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Original article

Determination of AGEs in mice femur by LC-MS/MS

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Abstract

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been used in the research on advanced glycation end-products (AGEs), enabling the measurement of several types of AGEs. However, there still remain many unknowns regarding how AGE accumulate in tissues during aging and age-related diseases, and the relationship between each biological tissue and AGE accumulation. We have established a method for the determination of AGEs in various tissues using mice as an animal model and have quantified AGEs in various soft tissues, turning out to be difficult to quantify AGEs in bone tissue, which is a hard tissue, unlike soft tissue processed easily. In this study, we attempted to quantify AGEs in mouse femur using LC-MS/MS. As a result, N^{ε}-(carboxymethyl)lysine (CML) and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) were clearly detected in mouse femur, and the amount of CML in femur was significantly higher than that in the crystalline lens. These results confirm that AGE content is high in tissues such as bone, where metabolism and inflammatory reactions occur actively through the action of osteoblasts and osteoclasts. The fact that AGEs in hard tissues as well as soft tissues of mice can now be quantified using LC-MS/MS is expected to make it more effortless to study AGEs in rodents in the future.

KEY WORDS: glycation, N^{ε}-(carboxymethyl)lysine (CML), N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), femur, lens

Introduction

It has been shown that advanced glycation end-products (AGEs), generated from non-enzymatic reactions between proteins and carbonyl compounds, accumulate in living organisms as well as in foods, and that their accumulation rate is correlated with aging and the progression of lifestyle-related diseases [1, 2)]. Among AGEs, N^{ε}-(carboxymethyl)lysine (CML) is an AGE produced in an oxidation-dependent manner [3, 4)] and has been shown to be an indicator of oxidative stress. Whereas, among Arg-derived AGEs, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), a major AGE of Arg-derived AGEs, is produced *via* glyceraldehyde-3-phosphate in the glycolysis system, and evaluated as an indicator of abnormal glucose metabolism since it is increased in diabetic patients [5)]. Thus, multiple concurrent measurements of AGE structures are important for assessing abnormalities in the body. Although it has been difficult to quantify AGEs *in vivo*, recent advances in AGE research have made it possible to measure several AGEs using liquid chromatography-tandem mass spectrometry (LC-MS/MS). It is now recognized that AGE accumulation in the crystalline lens changes with differences in blood glucose levels in rats and humans, and preventive effects and therapeutic strategies for cataracts targeting inhibition of AGE accumulation in the lens are expected. However, it is still unclear what tissues produce or accumulate AGEs, which increase with aging and diseases, and the relationship between each tissue and AGE accumulation. Therefore, we have established a method to quantify AGEs in various tissues using mice as an animal model. We have reported that, using mice, CML accumulation in the lens increases with the onset of diabetes [6)], and that AGE accumulate in the liver, kidney, and brain, and that their accumulation in the brain and kidney increases with aging [7)]. However,

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bone is a hard tissue, the processing for crushing and measurement is different from that for soft tissues, and the amount of sample obtained from mice is small due to their size, which is a drawback of AGE measurement in bone. In this study, we attempted to quantify AGEs in mouse femur, the largest bone in size, from mice.

Methods

Animal Experiments

Animal experiments were performed with the approval of the Tokai University Ethics Committee (approval number: #191036). Male Sea:ddY mice (5-weeks-old) were purchased and housed in a pathogen-free barrier-free facility (12-hour light/dark cycle) for 3 months. CE-2 (Clea, Tokyo, Japan) and water were fed ad libitum. After 3 months, lenses and femurs were sampled from mice under 30% isoflurane (v/v) anesthesia.

Measurement of CML and MG-H1 in each tissue

Lenses were homogenized in 1 mM diethylenetriamine pentaacetic acid (DTPA) solution and crushed, and protein quantification was performed by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The blood was removed from femurs. Then, femur samples ware lyophilized, and crushed using liquid nitrogen. The samples were then removed lipid and minerals to be used for the assay [8]. For measurements, 0.3 mg of lens protein and 1 mg of crushed femur were used. Sodium borate buffer (0.2 M boric acid, 2 mM DTPA, pH 9.1) was then added in equal volume to the sample, and $NaBH_4$ solution (2 mM NaBH4, 0.1 M NaOH) was added at one-tenth the volume of the sample and allowed to stand for 4 hours. Then, 20% trichloroacetic acid (TCA) solution was added, stirred, and the proteins were precipitated by centrifugation (12,000 rpm, 5 minutes, 4 °C) and the supernatant was discarded. Subsequently, pretreatment for LC-MS/MS measurements was performed according to the previous studies [6]. Specifically, 0.01 nmol of CML and MG-H1 internal standard (PolyPeptide Laboratories, Strasbourg, France) and 5 nmol of Lysine internal standard (Cambridge Isotope Laboratories, Inc, Tewksbury, MA, USA) was added to samples and hydrolyzed using 1 mL of 6 M HCl at 100 °C for 18 h. The mixture was then centrifuged and concentrated, followed by the removal of hydrochloric acid using a centrifugal concentrator. To the dried sample, 1.0 mL of ultrapure water was added to dissolve the sample in the solvent. The sample was then placed on a Strata-X-C column (Phenomenex, Torrance, CA, USA) to remove unnecessary components, washed with 2% formic acid, eluted with 7% ammonia, and allowed to dry. The dried sample was dissolved in 20% acetonitrile 0.1% formic acid solution and used for LC-MS/MS measurements.

LC-MS/MS conditions

For LC-MS/MS measurements, a TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for the MS section. A ZIC®-HILIC column (150 × 2.1 mm, 5 µm; Merck Millipore, Billerica, MA, USA) was used for the LC section [6]. Targets were separated by a gradient mode of mobile phase A (0.1% formic acid) and mobile phase B (acetonitrile in 0.1% formic acid). The flow rate was 0.2 mL/min and the column oven temperature was set at 40 °C.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Significant difference tests were calculated using the Mann-Whitney U test with $p < 0.05$ being considered a significant difference.

Results

Mice were housed for one week after purchase and kept for 3 months. The fasting blood glucose level of mice before autopsy was 111 ± 18 mg/mL. AGEs in mouse lens and femur bone were quantified by LC-MS/MS; the predicted sites of fragment ions used for CML and MG-H1 measurements are listed in ***Fig.1*** [9]. As a result, CML and MG-H1 were clearly detected in mouse femur, and the intensity of detection was approximately 20 times higher than that of CML and MG-H1 in lens (***Fig.2***). The CML content in mouse femur was significantly higher than that in lens (***Fig. 3***), even when corrected for the content of Lys in each tissue.

Discussion

Although the Maillard reaction was first reported in 1912, more than 100 years ago, it remains difficult to accurately assess its progression. Browning, changes in fluorescence intensity, and immunological techniques using anti-AGE antibodies have been used, however the quantification of biological AGEs is more difficult and therefore remains at the research level, limiting the clinical use of AGE measurements.

We have previously reported that AGEs are artificially generated by heating [10] and alkali treatment [11]. However, the measurement of AGEs by gas chromatography-mass spectrometer (GC-MS) requires several steps of derivatization, which is disadvantageous for the analysis of multiple samples.

In addition, as a biological sample, the lens of the eye has been frequently measured for the evaluation of biological AGEs because it is easy to crush and briefly yields soluble protein. However, it is technically more difficult to quantify AGEs in bone tissue than in lens due to the hardness of the bone. In this study, we attempted to quantify AGEs in mouse femur using LC-MS/MS, which does not require derivatization. As a result, we were able to measure AGEs in bone protein by delipidating, demineralization, and hydrochloric acid hydrolysis of femur bone.

Lens proteins in the eye have been considered to be one of the tissues with high AGE content due to their low turnover rate. However, CML levels in the femur were unexpectedly higher than in the lens, and MG-H1 levels were similar to those in the lens. Production of carbonyl compounds and reactive oxygen species is increased in metabolically active organs [5] and inflammatory responses [12].

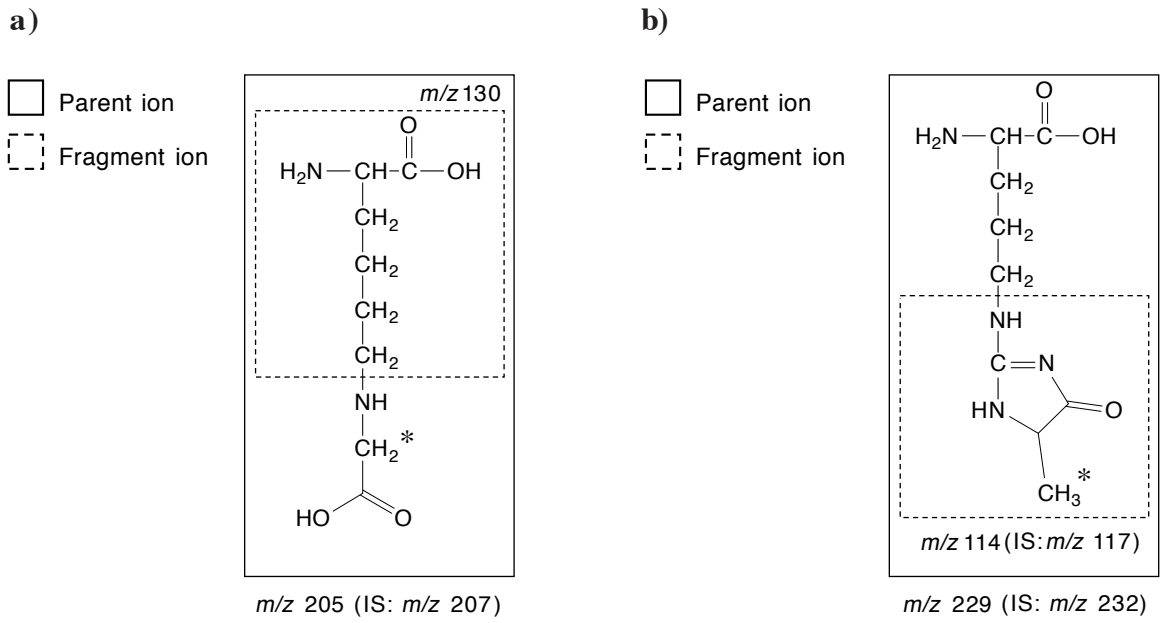

Fig. 1. **Structures of CML (a) and MG-H1 (b).**

The straight lines indicate the parent ion and the dotted lines the predicted sites of the measured fragment ions. CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

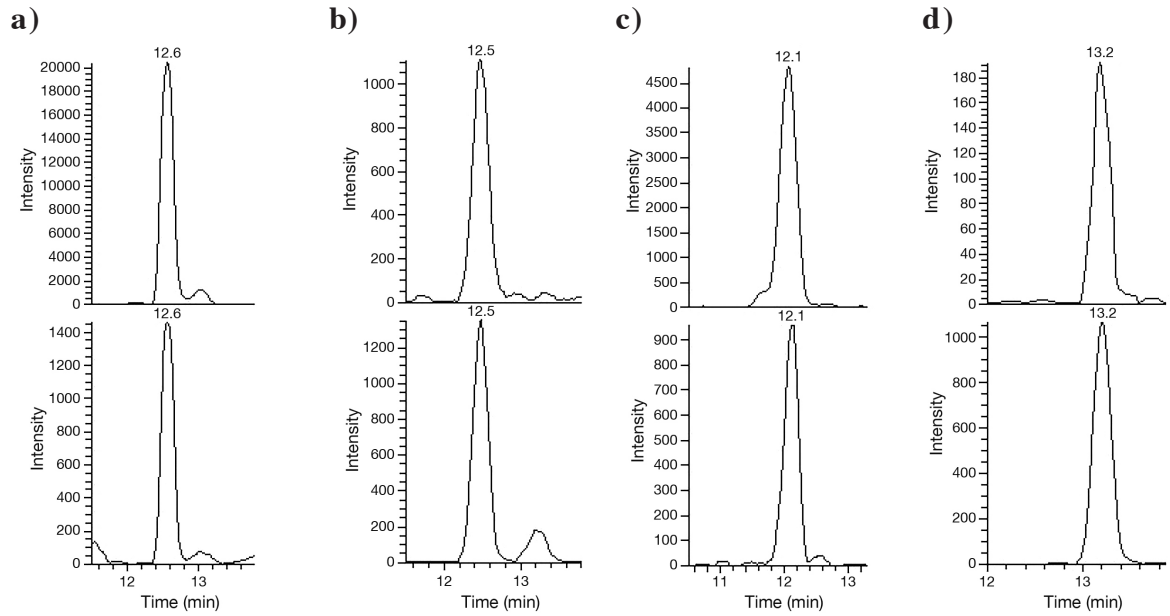

Fig. 2. **Chromatograms of AGEs in mouse tissue detected by LC-MS/MS.**

The upper peak represents AGEs in tissue, and the lower peak represents each AGE internal standard in tissue. CML peaks femur (**a**) and lens (**b**). MG-H1 peaks femur (**c**) and lens (**d**). AGEs, advanced glycayion endproducts; LC-MS/MS, liquid chromatography-tandem mass spectrometry; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

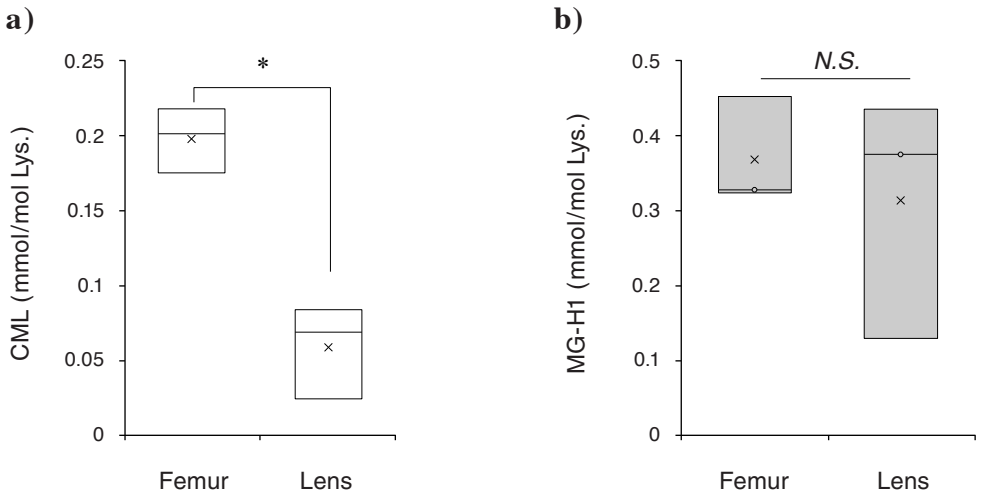

Fig. 3. **Comparison of CML and MG-H1 content between tissues.**

CML (**a**) and MG-H1 (**b**) content in mouse tissues in bone and lens, n = 3, significance level *p < 0.05. N.S., not significant; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

Since bone homeostasis is maintained by the balance between osteoblasts and osteoclasts, metabolism of bone is relatively active and generates AGEs [8, 13].

Suzuki *et al.* [13] reported that accumulation of CML in osteoblasts induces apoptosis, resulting in an increased risk of osteoporosis. In the present study, bone CML was higher than that in the lens, suggesting that AGE accumulation may affect bone homeostasis more than we had previously expected. The fact that AGEs can be quantified in hard tissues as well as soft tissues of mice by LC-MS/MS without derivatization is expected to make it more effortlessly to study AGEs in rodents.

Conflict of interest declaration

There are no relevant matters concerning this study.

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