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Research Paper

Glycation of bovine serum albumin by ascorbate *in vitro*: Possible contribution of the ascorbyl radical?

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ABSTRACT

Ascorbic acid (AA) has been reported to be both pro-and antiglycating agent. *In vitro*, mainly proglycating effects of AA have been observed. We studied the glycation of bovine serum albumin (BSA) induced by AA *in vitro*. BSA glycation was accompanied by oxidative modifications, in agreement with the idea of glycoxidation. Glycation was inhibited by antioxidants including polyphenols and accelerated by 2,2′-azobis-2-methyl-propanimidamide and superoxide dismutase. Nitroxides, known to oxidize AA, did not inhibit BSA glycation. A good correlation was observed between the steady-state level of the ascorbyl radical in BSA samples incubated with AA and additives and the extent of glycation. On this basis we propose that ascorbyl radical, in addition to further products of AA oxidation, may initiate protein glycation.

Introduction

One of unavoidable posttranslational protein modifications is the non-enzymatic glycosylation (glycation). Protein glycation is initiated by a nucleophilic addition reaction between the free amino group of a protein, lipid or nucleic acid and the carbonyl group of a reducing saccharide or aldehyde. This reaction forms a reversible Schiff base, which rearranges over a period of days to generate Amadori products. The Amadori products undergo dehydration and rearrangements followed by other reactions such as cyclization, oxidation, and dehydration to form more stable Advanced Glycation End Products (AGEs) [1,2].

Many substances affect protein glycation, some promoting and some inhibiting this process. One of such compounds is ascorbic acid (AA), a key exogenous non-enzymatic antioxidant present in plasma and within cells. In the general population the assumed optimal plasma AA concentration of 50 μM, as proposed by a consensus conference, can be achieved by the intake of 100 mg of vitamin C per day, which is the new recommendation of the Austrian, German, and Swiss Nutrition Societies [3]. AA has been reported to be both an anti- and proglycating agent. Numerous studies have demonstrated mainly pro-glycating activity of AA in *in vitro* systems and mainly anti-glycating activity of this compound *in vivo* [4–11].

A simple explanation of these divergent results implies that AA oxidation products i.e. dehydroascorbic acid (DHA) and compounds formed by its decomposition, and not AA itself are the glycating agents in AA-containing systems. Unlike AA, DHA is unstable both in the absence and in the presence of oxygen, and is a reactive electrophile, which generates further reactive degradation products over time in solution. These electrophilic products react with nucleophiles on proteins, specifically lysinyl and arginyl residues, resulting in structurally deleterious, non-enzymatic modifications of proteins. Increases in DHA degradation have been involved in the etiology of a variety of diseases, including age-related cataract [9,12], diabetes [13] and Alzheimer's disease [14]. These diseases have all been associated with increases in ROS production and protein glycation.

Although glycation of long-lived proteins is the most important from the physiological point of view, the effect of glycation on shorter-living proteins like plasma albumin is also of interest as their properties and functions may be affected by this process. Many *in vitro* glycation experiments have been done on human serum albumin and bovine serum albumin (BSA) which has high (76%) sequence homology to human serum albumin. The glycation of albumin induces several structural and functional modifications, including alterations in ligand binding [15,16].

We have found previously that AA enhanced BSA glycation induced by sugars, especially glucose, and induced glycation itself [17]. The aim of the present study was the characterization of the AA-induced glycation of BSA and of the effects of various

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compounds on this process.

Materials and methods

Materials

All basic reagents were from Sigma-Aldrich Company (Poznań, Poland) unless indicated otherwise. Genistein, 4-hydroxycinnamic acid, naringin, quercetin and genkwanin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Sample preparation

BSA (purity of 96%) was dissolved in 0.1 M sodium phosphate buffer, pH 7.4, at a concentration of 0.09 mM. AA was used at a concentration of 1 mM. The samples without or with selected additives were incubated in closed vials at a temperature of 37 °C for 6 days with 1 mM sodium azide as a preservative. The following water-soluble additives were used: 0.05, 0.1, 0.2, 0.5 and 1 mM nitroxides: 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), 4-hydroxy-TEMPO and 4-carboxy-TEMPO; oxidants: 0.1 mM H_2O_2, 5 mM 2,2′-azobis-2-methyl-propanimidamide hydrochloride (AAPH) and 1 mM $FeCl_3$; antioxidants (1 mM): captopril, tiron and trolox; metal chelators: ethylenediminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), diethylenetriaminepentaacetic acid (DTPA) and nitrilotriacetic acid (NTA); organic acids: 1-cyano-4-hydroxycinnamic acid, 4-hydroxycinnamic acid; polyphenols: caffeic acid, ellagic acid, ferulic acid, gallic acid and propyl gallate. Genistein, genkwanin, naringin, quercetin and rutin were dissolved in dimethylsulfoxide (DMSO) so the level of glycoxidation found in samples containing AA and DMSO was used as a reference in these cases. The above concentrations of selected anti-glycating compounds had been applied in our earlier *in vitro* experiments [17–20].

In studies of the effects of the effects of addition of superoxide dismutase (SOD) and catalase (CAT) on AA-induced BSA glycation, 10 μg/ml SOD and 10 μg/ml CAT were used.

AGE measurements

AGEs are a heterogeneous group of compounds with a characteristic fluorescence. AGEs were estimated by assessing the formation of AGE-derived fluorescence, termed glycophore, using the spectrofluorimetric method according to Henle et al. [21] and Münch et al. [22] at the excitation and emission wavelengths of 325 and 440 nm, respectively. Two hundred microliters of aliquots were applied to a 96-well plate. In order to check the validity of results based on the assay of fluorimetric indices of BSA oxidative damage, the AGEs content was also evaluated by enzyme-linked immunosorbent assay (ELISA) Kit for Advanced Glycation End Product (USCN Life Science Inc., Product no. CEB353Ge), according to the instruction of the manufacturer. Increase in the AGE content in samples incubated with AA only (in the absence of any potential protectors) with respect to a sample incubated with AA only or AA with DMSO (for compounds introduced from DMSO solutions) was assumed as 100%; the increase in samples containing selected anti-glycating protector was expressed as % of this value.

Content of dityrosine, kynurenine, and N′-formylkynurenine

The levels of dityrosine, kynurenine, *N*′-formylkynurenine and, to a lesser extent, kynurenine fluorescence are markers of protein oxidation [23]. The content of dityrosine, kynurenine, *N*′-formylkynurenine and kynurenine was estimated on the basis of their characteristic fluorescence at the wavelengths of 325/440 nm, 330/415 nm, 325/434 nm and 365/480 nm, respectively [24]. Two hundred microliters of aliquots were applied to a 96-well plate.

Content of amyloid cross-β structure

Formation of amyloid cross-β structure was measured using thioflavin T [25,26]. Briefly, 5 μl of 640 μM thioflavin T in 0.1 M sodium phosphate buffer, pH 7.4, were added to 95 μl of a sample. The fluorescence intensity was measured at excitation/emission wavelengths of 435/485 nm after 1-h incubation at room temperature. Fluorescence measurements were made in an Infinite 200 PRO multimode reader (Tecan Group Ltd., Männedorf, Switzerland).

Electron paramagnetic resonance (EPR) spectroscopy

ESR spectra of samples at "time 0" (ca 30 min after formulation of the incubation media) and after 6 days were analysed using a Bruker FT-EPR spectrometer ELEXSYS E580 (Teaching Center of Microelectronics and Nanotechnology, University of Rzeszów). 50 μl of each sample was introduced into a capillary tube for EPR measurement (non-heparinized microhematocrit tubes ~75 μL; 1.55 × 75 mm; Medlab Products, Raszyn, Poland). Sample capillaries were placed into a quartz EPR sample tube and centered in a standard rectangular microwave cavity under critical coupling conditions. The spectrometer operated at X-band (9.850537 GHz). The following settings were used: central field, 3507.00 G; modulation amplitude, 0.3 G; modulation frequency, 100 kHz; microwave power, 94.64 mW; power attenuation 2.0 dB; scan range, 50 G; conversion time, 25 ms; sweep time, 25.6 s. Two scans were typically accumulated to intensify the signals observed. The spectra were recorded and analysed using Xepr software. The signal was integrated twice to determine its area, and thus the concentration of the radical.

Statistical analysis

All the experiments were done at least in triplicate. Data were shown in the form of mean values and standard deviations. The statistical analysis of the data was performed using STATISTICA software package (version 10, StatSoft Inc. 2010, Tulsa, OK, USA, www.statsoft.com). Differences between means were analysed using Student's *t*–test for independent samples and were considered significant or highly significant at $^{\blacktriangle}p$-values < 0.05, $^{\#}p$-values < 0.01 and $^{*}p$-values < 0.001.

Results and discussion

Ascorbic acid is present in blood plasma at relatively low concentrations (about 50 μM) [3,27] but can be accumulated in some cells expressing SVCT1 or SVCT2 transporter up to millimolar concentrations [28,29]. We chose an intermediate concentration of 1 mM for routine studies of the AA-induced glycation *in vitro*.

Studies of the kinetics of AA-induced AGE formation showed a hyperbolic-type course of the reaction, reaching saturation after several days. The increase in the level of AGEs was parallelled by increases in the levels of dityrosine, *N*′-formylkynurenine and kynurenine, indicating that protein glycation was accompanied by oxidative changes, in agreement with the idea of glycoxidation. Interestingly, DMSO enhanced the rate of AA-induced glycoxidation. The measurements of the formation of amyloid cross-β structure were less reproducible showing a high day-to-day variation. DMSO inhibited rather than enhanced amyloid cross-β structure formation (Fig. 1). On the basis of kinetic data, the