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A Novel Biomarker for Hyperglycemia, MRX Isolated from Hydrolysate of Glycated Proteins

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Long-lived proteins can undergo non-enzymatic glycation to form highly crosslinked structures with characteristic fluorescence during aging and diabetes processes. In this paper, a typical fluorophore, named Maillard reaction product X (MRX), was isolated from the hydrolysate of glycated proteins. MRX could be formed by incubation of bovine serum albumin with glucose, followed by acid hydrolysis. The structure of MRX was determined to be 8-hydroxy-5-methyldihydrothiazolo[3,2-α]pyridinium-3-carboxylate. MRX was also found to be formed by the incubation of cysteine and arginine with glucose, followed by hydrolysis. We found the formation of MRX in the recently developed genetically diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats and compared them with that in the control Long-Evans Tokushima Otsuka (LETO) rats. Significantly higher levels of MRX were observed from the serum ($p < 0.005$) and urinary protein ($p < 0.001$) of OLETF rats in comparison with those of LETO rats. MRX must be a potential candidate as a biomarker for hyperglycemia. © 1998 Academic Press

The relationship between the metabolic disorder of diabetes and the pathogenesis of secondary complications, in particular microangiopathy, remains unclear. However, there is increasing evidence that chronic hyperglycemia is the major cause of these complications. The discovery of glucose-dependent chemical alterations of various proteins suggested that they could induce functional abnormalities of these proteins and thereby lead to the pathophysiology of diabetes. Glycation of hemoglobin A was the first example of a protein modification correlated with hyperglycemia of diabetes [1]. More recently, evidence for glycation of serum albumin [2, 3], lipoproteins [4] and transferrin [5] has been obtained. Also, glycation of tissue proteins such as lens crystalline [6] and collagen [7] has been reported.

Glucose degradation products can undergo non-enzymatic glycation with proteins to form advanced glycation end products (AGEs) during the development of micro- and macrovascular complications and also cataracts in diabetes [8] [9] [10]. Recently it has been shown that the AGEs are associated with neurofibrillary tangles and plaques of Alzheimer§ disease [11, 12], and their formation in proteins and lipoproteins was found to enhance oxidative stress [13, 14, 15].

The OLETF rats are a new animal model for human non-insulin dependence diabetes mellitus (NIDDM) that has been established to form an outbred Long-Evans strain from Charles River Canada [16, 17]. The OLETF rats develop a diabetic syndrome in nearly 100% of male rats at 25 weeks of age. The OLETF rats exhibit hyperglycemia and hyperinsulinemia during the early phase of the disease as a result of islet cell hyperplasia and peripheral insulin-resistance [16, 18]. As age progresses, the rats eventually develop hypoinsulinemia as a result of deterioration of the islet B cells. At 40 weeks of age, exudative changes occur in the kidneys, which closely resemble those of human NIDDM [16].

Here we report the first isolation and structural characterization of MRX, a novel type of biomarker for hyperglycemia, which was found in a hydrolysate of glycated proteins. In addition, we report the mechanism for MRX formation using an *in vitro* system. In this

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Abbreviations: HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrum; NMR, nuclear magnetic resonance; AGEs, advanced glycation end products; MRX, Maillard reaction product X; BSA, bovine serum albumin; N^{α}-*t*-Boc-cystine, N^{α}-*t*-butoxycarbonyl-L- cystine; N^{α}-*t*-Boc-arginine, N^{α}-*t*-butoxycarbonyl- L -arginine; TMS, tetramethylsilane; HMBC, ^{1}H-detected multiple-bond heteronuclear multiple quantum coherence spectrum; OLETF, Otsuka Long-Evans Tokushima Fatty; LETO, Long-Evans Tokushima Otsuka; STZ, streptozotocin.

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paper, we furthermore examined the presence of MRX in the hydrolysate of serum and urinary proteins in OLETF rats. We also detected and quantified MRX in the hydrolysate of urinary proteins in streptozotocin (STZ)-induced diabetic rats, which were the animal model of human insulin dependence diabetes mellitus.

MATERIALS AND METHODS

Materials. N^{α}-acetyl-cysteine, N^{α}-*t*-Boc-arginine, N^{α}-*t*-Boc-cystine and bovine serum albumin (essentially fatty acid free) were purchased from Sigma (St. Louis, MO). Glucose and guanidine hydrochloride were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animal. Male OLETF rats (8 weeks old, n = 8, 227-261 g body weight) and control LETO rats (8 weeks old, n = 8, 282-322 g body weight) were kindly donated by Tokushima Research Institute, Otsuka Pharmaceutical (Tokushima, Japan). Male Wistar rats (6 weeks old, n = 10, 126-135 g body weight) were obtained from Japan SLC, Inc. (hamamatsu, Japan). Five days later, STZ, freshly dissolved in 150 mg/2.5 ml physiological saline, was administered to rats (n = 5) at a dose of 60 mg/kg B.W. by a single intraperitoneal (*i.p.*) injection. All rats were housed under controlled light/dark conditions (lights on: 07:00-19:00) with the room temperature regulated to 23-25°C. Rats were allowed free access to commercial chow diet (Rodent diet CE-2, CLEA) and tap water. The blood glucose levels in OLETF and LETO rats aged 72 weeks were 418.4197.9 mg/dl (meanS.D.) and 185.616.8 mg/dl, respectively ($p < 0.001$). The blood glucose levels in STZ-treated and normal rats aged 11 weeks were 405.034.2 mg/dl and 138.36.2 mg/dl, respectively ($p < 0.01$).

General procedure. Separation of MRX was carried out on a Jasco Gulliver HPLC with a model 821-FP fluorescence detector at an excitation wave length of 340 nm and monitoring of emission at 402 nm. FAB-MS were measured with a JEOL JMS-DX-705L instrument. NMR spectra were recorded with a Bruker AMX600 (600 MHz) instrument, using TMS as an internal reference (δ0.00). Ultraviolet absorption spectra were measured with a Hitachi U-Best-50 spectrophotometer, and fluorescence spectra were recorded with a Hitachi F-2000 spectrometer.

The formation of MRX by incubation of BSA with glucose and by hydrolysis. BSA (4 mg/ml) in 0.1 M phosphate buffer (pH 7.4) was incubated with 0.1 M glucose at 37°C for 2 weeks. Aliquots were dialyzed against distilled water for 48 h with water changing every

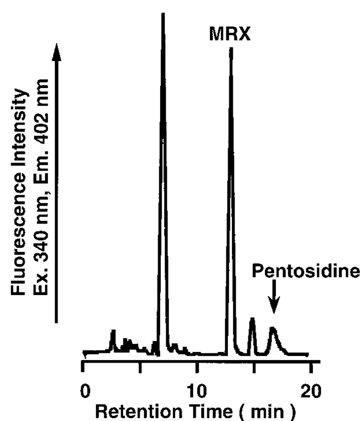

FIG. 1. HPLC analysis of hydrolysate of glycated BSA. Conditions for the HPLC are described in the experimental section.

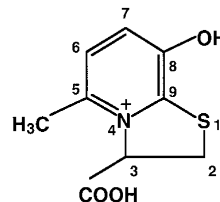

FIG. 2. Structure of MRX.

16 h or carried out by gel filtration chromatography (Sephadex G-100) with 0.01 M phosphate buffer (pH 7.4). The purified glycated BSA was freeze-dried and hydrolyzed with 6 N HCl at 105 C for 24 h. The acid hydrolysate of glycated BSA was dried and dissolved in 200 :l of water and filtered through a 0.45-:m filter (Nacalai tesque). Ten microliters of the solution was injected into HPLC. Separation was done in a Develosil ODS-HG-5 column (0.46 × 25 cm). To elute the column, 7 mM phosphate in water (flow rate, 1.0 ml/min.) was used. The eluent was monitored at 210 nm and the fluorescence at 340/402 nm. MRX was eluted between 13-14 min (Fig. 1).

Detection and quantification of MRX in the hydrolysate of proteins in rats. Urine was collected from OLETF rats aged 72 weeks and STZ-treated rats aged 11 weeks and dialyzed against distilled water for 48 h, changing water every 16 h. The dialyzate was freeze-dried and hydrolyzed with 6 N HCl at 105°C for 24 h. The hydrolysate was dried and dissolved with 200 μl water, which was analyzed for MRX by HPLC.

To 200 μl serum from each, cold 40% trichloroacetic acid (200 μl) was added. Precipitated proteins were separated by centrifugation at 11,000 x *g* for 10 min. The pellets were collected and washed with 200 μl diethyl ether. The resultant pellets were dried and subjected to acid hydrolysis with 6 N HCl at 105°C for 24 h. The hydrolysate was dried and redissolved with 200 μl water, which was analyzed for MRX by HPLC. Separation was performed in a similar manner as the hydrolysate of glycated BSA.

RESULTS

Structure of MRX

^{1}H-NMR spectrum exhibited the following signals; δ_H (D_2O): 2.60 (s, 3H, 5-CH_3), 3.96 (d, 1H, J = 11.9 Hz, H-2), 4.11 (dd, 1H, J = 11.9, 8.8 Hz, H-2), 5.83 (d, 1H, J = 8.8 Hz, H-3), 7.34 (d, 1H, J = 8.4 Hz, H-6), 7.60 (d, 1H, J = 8.4 Hz, H-7). ^{13}C-NMR spectrum showed the following signals; δ_C (D_2O): 19.9 (5-CH_3), 34.4 (C-2), 73.1 (C-3), 125.3 (C-6), 129.5 (C-7), 146.3 (C-5), 149.6 (C-9), 150.6 (C-8), 171.1 (3-COOH). The methylene was correlated with the carbonyl carbon in the HMBC spectrum. The 2D spectrum also showed the correlation of H-2 and C-3, of H-3 and C-2, and of H-3 and C-9. The molecular formula, $C_9 H_{10} O_3$ N S, was obtained by high-resolution FAB-MS of MRX. The material was optically active, $[\alpha]_D^{24} = -130°$ (c 1.2 in 0.1 N NaOH). MRX was identified as 8-hydroxy-5-methyldihydrothiazolo[3,2-a] pyridinium-3-carboxylate (Fig. 2). This compound was reported as a kinase inhibitor prepared from liver hydrolysate [19]. MRX was a fluorescent substance with an excitation λmax value of 340 nm and an emission λmax value of 402 nm, and it was proposed that MRX must be the pyridium ionic form and con-

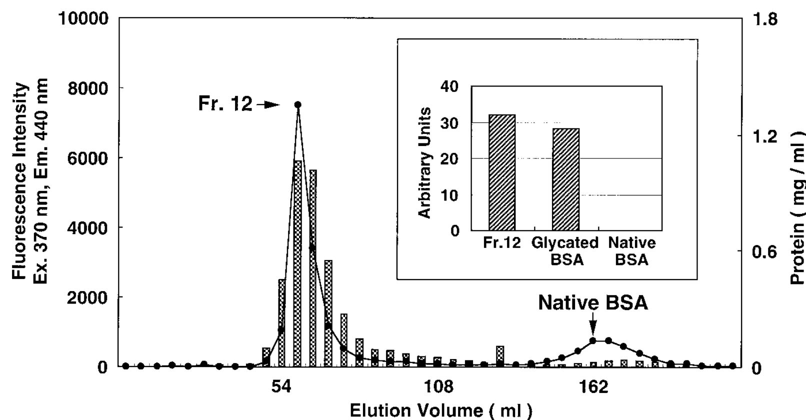

FIG. 3. Gel filtration of glycated BSA. (●): protein, (▩): fluorescence. The inset shows the formation of MRX. Fr. 12 shows the polymer of glycated BSA obtained by gel filtration. Glycated BSA in insert graph is obtained by dialysis from BSA incubated with 300 mM glucose for 2 weeks at 37°C.

densed-ring compound of cysteine and glucose. Furthermore, since the pyridium ring was formed by the reaction of the α-amino group, it could be formed secondarily by hydrolysis.

In Vitro Analysis for the Formation of MRX

In order to clarify that MRX was formed from glucose bound to proteins, glycated BSA was purified by gel filtration chromatography (Sephadex G-100). Fr. 12 in Figure 3 shows the trimer or tetramer of glycated BSA obtained by gel filtration chromatography. Fr. 12 had a large quantity of proteins and fluorescence. Because MRX was formed from Fr. 12 by hydrolysis as well as dialysate of glycated BSA (insert graph), glucose which was not bound to the BSA did not contribute to the formation of MRX. It is suggested that the precursors of MRX must be present in glycated proteins and play the role of cross-linkers.

BSA incubated with glucose showed a time-dependent increase in the formation of MRX (Fig. 4). Two weeks incubation of BSA with 300 mM glucose showed six times as large as 10 mM glucose in the formation of MRX. Instead of BSA, red blood cells incubated with glucose were also found to form MRX in time-dependence (data not shown). MRX was also formed by incubation of N^{α}-acetyl-cysteine and N^{α}-*t*-Boc-arginine with glucose. Furthermore, this compound was formed from N^{α}-*t*-Boc-cystine or glutathione and N^{α}-*t*-Boc-arginine with glucose (data not shown). Moreover, when guanidine hydrochloride was substituted for N^{α}-*t*-Boc-arginine incubated with glucose and N^{α}-acetyl-cysteine, MRX was formed by hydrolysis. These reaction mixtures were analyzed by HPLC. The peak (retention time 13-14 min) of the reaction mixtures was collected and identified by ^{1}H-NMR. However, N^{α}-*t*-Boc-arginine or guanidine hydrochloride addition after incubation of N^{α}-acetyl-cysteine with glucose did not form MRX by hydrolysis (data not shown). The formation of MRX was observed only in the presence of N^{α}-*t*-Boc-arginine or guanidine hydrochloride at the same time during the incubation of thio-amino acid with glucose. These results suggested that the guanidino group is necessary for the formation of the precursors of MRX.

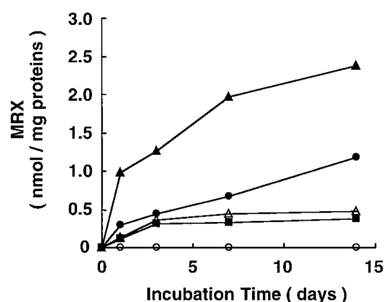

FIG. 4. Formation of MRX by incubation of 4 mg/ml BSA with 0 (○), 10 (■), 50 (△), 100 (●), and 300 (▲) mM glucose.

To speculate on the precursor of MRX, *S*-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-L-cysteine methyl ester was synthesized following the previously reported method [20, 21]. MRX was formed in a 4.35% yield, when this thio-glucoside was hydrolyzed. Neither N^{α}-*t*-Boc-arginine nor guanidine hydrochloride was required in the hydrolysis. The guanidino group, therefore, could be required for the formation or the stability of the precursors of MRX. However, it is not clear what kind of mechanism is involved in the contribution of the guanidino group in the formation of the precursor of MRX.

Detection and Quantification of MRX in the Hydrolysates of Proteins in Rats

OLETF rats, a genetic model of spontaneous development of NIDDM, exhibit hyperglycemic obesity with

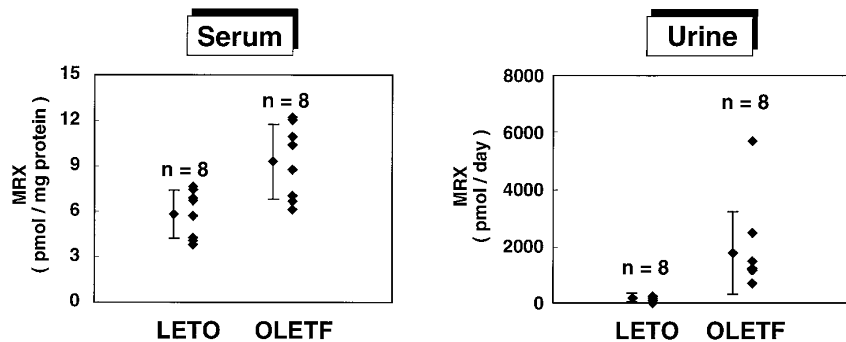

FIG. 5. Detection of MRX by HPLC in the hydrolysate of serum and urinary proteins in OLETF rats and LETO rats. Conditions for HPLC are described in the experimental section.

hyperinsulinemia and insulin resistance similar to that in humans. The body weight of OLETF rats increased more rapidly than that of normal, LETO rats and decreased slowly since they were 40 weeks old. The blood glucose levels of OLETF rats were 2.3 times those of LETO rats. We quantified the excretion of MRX in the hydrolysates of diabetic rats= serum and urinary proteins. The protein fractions were collected and hydrolyzed, and the formation of MRX in each hydrolysate was determined by HPLC. The retention time of the peak of MRX in the hydrolysates was identical to that of purified MRX isolated from glycated BSA. MRX isolated from OLETF rats= serum and urinary proteins was identified by spectroscopic characterization. The serum of OLETF rats showed significantly higher levels of MRX ($p < 0.005$) compared with that of normal subjects. The levels of MRX in the hydrolysate of LETO and OLETF rats= serum proteins were 5.92.7 and 9.24.4 pmol per mg protein, respectively (Fig. 5). The levels of MRX in the urinary proteins of OLETF rats were 109.9 times those of LETO rats ($p < 0.001$). The levels of MRX in the hydrolysates of LETO and OLETF rats= urinary proteins were 16.98.6 and 1854.51577.1 pmol per day, respectively. The difference in the levels of pentosidine [22] in the hydrolysate of serum and urinary proteins between OLETF and LETO rats was not regarded as significant (data not shown). The levels of MRX in the urinary proteins of STZ-treated rats were 18.5 times those of normal rats. The MRX levels in the hydrolysate of urinary proteins in normal rats were 40.411.3 pmol per day and in STZ-treated rats were 745.9587.0 pmol per day ($p < 0.01$). The blood glucose levels of STZ-treated rats were also significantly higher than those of normal rats ($p<0.01$). The levels of MRX in both OLETF and STZ-treated rats were proportional to those of blood glucose (Fig. 6).

DISCUSSION

The methods employed in this study resulted in the isolation of a novel type of an AGE, termed MRX, from the incubation of glucose, N^{α}-acetyl-cysteine and N^{α}-*t*-Boc-arginine. Although glycation of lysine and arginine has been studied by many investigators [22, 23, 24, 25], there is little agreement as to the contributions of glycation of the cysteine residue. By our detailed examination, MRX was found to be formed from the reactant of glucose with the cysteine residue in the proteins by glycation. MRX has already been isolated and identified after acid hydrolysis from bovine liver, which was shown to inhibit the breakdown of bradykinin by kininases [19]. MRX would be the condensed-ring compound formed secondarily by hydrolysis and not present in the body. However, this compound arose by hydrolysis from both the oxidized form glutathione and N^{α}-*t*-Boc-cystine during the course of incubation with glucose and N^{α}-*t*-Boc-arginine. We studied the mechanism for MRX formation on the basis of these results. Our data suggested that MRX must be formed secondarily by hydrolysis from a precursor of MRX which is present in glycated cysteine, because we found that MRX was detected in the glycated BSA purified by gel filtration (Fig. 3). As shown in Fig. 3, MRX formed only from the hydrolysate of polymerized BSA; therefore, it is suggested that the precursors of MRX must play a role as cross-linkers. Furthermore, because

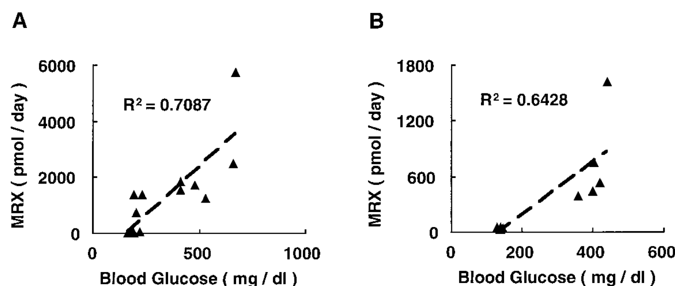

FIG. 6. Relationship between the MRX levels and the blood glucose levels in OLETF and LETO rats (A) and normal and STZ-treated rats (B). The formation of MRX in the hydrolysate of urinary proteins were detected by HPLC. HPLC conditions are the same as in Fig. 5.

MRX was not formed from the incubation of glucose and cysteine without arginine or guanidine, the guanidino group would be necessary for the formation of the precursor of MRX.

Recently, we succeeded in synthesizing *S*-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-L-cysteine methyl ester [20, 21], one of the potential candidates for the precursor of MRX. In fact, MRX was formed by the hydrolysis of this thio-glucoside in a 4.35% yield without arginine or guanidine in preparation. These results suggested that the precursor of MRX must have the thio-glucoside moiety in the structure, and the guanidino group plays an important role for attacking to cysteine residue by oxidized glucose, although it is still under investigation how the guanidino group contributes to the reaction of oxidized glucose with the SH group of cysteine. The direct role of glucose in cross-linking positions of long-lived proteins has not yet been established *in vivo*, because of the difficulties in identifying the structures of oxidized glucose and precursors in the glycated proteins both *in vivo* and *in vitro*. Now, we have been involved in the isolation and characterization of the precursors of MRX in glycated proteins.

MRX was not detected in the hydrolysate of native BSA, but was found in a large quantity in that of glycated BSA. This result indicated that MRX is one of the main acid-stable fluorescent products formed from the glycated proteins. We examined MRX by hydrolysis in proteins excreted by rats. The levels of MRX in the hydrolysate of serum and urinary proteins in OLETF rats were 1.6 and 109.9 times those of LETO rats, respectively. The blood glucose levels in OLETF rats were increased 2.3 folds compared with LETO rats. The levels of pentosidine [22], one of the most well-known AGEs, in the hydrolysates of proteins were not significantly different between OLETF and LETO rats. The levels of blood glucose and MRX in the hydrolysate of urinary proteins in STZ-treated rats were also significantly higher than those of normal rats. From these results, we conclude that the precursors of MRX must be formed under high glucose conditions, and more detailed relationship between the level of the precursor of MRX and hyperglycemia should be studied.

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