Mannheim (Mannheim, Germany). D-Glucose, glyoxylic acid, anhydrous $FeCl_2$, Cu, Zn-superoxide dismutase (Cu, Zn-SOD) and catalase were purchased from Wako (Osaka, Japan). $NaCNBH_3$ was obtained from Aldrich (Milwaukee, WI). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody was purchased from Kirkegaard Perry Laboratories (Gaithersburg, MD). Microtitration plates (96-well, Nunc Immunoplate II) were purchased from Nippon Inter Med (Tokyo, Japan). All other chemicals were of the best grade available from commercial sources.

Preparation of glycated HSA and non-glycated HSA. A special attention was paid in the present study to obtain highly glycated HSA (high contents of Amadori products) but an extremely low level

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Abbreviations used: AGE(s), advanced glycation end products; ROS, reactive oxygen species; HSA, human serum albumin; CML, N^{ϵ}-(carboxymethyl)lysine; O_2^-, superoxide anion radical; H_2O_2, hydrogen peroxide; $^{\cdot}OH$, hydroxyl radical; Boc-fructose-lysine, N^{α}-t-butyloxycarbonyl-N^{ϵ}-(1-deoxy-D-fructos-1-yl)-L-lysine; ELISA, enzyme-linked immunosorbent assay.

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of CML (14, 20, 21). Briefly, 50 mg/ml of HSA was incubated for 7 days at 37°C with 1.6 M glucose in 10 ml of 50 mM sodium phosphate buffer (pH 7.2) in the presence of 1 mM diethylenetriaminepentaacetic acid (DTPA), followed by dialysis against 50 mM sodium phosphate buffer (pH 7.2). To prepare non-glycated HSA, 1 g of HSA dissolved in 300 ml of 0.5 M glycine-NaOH buffer with 2% $MgCl_2$ (pH 8.5) was mixed with 500 ml of phenyl boronic acid resin (PBA-60, Amicon, Beverly, MA). After mixing for 2 h at room temperature, solution was filtrated through a glass filter. The solution thus obtained was again incubated with another portion of the same resin, followed by dialysis against 50 mM sodium phosphate buffer (pH 7.2). The finally obtained solution was concentrated to 30 mg/ml by an ultrafiltration system and used for the experiments. The level of fructosamine in the non-glycated HSA preparation was determined by Fructosamine assay kit (Boehringer Mannheim).

Preparation of N^{α}-t-butyloxycarbonyl-N^{ϵ}-(1-deoxy-D-fructos-1-yl)-L-lysine (Boc-fructose-lysine). Boc-fructose-lysine was synthesized as a model Amadori compound according to Njoroge et al. (22). Briefly, 1 g of N^{α}-t-butyloxycarbonyl-L-lysine prepared from N^{α}-t-butyloxycarbonyl-N^{ϵ}-carbobenzoxy-L-lysine (Kokusan Chemical Works, Japan) was refluxed for 2 h with 2 g of glucose in 100 ml of methanol. The reaction mixture was evaporated *in vacuo* and purified by silica gel column chromatography (methanol/ acetic acid/ ethyl acetate: 60: 1: 39) to give 0.67 g of Boc-fructose-lysine. The purity was assessed by a reverse phase high performance liquid chromatography (HPLC) (column: Shimadzu Techno Research STR-ODSII; eluent: 30% methanol in 0.1% TFA), and the structure was confirmed by ^{1}H-NMR (Bruker ARX-500) and sputtered ion mass spectrometry (Hitachi M-80B).

Generation of reactive oxygen species. Glycated HSA or non-glycated HSA (2 mg/ml) was incubated for 1 h at 37°C with 0.4 mM anhydrous $FeCl_2$ in the absence or presence of indicated concentrations (0.1 mM to 1.0 mM) of H_2O_2 in 0.1 ml of 50 mM sodium phosphate buffer (pH 7.2), followed by extensive dialysis at 4°C against phosphate buffered saline (PBS). To examine the effect of O_2^-, glycated HSA or non-glycated HSA (2 mg/ml) was incubated for 1 h at 37°C with 0.5 mM hypoxanthine and xanthine oxidase (1.0 units/ml) in 0.1 ml of 50 mM sodium phosphate buffer (pH 7.2).

Preparation of CML-HSA. CML-HSA was prepared as described previously (13, 23). Briefly, 175 mg of HSA was incubated at 37°C for 24 h in 1 ml of 0.2 M sodium phosphate buffer (pH 7.8) with 0.15 M glyoxylic acid and 0.45 M $NaCNBH_3$, followed by dialysis against PBS. CML-HSA thus prepared contained 12.94 mol/ mol of HSA upon the amino acid analysis described below.

Immunological estimation of CML contents. Monoclonal anti-AGE antibody termed 6D12 was prepared in mice using AGE-modified bovine serum albumin (2). 6D12 purified by protein A affinity chromatography to IgG_1 was used. Our recent study revealed that 6D12 recognizes a CML-protein adduct (13). The amounts of CML-protein adducts in glycated and non-glycated HSA were determined at room temperature as described previously by competitive enzyme-linked immunosorbent assay (ELISA) using 6D12 (2). Briefly, each well of a 96-well microtiter plate was coated with 0.1 ml of 1 μg/ml CML-HSA in 50 mM sodium carbonate buffer (pH 9.6), and incubated for 1 h. The wells were washed three times with PBS containing 0.05% Tween 20 (washing buffer). The wells were blocked with 0.5% gelatin in 50 mM sodium carbonate buffer (pH 9.6) for 1 h. After washing three times with washing buffer, the wells were incubated for 1 h with the sample to be tested (50 μl) and 0.1 μg/ml of 6D12 (50 μl). The wells were incubated with HRP-conjugated anti-mouse IgG antibody, followed by reaction with 1, 2-phenylenediamine dihydrochloride. The reaction was terminated by 1.0 M sulfuric acid, and the absorbance at 492 nm was read on a micro-ELISA plate reader. The immunoreactivity of CML-HSA, whose CML content was 12.94 mol/ mol of HSA, was defined as 100 arbitrary unit (A.U.). The immunoreactivity of each sample was expressed as the immunoreactivity relative to CML-HSA. For experiments using ELISA, the same set of experiment was repeated at least three times and the representative data were shown in each figure.

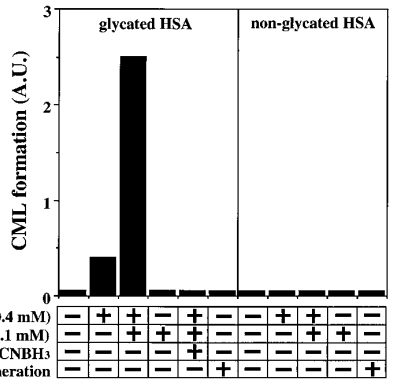

FIG. 1. Effects of Fe^{2+} and H_2O_2 on CML formation from glycated HSA. Glycated HSA and non-glycated HSA (2 mg/ml) were incubated in the presence or absence of 0.4 mM $FeCl_2$ and 0.1 mM H_2O_2 in 50 mM sodium phosphate buffer (pH 7.2) for 1 h at 37°C. Prior to incubation with Fe^{2+} and H_2O_2, the glycated HSA was pre-treated with $NaCNBH_3$ to reduce Amadori products into hexitollysine and dialyzed against 50 mM sodium phosphate buffer (pH 7.2). To examine the effect of O_2^-, glycated HSA or non-glycated HSA (2 mg/ml) was incubated for 1 h at 37°C with 0.5 mM hypoxanthine and xanthine oxidase (1.0 units/ml) in 50 mM sodium phosphate buffer (pH 7.2). CML formation in each sample was determined by competitive ELISA using 6D12 and was expressed as the immunoreactivity relative to CML-HSA. The immunoreactivity of CML-HSA was defined as 100 arbitrary units (A.U.). Experimental details are described under "Materials and Methods."

Amino acid analysis. CML contents of modified proteins were quantitated by amino acid analysis as described (13). After acid hydrolysis with 6 N HCl for 24 h at 110°C, samples were subjected to an amino acid analyzer (Model 835, Hitachi Co., Tokyo) using an ion exchange HPLC column (#2622 SC, 4.6 × 60 mm, Hitachi Co.) and ninhydrine post-column detecting system. Hippuryl-CML was prepared by incubating hippuryllysine with glyoxylic acid and $NaCNBH_3$ as described (13) and was used as a standard CML. The identity of CML detected by HPLC was confirmed by fast atom bombardment (FAB) mass spectrometry.

RESULTS

Effects of reactive oxygen species on CML formation from glycated HSA. Glycated HSA was prepared by 7-days incubation at 37°C with 1.6 M glucose in the presence of 1 mM DTPA in 50 mM phosphate buffer (pH 7.2). Amino acid analyses showed that 21.9 out of 59 lysine residues were covalently modified by glucose in glycated HSA, while its CML content was less than a detectable level (<0.01 mol of CML/mol of HSA). On the contrary, the level of Amadori compounds of non-glycated HSA determined as fructosamine was negligible (10.9 μmol/l).

Using these glycated HSA and non-glycated HSA preparations, we determined which type of reactive oxygen species was involved in the CML formation from glycated HSA (Fig. 1). Upon incubation with 0.4 mM Fe^{2+} for 1 h, the glycated HSA dramatically increased

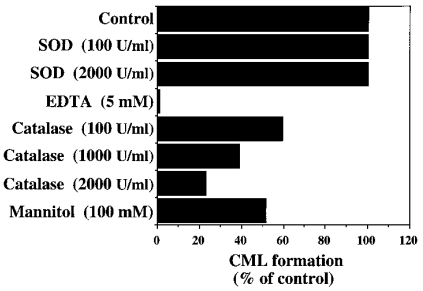

FIG. 2. Effects of several inhibitors on the Fe^{2+}-induced CML formation from glycated HSA. Glycated HSA was incubated at 37°C for 1 h with 0.4 mM Fe^{2+} in the absence or presence of indicated amounts of SOD, EDTA, catalase, or mannitol. Samples were dialyzed and their CML contents were determined by a competitive ELISA as described under "Materials and Methods." CML contents were expressed as % of control.

its reactivity to the anti-CML antibody up to 0.5 A.U. Amino acid analyses showed that this level corresponded to 0.10 mol of CML/mol of HSA. Addition of 0.1 mM H_2O_2 to this system resulted in a further increase in the immunoreactivity by 6.3-fold (2.5 A.U.) and CML content was 0.19 mol/mol of HSA. However, the incubation with H_2O_2 alone instead of Fe^{2+} failed to generate CML. When Amadori adducts of glycated HSA were reduced with $NaCNBH_3$ into hexitollysine in advance, CML was not generated by the incubation with Fe^{2+} and H_2O_2. In contrast, the CML formation from non-glycated HSA was negligible (Fig. 1). We also examined the effect of O_2^-. However, O_2^- had no effect on CML formation from glycated HSA (Fig. 1). Under the identical conditions, 52 μM of O_2^- was generated by the hypoxanthine/ xanthine oxidase system when determined by ESR spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO).

Three points became clear from these results. First, CML formation from glycated HSA occurred only when $FeCl_2$ was added to the system. Secondary, this was enhanced by addition of H_2O_2. And finally, CML generation did not occur from non-glycated HSA or $NaCNBH_3$-reduced glycated HSA. Thus, it is likely that Amadori product of glycated HSA is converted to CML by the action of $^{\cdot}OH$.

Effects of a metal chelator and inhibitors for reactive oxygen species on the Fe^{2+}-induced CML formation from glycated HSA. This notion was further tested by determining the effect of several inhibitors for reactive oxygen species on the Fe^{2+}-induced CML formation from glycated HSA. As shown in Fig. 2, SOD, an O_2^- scavenger, failed to inhibit the formation of CML. On the other hand, EDTA, a chelator of transition metal ion, exhibited a marked inhibition (>95%). Catalase, a H_2O_2 scavenger, also showed significant inhibition in a dose-dependent manner. The maximum inhibitory effect of mannitol used as a $^{\cdot}OH$ scavenger was significant (about 50%), but weaker than those of EDTA or catalase. Since the Fe^{2+}-induced CML formation from glycated HSA were sensitively inhibited by EDTA, catalase and mannitol, these results strengthened our notion that $^{\cdot}OH$ generated probably by the reaction of Fe^{2+} with H_2O_2 which is originated from glycated HSA, plays a major role in CML formation from glycated proteins.

Dose-dependent effects of H_2O_2 on Fe^{2+}-induced CML formation. Next, dose-dependent effects of H_2O_2 on the CML formation were determined. As shown in Fig. 3, the incubation of glycated HSA with 0.4 mM $FeCl_2$ for 1 h at 37 °C led to the CML formation (0.4 A.U.). Parallel incubation in the presence of 0.1 mM H_2O_2 gave rise to a 6.3-fold increase in CML formation (2.5 A.U.). Upon further increases of H_2O_2 concentrations to 0.5 and 1.0 mM, CML levels generated from glycated HSA were enhanced by 20-fold (8.0 A.U.) and 25-fold (10.0 A.U.), respectively. H_2O_2 alone even at the highest concentration again failed to generate CML from glycated HSA. From the dose-dependent effect of H_2O_2 in the presence of $FeCl_2$, it is evident that $^{\cdot}OH$ generated through the Fenton reaction between Fe^{2+} and H_2O_2 is responsible for CML formation from Amadori products of glycated HSA.

Effects of Fe^{2+} and H_2O_2 on CML formation from Boc-fructose-lysine. Finally, we performed experiments using Boc-fructose-lysine as a model Amadori compound to confirm the contribution of $^{\cdot}OH$ to CML formation. When 1 nmol of Boc-fructose-lysine was subjected to acid hydrolysis and amino acid analysis, 0.811 nmol of lysine residue was recovered as one of decomposition products, whereas an amount of CML was negligible (<0.005 nmol) (Fig. 4a). However, after incubation of Boc-fructose-lysine with 0.4 mM Fe^{2+} for 1 h, CML became a detectable level of 0.018 nmol (Fig. 4b). Addition of 0.1 mM H_2O_2 to this system resulted in a further increase of CML to 0.110 nmol (Fig. 4c), while lysine residue, a major decomposition product derived

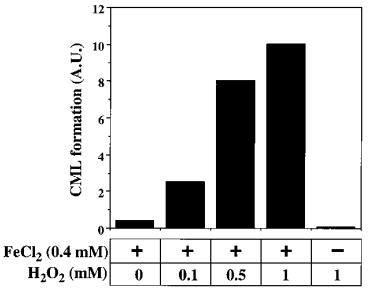

FIG. 3. Dose-dependent effects of H_2O_2 on the Fe^{2+}-induced CML formation. Glycated HSA (2 mg/ml) was incubated at 37°C for 1 h with 0.4 mM $FeCl_2$ and indicated concentrations of H_2O_2 in 50 mM sodium phosphate buffer (pH 7.2). Samples were dialyzed and their CML contents were determined by competitive ELISA and expressed as arbitrary units (A. U.), a value relative to CML-HSA defined as 100 arbitrary units (A.U.) as in Fig. 1.

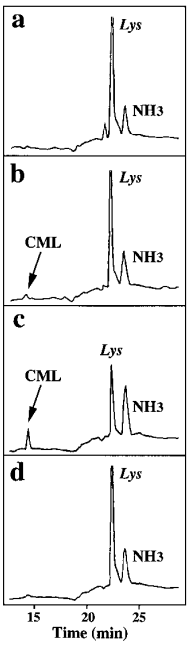

FIG. 4. Effects of Fe^{2+} and H_2O_2 on CML formation from Boc-fructose-lysine. Boc-fructose-lysine was incubated at 37°C for 1 h in the presence or absence of 0.4 mM $FeCl_2$ and 0.1 mM H_2O_2 in 50 mM sodium phosphate buffer (pH 7.2). Each sample was subjected to acid hydrolysis and applied to amino acid analysis as described under "Materials and Methods." Lysine (Lys) derived from acid hydrolysates of Boc-fructose-lysine was eluted at 22.09 min (a). When Boc-fructose-lysine was incubated with 0.4 mM $FeCl_2$, a CML peak eluted at 14.15 min which is identical to standard CML could be detected (b). Addition of 0.1 mM H_2O_2 and 0.4 mM $FeCl_2$ to this system resulted in a further increase in the CML peak (c). On the contrary, incubation of Boc-fructose-lysine with 0.1 mM H_2O_2 alone did not give a positive CML peak (d). NH_3 represents a peak of ammonia.

from Boc-fructose-lysine, decreased from 0.811 to 0.414 nmol (Fig. 4c). In contrast, however, incubation of Boc-fructose-lysine with 0.1 mM H_2O_2 alone did not generate a significant level of CML (<0.005 nmol) (Fig. 4d). These results obtained by the experiments using Boc-fructose-lysine support the contention that fructose-lysine is converted to CML by the action of $^{\cdot}OH$.

DISCUSSION

It has long been known that, since CML formation from proteins upon incubation with glucose is inhibited by antioxidants or antioxidative conditions, oxidation plays an important role in CML formation (20). In the present study, to examine effects of reactive oxygen species on CML formation from Amadori product, CML generated from glycated HSA was determined both by the immunochemical method using 6D12 and the amino acid analysis. When glycated HSA was incubated with 0.4 mM Fe^{2+} for 1 h, a significant amount of CML became detectable by competitive ELISA using 6D12 which was enhanced by the addition of H_2O_2, whereas H_2O_2 alone or O_2^- had no effect on the CML formation from glycated HSA.

There could be two steps for Fe^{2+}-induced CML formation from an Amadori product; the first step is interaction of Fe^{2+} with an Amadori product and the second one is its subsequent cleavage into CML and erythronic acid. Sakurai et al. (24) proposed that enediol chelated with Fe^{3+} might be oxidized to 2, 3-dicarbonyl of glycated HSA. However, a mechanism of subsequent cleavage of 2, 3-dicarbonyl compound into CML and erythronic acid is not clearly known. Since the Fe^{2+}-induced CML formation was enhanced by the addition of H_2O_2, it seems reasonable to expect that $^{\cdot}OH$ generated by Fenton reaction between Fe^{2+} and H_2O_2 derived endogenously from Amadori products might play an important role in decomposition of glycated HSA to form CML.

Based on the observation that glycated paired helical filament tau was able to generate O_2^- and this reaction was inhibited by the addition of SOD, Yan et al. (25) proposed O_2^- production from glycated paired helical filament tau. Consistent with this notion, O_2^- generated from glycated RNase was demonstrated by electron paramagnetic resonance (EPR) measurements (26). Dismutation reaction is known to convert O_2^- into H_2O_2. It is also suggested that formation of O_2^- and H_2O_2 were generated from Amadori compound even under physiological conditions (15). Taniguchi and his co-workers demonstrated that hydroxyl radicals produced from glycated Cu, Zn-SOD are responsible for site-specific fragmentation of the enzyme as well as its random fragmentation, indicating that $^{\cdot}OH$ is produced through Fenton reaction of Cu^{2+} with H_2O_2 released from the glycated Cu, Zn-SOD (27, 28). Under the present conditions, a significant amount of CML became detectable when glycated HSA was incubated with Cu^{2+} instead of Fe^{2+}, which was also greatly enhanced by the addition of H_2O_2 (data not shown). It is likely therefore that endogenous H_2O_2 is mainly produced from O_2^- which is originally derived from glycated HSA.

To clarify a direct role of $^{\cdot}OH$ in the Fe^{2+}-induced CML formation from glycated HSA, we tested effects of several radical scavengers on CML formation. Results clearly showed that Fe^{2+}-induced CML formation was significantly inhibited by the addition of catalase, whereas SOD had no effect on it (Fig. 2). On the other hand, mannitol, a well-known $^{\cdot}OH$ scavenger, did decrease CML formation to 52% of control. The partial inhibitory effect of mannitol could be explained by the possibility that hydroxyl radicals generated in the vicinity of metal-binding sites of glycated HSA such as enediol can react readily with 2,3-dicarbonyl before they are diffused and quenched by mannitol (29, 30). Chevion suggested the possibility that Fenton reaction occurs preferentially at or near metal-binding sites of proteins where reactive oxygen species can generate

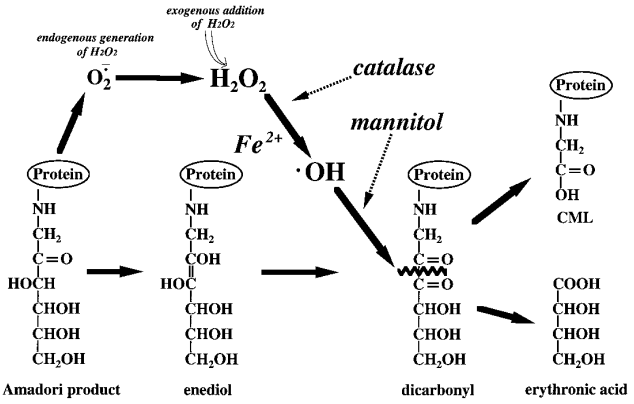

FIG. 5. Proposed mechanism of CML formation by the action of hydroxyl radical.

(31). Stadtman (32) also suggested that the metal ion-catalyzed oxidation of amino acids is a "caged" process, since the oxidation is not significantly inhibited by ·OH scavengers.

To evaluate a potential participation of ·OH, different concentrations of H_2O_2 were incubated with glycated HSA in the presence of 0.4 mM Fe^{2+}, followed by determination of CML by competitive ELISA. The result clearly indicated a major role of ·OH in CML formation, since Fe^{2+}-induced CML formation was enhanced dose-dependently by the addition of H_2O_2, but 1 mM H_2O_2 alone had no effect on it (see Fig. 3).

The results obtained with glycated HSA was confirmed by experiments using Boc-fructose-lysine. When Boc-fructose-lysine was incubated with 0.4 mM Fe^{2+} for 1 h, a significant amount of CML became detectable by amino acid analysis which was enhanced by the addition of H_2O_2, whereas H_2O_2 alone had no effect on the CML formation from Boc-fructose-lysine.

Based on the present data, a possible mechanism of Fe^{2+}-derived CML formation from glycated HSA was illustrated in Fig. 5. O_2^- generated from Amadori compound is converted to H_2O_2 by nonenzymatic dismutation reaction. Further reaction of this endogenous H_2O_2 molecule with Fe^{2+} by Fenton reaction gives rise to hydroxyl radicals. A carbon chain between C-2 and C-3 of dicarbonyl which has been converted from an Amadori compound through enediol, is then cleaved by these hydroxyl radicals, followed by cleavage into CML and erythronic acid. During these reactions, catalase and mannitol can inhibit the CML formation as a H_2O_2 scavenger and a ·OH scavenger, respectively, whereas exogenously added H_2O_2 results in acceleration of Fe^{2+}-induced CML formation by increasing ·OH production.

The chemical conversion of α-dicarbonyl compounds into carboxylic acid is known as Baeyer-Villiger reaction in which two carboxylic acids are generated from α-dicarbonyl by incubation with ~40 M H_2O_2 (33). However, the participation of this mechanism in the present study is unlikely because 1 mM H_2O_2 failed to generate CML from glycated HSA (Fig. 3).

The present study has demonstrated that ·OH 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. Since the presence of CML has been demonstrated in a variety of tissue proteins, ·OH is also expected to be involved in CML formation as well as long-term oxidative damage *in vivo*.

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