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Preferential recognition of Amadori-rich lysine residues by serum antibodies in diabetes mellitus: Role of protein glycation in the disease process

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ABSTRACT

This study analyzes effect of glycation on proteins rich in lysine residues as hyperglycemia induced protein glycation has been mainly reported in diabetes mellitus at the intrachain lysine residues leading to the formation of Amadori modified proteins. We have studied the effect of glucose on poly-L-lysine (PLL), a homopolymer of lysine residues. Levels of Amadori products in the glycated PLL were evaluated by fructosamine assay and the presence of 5-hydroxymethylfurfural (HMF) in the glycated PLL was analyzed by thiobarbituric acid assay. Fluorescence and FT-IR spectroscopy were applied to characterize the modified PLL. Binding characteristics of experimentally induced antibodies against glycated PLL and the presence of antibodies against glycated PLL in the sera of diabetes patients was evaluated by solid phase enzyme immunoassays. The fructosamine assay showed significantly high yield of early glycation (Amadori) products in the glycated PLL, which was confirmed by increased yield of HMF from Amadori products of glycated PLL. Loss in fluorescence intensity and appearance of a new band corresponding to Amadori products were observed in FT-IR spectrum of the glycated PLL. Glycated PLL was found to be highly immunogenic in rabbits as compared to the native form. Serum antibodies from diabetes patients showed appreciably high recognition of the glycated PLL. The results conclusively show the glycation induced damage to the lysine molecules and specific recognition of Amadori-lysine residues by serum antibodies from diabetes patients. The glycated lysine residues may serve as a diagnostic biomarker for early glycation process in diabetes mellitus.

1. Introduction

Nonenzymatic glycation of proteins is increased in diabetes mellitus due to hyperglycemia and causes several complications. Interaction of glycated proteins with beta cell receptor and/or pancreatic β-cell destruction by islet cell autoantibodies may hold a key to insulin synthesis [1]. The glycation primarily occurs at intrachain lysine residues of proteins [2] and involves the condensation reaction of the carbonyl group of reducing sugar aldehydes with α- and ε-amino groups of lysine residues. The nucleophilic addition reaction rapidly forms Schiff base (an aldimine) and then through acid–base catalysis these labile adducts undergo rearrangements to form more stable early glycation product known as Amadori product (a fructosamine) [3]. Irreversible chemical reactions in Amadori products generate advanced glycation end products (AGEs), which may be fluorescent with cross-linking properties, such as pentosidine and crosslines, or nonfluorescent and noncrosslinking, such as N^{ε}-(carboxymethyl)lysine (CML)] or non-fluorescent crosslinked product as pyrrline [4].

Structural characterization of AGEs has been reported by numerous investigators, but only a few studies have focused on characterization of Amadori products. In this study, we report characterization of Amadori products that were formed in a high concentration during incubation of PLL with glucose for a specified time. Antigenicity of native and glycated PLL was probed in an animal model. Serum samples of diabetes patients were also investigated for the presence of antibodies against PLL, specifically glycated PLL.

2. Subjects and methods

2.1. Reagents

Poly-L-lysine (PLL), human serum albumin (HSA), histone, anti-rabbit/anti-human IgG-alkaline phosphatase conjugate, *p*-nitro-phenyl phosphate, Tween-20, and Freund's complete and incomplete adjuvants were purchased from Sigma (St. Louis, MO). D-glucose and sodium borohydride ($NaBH_4$) were obtained from Merck (Darmstadt, Germany). Polystyrene flat bottom microtiter enzyme-linked immunoabsorbent assay (ELISA) plates and modules were from Nunc (Roskilde, Denmark). All other chemicals and reagents used were of highest analytical grade available.

2.2. Collection of human blood and serum samples

Blood was collected from voluntary donors with a history of diabetes mellitus (both type 1 and type 2 diabetic subjects) attend-

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ing outdoor and indoor in the J.N. Medical College Hospital, A.M.U., Aligarh. Samples from normal healthy subjects were collected for use as control. Informed consent was obtained from each patient or from patients' family members. The study protocol was approved by the local ethics review committee. Subjects' data has been presented in Table 1.

Sera, separated from the blood samples, were decomplemented by heating at 56°C for 30 minutes and stored in aliquots at −20°C with 1% sodium azide as preservative.

2.3. Preparation of Amadori-rich glycated PLL

Methods described elsewhere [5–7] were adapted with slight modification to prepare glycated PLL containing extremely high level of Amadori products. Briefly, PLL (1 mg/ml) was incubated with 50 mmol/l glucose for 7 days at 37°C in 20 mmol/l sodium phosphate buffer (pH 7.4) in the presence of 5 mmol/l diethylenetriaminepentaacetic acid (DTPA). After incubation, it was extensively dialyzed against phosphate buffer (pH 7.4). Equal concentration of PLL dissolved in the same buffer served as control.

Glycation of IgG, HSA, and histone was performed by the same method.

2.4. Reduction of glycated PLL with $NaBH_4$

The glycated PLL was reduced with $NaBH_4$ in phosphate buffer, pH 8.0, as per a previously described procedure [8]. Excess borohydride was destroyed by slow addition of 1N HCl.

2.5. Determination of Amadori products

The level of Amadori products, measured as fructosamine in the dialyzed preparation, was determined by NBT reduction assay [9] after some modifications. Native and glycated PLL samples (100 μl each) were respectively added to 96-well microtiter plates in duplicate wells. A 100-μl quantity of NBT reagent (250 μmol/l in 0.1 mol/l carbonate buffer, pH 10.35) was added to each well and incubated at 37°C for 2 hours. The color was read in a microplate reader at 525 nm. Amadori products were determined using an extinction coefficient of 12640 $cm^{-1} \cdot mol^{-1}$ for monoformazan [10].

2.6. Determination of hydroxymethylfurfural (HMF) in glycated PLL

Briefly, 1 ml each of the native and glycated PLL samples were mixed with 1 M oxalic acid and incubated at 100°C for 2 hours [11]. The protein from the assay mixture was removed by precipitation with 40% trichloroacetic acid. Thiobarbituric acid (0.05 mol/l) was added to protein-free filtrate and incubated at 40°C for 40 minutes. The color was developed and the amount of HMF was calculated using molar extinction coefficient value of 4×10^4 $cm^{-1} \cdot mol^{-1}$ at 443 nm for HMF.

2.7. ROS modification of glycated PLL

The glycated PLL was modified by hydroxyl radical as described earlier with minor modifications [12]. Briefly, glycated PLL (in 20 mmol/l sodium phosphate buffer, pH 7.4) was incubated with 5 mmol/l $FeCl_2$ and 10 mmol/l H_2O_2 for 1 hour at 37°C. The assay mixture (dissolved in 20 mmol/l sodium phosphate buffer) was then extensively dialyzed against phosphate-buffered saline, pH 7.4. Formation of N^{ε}-(carboxymethyl)lysine (CML) in the dialyzed preparation was confirmed by ^{1}H-NMR (Bruker DRX-300) spectrometry.

Table 1
Characteristics of diabetic and normal human study subjects

Variable	Control subjects	Type 1 diabetic subjects	Type 2 diabetic subjects
n	10	22	12
Age (y)	42 ± 2	25 ± 8	52 ± 12
Sex (male:female)	6:4	14:8	6:6
BMI (kg/m^2)	22.8 ± 4.1	24.4 ± 3.7	26.1 ± 4.8
Blood glucose (mg/dl)	109.2 ± 7.5	354.0 ± 28.6	279.5 ± 32.4
HbA_1c (%)	4.9 ± 0.4	8.3 ± 1.2	7.1 ± 1.4
Serum fructosamine (μmol/l)	198.3 ± 5.1	366.5 ± 17.3	301.6 ± 13.8

BMI= body mass index.

2.8. Fluorescence studies

The native and glycated PLL were subjected to fluorescence studies on a Shimadzu (RF 5301-PC) spectrofluorophotometer at 25° ± 0.1°C in a 1-cm path length cuvette. The slit width was fixed at 5 nm. Native and glycated PLL samples were excited in the range of 280–410 nm and the emission intensities were recorded in the range of 300–500 nm. The possible presence of AGEs in the glycated PLL was verified with AGE-specific fluorescence at 440 nm after excitation at 370 nm [13]. Loss of fluorescence intensity (FI) was calculated using the following equation: % Loss of FI = ($FI_{\text{native PLL}} - FI_{\text{glycated PLL}}$ / $FI_{\text{native PLL}}$) × 100.

2.8. FT-IR measurements

The preparation was then subjected to spectral recording on a Shimadzu FT-IR spectrophotometer (8201-PC). Samples to be analyzed on FT-IR spectrophotometer were first lyophilized and prepared as KBr pellets.

2.9. Induction of antibodies against Amadori-rich glycated PLL

A previously described protocol was followed to induce antibodies against the glycated PLL in randomly bred New Zealand white female rabbits [14]. Briefly, rabbits (n = 4; two each for the native and glycated PLL) were immunized intramuscularly at multiple sites with 100 μg of the respective antigens emulsified with an equal volume of Freund's complete adjuvant. The animals were boosted with repeated dose of antigens in Freund's incomplete adjuvant at weekly intervals for 6 weeks. To evaluate antibody titer, test bleeds were performed after alternate booster dose. Once a desired level of antibody was obtained, the animals were bled and the separated serum was heated at 56°C for 30 minutes to inactivate complement proteins.

2.10. ELISA

ELISA was performed on polystyrene plates [15] with slight modification. Polystyrene polysorp immunoplates were coated with 100 μl of the native or glycated PLL (10 μg/ml) in 0.05 mol/l carbonate–bicarbonate buffer, pH 9.6. The plates were incubated for 2 hours at 37°C and overnight at 4°C. Each sample was coated in duplicate and half of the plate, devoid of antigen coating, served as control. Unbound antigen was washed three times with TBS-T (20 mmol/l Tris, 150 mmol/l NaCl, pH 7.4 containing 0.05% Tween-20), and unoccupied sites were blocked with 2% fat-free skimmed milk in TBS (10 mmol/l Tris, 150 mmol/l NaCl, pH 7.4) for 6 hours at 37°C. After incubation, the plates were again washed three times with TBS-T. Test serum was added to antigen-coated wells and reincubated for 2 hours at 37°C and overnight at 4°C. Bound antibodies were assayed with anti-rabbit/anti-human IgG-alkaline phosphatase conjugate using *p*-nitrophenyl phosphate as substrate. Absorbance (A) of each well was monitored at 410 nm on an automatic microplate reader, and the mean of duplicate readings for each sample was recorded. Results have been expressed as a mean of $A_{test} - A_{control}$.

2.11. Competition ELISA

The antigen-binding specificity of the antibodies was evaluated by competition ELISA [16]. Varying amounts of inhibitor (0–20 μg/ml) were mixed with a constant amount of antiserum or IgG. The mixture was incubated at room temperature for 2 hours and