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Monitoring of the human serum albumin carbonylation level through determination of guanidino group content

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ABSTRACT

Carbonylation of the protein amino, guanidine, and thiol groups with α-oxoaldehydes (which are produced in higher quantities in diabetes, uremia, oxidative stress, aging, and inflammation) is one of the important causes of vascular complications. For monitoring of the human serum albumin (HSA) carbonylation level, a spectrophotometric method based on the formation of colored adduct between guanidine group and thymol–sodium hypobromite reagent in the alkaline medium was investigated. Beer's law is obeyed in the concentration range of Arg and protein guanidine groups from 1 to 40 mM. Precision of the method (relative standard deviation) was in the range of 0.9 to 2%. Accuracy was examined by the standard addition method (recovery ~100%). The method was applied for monitoring of the carbonylation level of HSA with methylglyoxal in vitro and of HSA isolated (using affinity chromatography) from sera of 21 patients with type 2 diabetes and 12 healthy persons. The content of guanidine groups in HSA isolated from diabetics (19.64 ± 1.07 mM/mM albumin) was significantly lower ($P < 0.001$) in comparison with a control group (21.87 ± 1.02 mM/mM albumin). The method is simple and fast, has good accuracy and precision, and is suitable for clinical practice as well for in vitro protein carbonylation experiments.

Dicarbonyl compounds (α-oxoaldehydes: glyoxal, methylglyoxal [MG],[1] 3-deoxyglucosone, and glycolaldehyde) are produced in higher quantities in diabetes, uremia, arteriosclerosis, oxidative stress, aging, and inflammation [1–4]. They are highly reactive and act as potent protein [2] and nucleic acid modifying agents, leading to carbonylation of protein. Modification of proteins with MG and glyoxal is dramatically faster than that with glucose even at lower concentrations and with shorter incubation periods [5].

Human serum albumin (HSA) is the most abundant protein in plasma (~0.6 mM). It participates in the maintenance of colloid osmotic blood pressure, and it binds and transports fatty acids, hormones, bilirubin, vitamins, metal cations, and drugs. In addition, HSA has an antioxidant role in plasma, scavenging oxidant species [6]. Modification of albumin through carbonylation and oxidative stress have been shown to contribute to vascular complications in patients with diabetes [7]. Patients with diabetes develop micro and major vascular complications [8], and in this disease there are a number of equally tenable hypotheses on the origins of vascular tissue damage, including protein glycation and the subsequent formation of advanced glycation end products (AGEs). These reactions lead to changes in serum albumin structure and, therefore, to the altered biological function.

Lysine, arginine [9], and cysteine [2] residues present on protein surfaces participate in protein modification with α-oxoaldehydes. Some products of Lys and Cys carbonylation are well characterized: mono adducts (N_ε-carboxymethyllysine [CML] and N_ε-carboxyethyllysine [CEL], *S*-(carboxymethyl)cysteine, and *S*-(carboxyethyl)cysteine, which are used as markers of glycation, i.e., of secondary complications development in diabetes) and protein crosslinks (3-deoxyglucosone lysine dimer [DOLD], methylglyoxal lysine dimer [MOLD], and glyoxal lysine dimer [GOLD], which are significant in terms of tissue damage in aging and diabetes) [10–14]. Carbonylation of the guanidine group with MG lead to formation of *N*-(carboxymethyl)arginine, which is subsequently transformed to 1,5-dihydroimidazolone and slowly oxidized to 5-methylimidazol-4-one [2]. These dicarbonyl-derived AGEs represent the major chemical modifications that accumulate in tissue proteins with age and in chronic diseases such as diabetes and atherosclerosis [15].

Currently, there is no commonly accepted or widely used method to detect AGEs, nor are there commercially available kits for use in diagnostic procedures. The most common methods used

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[1] *Abbreviations used:* MG, methylglyoxal; HSA, human serum albumin; AGE, advanced glycation end product; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; HbA_{1C}, glycated hemoglobin; SD, standard deviation; BSA, bovine serum albumin; RSD, relative standard deviation.

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for detection are high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry [16]. The aim of our research was to develop a method that allows easy monitoring of changes of the amino acids side chains on the surface of HSA molecules in carbonyl stress and, therefore, the assessment of chemical damages and biological property changes of these molecules. Because amino and guanidine groups are highly abundant on the surface of HSA (59 Lys amino groups and 24 Arg guanidine, 1 Cys side residue per protein molecule) [17], the changes in their levels should be proportional to the amount of carbonylation. For the estimation of carbonylation level of protein amino groups, the spectrophotometric method based on the reaction of amino group with *p*-benzoquinone was proposed [18]. Although this method is simple, precise, and accurate, and the interference of thiol group is minimized, sometimes the correction for this influence is necessary. In addition, it is very useful to get better insight into protein modification (i.e., into the changes of structure and function of proteins) through analysis of the content of several reactive groups on the protein surface.

Therefore, in this study, a spectrophotometric method suitable for monitoring of the HSA guanidino group content changes during in vitro experiments of carbonylation and during carbonyl stress in patients with diabetes was investigated. The method is based on the Sakaguchi reaction, that is, on the coupling of thymol and hypobromite to the guanidyl group in the presence of high concentration of sodium hydroxide [19]. The principal product of semicarbazone quinone structure (established by Dupin et al. [20]) is ionized and colored. During in vitro carbonylation, the amino group content was also determined and the influence of both amino and guanidine group changes on HSA properties was considered.

Materials and methods

Chemicals

All chemicals were purchased from Sigma (Steinheim, Germany) unless otherwise noted. The 20% solution of HSA was purchased from Baxter (Vienna, Austria), Cibacron Blue F3G-A Sepharose CL-6B was purchased from Pharmacia Biotech (Uppsala, Sweden), and bromine was purchased from Merck (Darmstadt, Germany). All chemicals used were analytical grade. Deionized water was used for all experiments. Professional laboratory glassware and plastics (sample tubes, Pasteur pipettes, and pipette tips, Brand, Wertheim, Germany) were used.

Guanidine group assay

A spectrophotometric determination of guanidine groups was performed as follows. To 10 μl of sample, 2 ml of thymol solution (0.01% thymol in 0.5 M NaOH) was added. Absorbance on 470 nm was measured (on a Beckman DU-50 spectrophotometer) after the addition of 100 μl sodium hypobromite (2% bromine in 5% NaOH) against reagent blank. Sodium hypobromite solution is stable for 1 month if kept at +4 °C.

Monitoring of the guanidine group changes during in vitro incubation of HSA with MG

HSA solutions (0.5 mM) were prepared in 0.1 M phosphate buffer (pH 7.4) and incubated in capped vials at 37 °C with 41 mM MG final concentration for 8 h. All solutions were sterile filtered prior to incubation. Aliquots of the reaction mixtures were taken at predetermined intervals during incubation. The excess of MG in those aliquots was removed by successive dilutions with 0.02 mM phosphate buffer (pH 7.4) (1:10, v/v) and concentrations using a filtration tube (molecular weight cutoff [MWCO] of 10,000, Amicon Ultra-15, Millipore, Carrigtwohill, Ireland), which were repeated four times. Albumin concentrations were measured by the biuret method [21], with 20 μl of the sample being added to 1 ml of biuret reagent. Absorbance at 546 nm was measured against the reagent blank after 30 min. The standard curve was created with HSA in the concentration range from 0.015 to 1.5 mM ($y = 0.3333x - 0.00364$, $r = 0.9998$, $P < 0.001$). For each aliquot of the reaction mixtures, the content of reactive guanidine (and amino) group was determined in triplicate.

In addition, the modifications of albumin during the incubation with MG were analyzed using native polyacrylamide gel electrophoresis (PAGE, 9% acrylamide) according to the protocol of Hoeffer Scientific Instruments [22]. Here, 7 μl of protein samples (0.05–0.15 mg/ml) was added to the gels. Gels were stained with Coomassie Brilliant Blue G-250 (CBB).

Quantification of guanidino groups in HSA isolated from sera

A total of 21 type 2 diabetic patients, who were hospitalized due to poor metabolic control, were selected for this study. Glycated hemoglobin (HbA_{1c}) was measured by the immunoturbidimetric method (Architect ci8200, Abbott, Chicago, IL, USA) [23]. A control group consisted of 12 healthy volunteers of appropriate age and sex. All persons gave their informed consent to participate in this study, which was approved by the local ethics committee. The investigations were performed in accordance with the principles of the Declaration of Helsinki.

HSA was isolated from sera of diabetic patients and healthy persons using affinity chromatography (Cibacron Blue F3G-A Sepharose CL-6B). A column of blue Sepharose (4.3 × 0.8 cm) was equilibrated with 0.02 M sodium phosphate (pH 7.2). Samples of 250 μl human serum were applied, and the column was washed with 14 ml of 0.02 M sodium phosphate buffer (pH 7.2). The bound albumin was eluted with 1.5 M NaCl in 0.02 M sodium phosphate buffer (pH 7.2). The first 6 ml of albumin fractions was collected and concentrated by ultrafiltration using a filtration tube. The content of guanidine groups in isolated albumin fractions was determined as described above.

Amino group assay

The spectrophotometric determination of protein amino group content was performed as follows. First, 100 μl of the sample, 1360 μl of 0.1 M potassium phosphate buffer (pH 7.4), and 40 μl of 0.1 M *p*-benzoquinone in dimethyl sulfoxide were mixed and incubated at 37 °C for 15 min. The absorbance at 480 nm was then measured against the sample blank promptly after incubation. The standard curves were created with HSA in the amino group concentration range from 0.2 to 2.0 mM (in probe), which corresponded to the real sample levels of amino groups of 3 to 30 mM ($y = 0.02182C + 0.02611$, $r = 0.9988$, $P < 0.0001$).

Statistical analysis

The statistical analysis was performed by Student's *t* test. Linear regression analysis and Pearson's correlation coefficient were used to compare the biochemical variables. Each result is expressed as the mean ± standard deviation (SD) from a minimum of three experiments.