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Zinc inhibits glycation induced structural, functional modifications in albumin and protects erythrocytes from glycated albumin toxicity

Rashmi Tupe[a,*], Amruta Kulkarni[a], Krishna Adeshara[a], Neena Sankhe[a], Shamim Shaikh[a], Sayli Dalal[b], Siddharth Bhosale[b], Sushama Gaikwad[b]

[a] Biochemical Sciences Division, Rajiv Gandhi Institute of IT and Biotechnology, Bharati Vidyapeeth University, Katraj, Pune 411046, India
[b] Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune 411008, India

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ABSTRACT

The present work aims to investigate the concentration and time dependant effect of zinc on the *in vitro* non enzymatic modifications of albumin by diabetic levels of glucose. Further, preventive and curative effect of zinc was studied by adding zinc before and after initiation of glycation respectively. Glycation of albumin was done at different concentrations of zinc (125, 250 and 500 μM) at different time intervals (21, 28 and 35 days) with appropriate controls. The antiglycation potential of zinc was assessed by estimating different markers of albumin glycation (fructosamines, carbonyls, bound sugar, AGEs), structural modifications (free amino, thiol group, β amyloid, native PAGE, ANS binding, fluorescence lifetime decay and CD analysis) and functional properties (antioxidant activity, hemolysis). Zinc at highest concentration (500 μM) significantly reduced modifications of albumin which was comparable to aminoguanidine and also protected secondary and tertiary structure of albumin after 28 days of incubation. Zinc exhibited significant protective effect on erythrocytes by inhibiting hemolysis. Thus the present study indicate preventive mode of albumin glycation inhibition by zinc.

1. Introduction

Chronic hyperglycemia is major factor responsible for development of diabetic complications. In such a hyperglycemic condition, glucose or glucose derived metabolites irreversibly and nonenzymatically modify extracellular and intracellular macromolecules. Formation and accumulation of advanced glycation end products (AGEs) is well characterized and best understood example of such modifications [1]. These modifications involve condensation of sugar aldehydes and ketones with free amino group through nucleophilic additions, resulting in rapid formation of Schiff bases followed by formation of more stable amadori products which further go degradation into highly reactive carbonyl compounds that again react with free amino groups to form intermediate amadori products and AGEs [2,3]. As AGEs irreversibly attached to macromolecules, their level does not decline even though hyperglycemia is corrected as these products continue to accumulate at unstable rates over the life span of diabetic tissue component [4]. Glycation is also associated with increased production of reactive oxygen species and reduced cellular antioxidant defense leading to oxidative stress followed by diabetic complications. In addition to causing cytotoxicity to variety of cells, major pathological consequence of glycation is free radical induced erythrocytes membrane peroxidation which includes increased membrane rigidity, decreased cellular deformability, reduced erythrocyte survival and lipid fluidity [5]. Therefore inhibition of glycation is an efficient approach to prevent cytotoxicity and reduce such complications.

Serum albumin (~66 KDa), most abundant protein in blood plasma (concentration in range of 35–54 mg/mL), functions as a major transport protein for numerous endogenous and exogenous substances. Albumin folds into three homologous domains, with each domain subdivided into two sub domains [6]. It has been reported that albumin has a variety of metal sites with different specificities [7]. Because of its long half life (≈21 days) and high concentration, albumin is highly sensitive to glycation. The glycation process induces structural modification of albumin, such as increase in molecular weight, decrease in free thiol and amino groups *etc.* depending upon extent of glycation [8]. These modifications affect the functional, transport and antioxidant properties of albumin [9,10].

* Corresponding author at: Rajiv Gandhi Institute of IT and Biotechnology, Bharati Vidyapeeth University, Katraj, Pune 411046, Maharashtra, India.
Tel.: +91 20 9922263074.
E-mail address: rashmitupe@gmail.com (R. Tupe).

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Zinc, an essential element of body, is critical for the development and functioning of cell, immune system and for transmission of genetic information, with catalytic and structural roles in wide range of proteins [11]. About 80% of plasma zinc is bound to albumin at two inter domain specific binding sites and albumin plays major role in zinc transport to various cells and tissues [12,13]. Plasma concentration of zinc is low in people with diabetes due to loss of zinc through urine. We have previously reported the inhibitory effect of zinc on albumin glycation [14,15] which is supported by other studies [16]. This finding prompted us to conduct a more thorough examination of zinc in glycation induced albumin modifications.

In present study we sought to investigate the concentration and time dependant effect of zinc on *in vitro* glycation of albumin by diabetic levels of glucose. Additionally to examine preventive or curative role of zinc in glycation, zinc was added before and after albumin glycation.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA, Fraction V), sodium azide, aminoguanidine (AG), nitro blue tetrazolium (NBT), 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), p-benzoquinone, 1-anilininaphthalene-8-sulphonic acid (ANS) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were obtained from Sigma (St. Louis, MO, USA). Glucose and zinc sulphate were obtained from Merck (Germany). All other reagents were of analytical grade.

2.2. Glycation of albumin

Glycation was initiated by incubating BSA (42 g/L) with a 15 mM glucose solution was prepared in phosphate buffer saline (PBS), pH 7.4. Glycation was carried out in presence of zinc. Glycated BSA samples along with zinc were maintained in three different sets. In first set, glycated BSA was incubated with different concentrations of zinc (125 μM, 250 μM and 500 μM) for 21, 28 and 35 days at 37 °C in dark. In second set glycation was initiated by adding glucose in BSA and zinc solution after 10 days of incubation period (pre-incubation) and in third set zinc was added to glycated BSA after 10 days followed by 21 days of incubation (post-incubation). All the solutions were made in PBS and filtered through 0.22 μm membrane filter into sterile filtered storage vials. Positive control (BSA + Glucose), negative control (BSA) and standard glycation inhibitor *i.e.* BSA + Glucose + AG, were maintained for each set. At the end of appropriate incubation period, unbound glucose was removed by dialysis against PBS and dialysate were used for further analysis of protein by Lowry's method [17], glycation markers and functional modifications. All additions were performed aseptically in triplicates.

2.3. Estimation of different glycation markers

The concentration of frutosamines, carbonyls and bound glucose in glycated albumin samples and controls were determined by reported methods [18–20]. The formation of AGEs in glycated albumin samples was assessed according to Tupe and Agte [15] where the fluorescence was measured at an excitation and emission wavelength of 370 nm and 440 nm respectively on Perkin Elmer Luminescence spectrometer (LS50B, USA). The results were expressed as arbitrary units (AU).

2.4. Estimation of structural modifications of albumin

Free thiol groups, free amino groups and β amyloid aggregation in glycated albumin samples were estimated by reported methods [21–23]. Formation of high molecular weight (HMV) aggregates after glycation was assessed by using 8% native polyacrylamide gel electrophoresis according to Laemmli's method [24].

2.4.1. ANS binding

Analysis of hydrophobic region in glycated albumin samples was performed using ANS [25]. Glycated samples were diluted appropriately in PBS and 5 μL of ANS (2.5 mM in 50 mM sodium phosphate buffer, pH 7.4) was added. ANS–protein complex fluorescence was measured at 440 nm with an excitation wavelength of 355 nm.

2.4.2. Fluorescence lifetime decay of tryptophan

Lifetime measurements were carried out on Edinburgh Instruments' FLS-920 single photon counting spectrofluorimeter. The diluted Ludox solution was used for measuring Instrument Response Function (IRF). The sample was excited at 295 nm and emission was recorded at 339 nm. The resultant decay curves were analyzed by a reconvolution fitting program supplied by Edinburgh Instruments.

2.4.3. CD analysis

The CD spectra of all the samples were recorded on a J-815 Spectropolarimeter with a PTC343 Peltier unit (Jasco, Tokyo, Japan) at 25 °C in a quartz cuvette. Each CD spectrum was accumulated from three scans at 100 nm/min with a 1 nm slit width and a time constant of 1 s for a nominal resolution of 0.5 nm. Far UV CD spectra of all the samples (0.2 mg/mL) were collected in the range of wavelengths of 190–250 nm using a cell path length 0.2 cm for monitoring the secondary structure. The tertiary structure of the glycated BSA (1 mg/mL) was monitored with near UV CD spectra in the wavelength 250–300 nm using path length 0.5 cm. Results were expressed as mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$.

2.5. Estimation of functional modifications of albumin

2.5.1. Antioxidant potential

To analyze the antioxidant potential of albumin, free radical scavenging activity of glycated samples was determined using ABTS assay [26]. Glycated samples (20 μL) were incubated with ABTS working solution (980 μL) and readings were recorded at 0 min and 6th min at 734 nm.

2.5.2. Protection to erythrocytes

The inhibition of oxidative stress induced erythrocyte hemolysis by glycated albumin was evaluated according to procedure described by Ajila and Rao with slight modifications [27]. Fresh human blood was collected in EDTA coated tubes and centrifuged at 3000 rpm for 10 min to remove plasma, followed by erythrocyte washing with 0.15 M sodium chloride solution (thrice). Cells were then suspended in PBS (pH 7.4) for final yield of erythrocytes suspension was 1×10^8 erythrocytes/mL. The erythrocytes were treated with 100 μM H_2O_2 and without glycated samples to obtain complete hemolysis (This was considered as 100% hemolysis). To 100 μL of erythrocyte suspension 100 μL glycated samples was mixed and 100 μL of H_2O_2 (100 μM) was added followed by incubation at 37 °C for 3 h. The absorbance of the supernatant was measured at 540 nm by employing ELISA plate reader (BioTek Elx800).