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Methyglyoxal administration induces modification of hemoglobin in experimental rats: An in vivo study

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ABSTRACT

Methylglyoxal, a highly reactive α-oxoaldehyde, increases in diabetic condition and reacts with proteins to form advanced glycation end products (AGEs) following Maillard-like reaction. In the present study, the effect of methylglyoxal on experimental rat hemoglobin in vivo has been investigated with respect to structural alterations and amino acid modifications, after external administration of the α-dicarbonyl compound in animals. Different techniques, mostly biophysical, were used to characterize and compare methylglyoxal-treated rat hemoglobin with that of control, untreated rat hemoglobin. In comparison with methylglyoxal-untreated, control rat hemoglobin, hemoglobin of methylglyoxal-treated rats (32 mg/kg body wt. dose) exhibited slightly decreased absorbance around 280 nm, reduced intrinsic fluorescence and lower surface hydrophobicity. The secondary structures of hemoglobin of control and methylglyoxal-treated rats were more or less identical with the latter exhibiting slightly increased α-helicity compared to the former. Compared to control rat hemoglobin, methylglyoxal-treated rat hemoglobin showed higher stability. Peptide mass fingerprinting analysis revealed modifications of Arg-31α, Arg-92α and Arg-104β of methylglyoxal-treated rat hemoglobin to hydroimidazolone adducts. The modifications thus appear to be associated with the observed structural alterations of the heme protein. Considering the increased level of methylglyoxal in diabetes mellitus as well as its high reactivity, AGE-induced modifications may have physiological significance.

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1. Introduction

Reducing sugars react with proteins by Maillard reaction to form advanced glycation end products (AGEs) involving Schiff base and Amadori products [1]. The non-enzymatic modification of proteins may be significant with increased level of blood glucose over prolonged periods of time in diabetes mellitus. Protein glycation reactions leading to AGEs are thought to be the root causes of different diabetic complications, including oxidative stress [2]. Besides glucose, other glycating agents include fructose [3], glyoxal [4], methylglyoxal [5], 3-deoxyglucosone [6] etc. Findings from our laboratory indicate that glycation of hemoglobin by glucose [7,8], fructose [9] and methylglyoxal [10] promote iron release and free radical-mediated oxidative reactions.

Methylglyoxal (MG), a highly reactive α-oxoaldehyde, is mainly derived from triose phosphates D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate during glycolysis in eukaryotic cells, and its blood level increases in both type 1 and type 2 diabetes mellitus [11–13]. The median concentration of MG is increased by 5–6-fold and 2–3-fold in blood samples of diabetic patients with Type 1 and Type 2 diabetes mellitus, respectively [14], and the formation of MG-derived AGEs is increased accordingly. MG-derived AGEs have been reported with different proteins namely insulin [5], human serum albumin [15], cytochrome *c* [16], α-synuclein [17], superoxide dismutase [18], etc. We have reported MG-induced structural alterations of myoglobin in recent studies [19,20], etc.

In vitro reaction of hemoglobin with MG has been reported in some earlier studies. In a brief report, Chen et al. have shown that MG interacts with hemoglobin with modifications of arginine residues forming hydroimidazone (MG-H1) [21]. Gao and Wang have found that the sites and extents of MG modification of arginine residues are correlated with solvent accessibility of these residues [22]. We have recently reported interaction of MG with hemoglobin leading to modification of arginine residues and subsequent changes in structural and functional properties of the heme protein [10]. Besides several in vitro studies, a number of in vivo animal studies on MG have been published. The α-oxoaldehyde has been reported to induce diabetes-like complications and retinal injury in experimental rats [23,24]. MG administration has been also shown to cause glucose intolerance, brain mitochondrial impairment and peritoneal fibrosis in rats [25–27]. Although in vitro interaction of MG with hemoglobin has been shown in previous studies, till date no studies describing the effect of MG on hemoglobin in vivo have been reported. Considering this, in the present study, we have administered MG in experimental rats and investigated its effect on rat

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hemoglobin in vivo with respect to structure and stability changes, as well as site(s) and nature of amino acid modifications.

2. Materials and methods

2.1. Materials

MG, Sephadex G-100, acrylamide, Coomassie R250, sequencing grade trypsin, α-cyano-hydroxycinnamic acid matrix (CHCA), 1-anilino-naphthalene-8-sulfonate (ANS), were purchased from Sigma Chemical Company, USA. All other reagents were AR grade and purchased locally.

2.2. Methods

Animal experiments were performed in accordance with regulations specified and monitored by the Institutional Ethics Committee. Male Wister rats (weighing 70–80 g) were divided in four groups - I, II, III and IV, consisting of four rats in each group. Group I rats were not treated with MG and denoted as control group. Groups II, III and IV rats were respectively injected with 8, 16 and 32 mg/kg body weight MG intravenously and were denoted as MG-treated groups. All animals were fed with standard diet and water ad libitum. Whole blood was collected from each group after one week by heart puncture and hemolysates prepared.

2.3. Separation of hemoglobin by size-exclusion column chromatography

Hemolysates prepared from different groups of rats were subjected to native PAGE. Total

hemoglobin was purified from hemolysates using Sephadex G-100 column chromatography [28] and subjected to native gel electrophoresis. The concentration of hemoglobin was determined from Soret absorbance using an extinction coefficient (ε_{415nm}) of 125 mM^{-1} cm^{-1} (heme basis) [29].

2.4. MALDI-TOF mass spectrometric study

Hemoglobin obtained from control group I and MG-treated group IV rats were subjected to mass spectral analysis using the linear positive ion mode of MALDI-TOF MS after digestion with sequencing-grade trypsin in solution at 37 °C for 16 h using enzyme: protein ratio 1:100 (*w*/*w*). The digested samples (0.5 μl each) were loaded directly to the MALDI plate, mixed with 0.5 μl of saturated CHCA solution (prepared in 50% acetonitrile and 0.1% trifluoroacetic acid) and allowed to dry and crystallize. Mass spectra were recorded in a 4800 Proteomics Analyzer (MALDI-TOF/TOF mass spectrometer, Applied Biosystems) using the linear positive ion mode of MALDI-TOF MS at 20 kV acceleration voltage. Identification of MG-modified peptides and specific MG-derived AGE adducts was performed as described earlier [19,20]. Purified hemoglobin of group I and group IV rats were used for further experiments.

2.5. Absorbance spectroscopy

Absorbance spectra of control and MG-treated rat hemoglobin (3 μM each) were recorded in a UV/VIS Spectrophotometer (Hitachi U 2000) using 1 ml quartz cuvette of path length 1 cm in the region 250–600 nm, taking 3 μM protein in each case.

2.6. Spectrofluorimetric study

Fluorescence emission spectra of purified hemoglobin of control and MG-treated rats were recorded in the region 320–400 nm with excitation at 280 nm in a spectrofluorimeter (Hitachi F-3010). Protein concentration was adjusted to 3 μM for recording each spectrum.

For ANS binding study, the samples (8 μM each) were incubated with 20 μM ANS for 10.

minutes at room temperature and the fluorescence emission spectra (450–600 nm) were recorded with excitation at 370 nm.

2.7. CD study

CD spectra of the samples (3 μM each) were recorded in the far UV region (190–250 nm) in a spectropolarimeter (Jasco 600). The α-helical contents were calculated following the method of Chen et al. [30].

2.8. Stability studies

DSC study was carried out to measure thermal stability of samples. The melting profiles were recorded in a VP-DSC Microcalorimeter by heating the samples (1 °C/min) over a definite temperature range. Before introduction into the calorimetric cells, the protein samples were thoroughly degassed.

Conformational stability of samples was measured by chemical denaturant-induced unfolding study. Fluorescence emission spectra (300–400 nm) of samples (3 μM each) were recorded with excitation at 280 nm after overnight incubation with 500 mM guanidine hydrochloride.

3. Results and Discussion

3.1. Separation of hemoglobin

The native gel profile of hemolysates obtained from different groups of rats (I–IV) was found to be more or less identical, as shown in Fig. 1A (lanes 1–4). Hemoglobin was purified from the hemolysates of different groups by size-exclusion chromatography. Fig. 1B shows gel electrophoretic profile of hemoglobin purified from different groups. Purified hemoglobin of group I and group IV rats were subjected to further experiments.

3.2. Mass spectrometric study

The tryptic mass fingerprint spectrum of control (group I) rat hemoglobin is shown in Fig. 2A. In the spectrum of MG-treated (group IV) rat hemoglobin (Fig. 2B), the peptides with *m*/*z* values 1141.67 Da, 1180.64 Da and 1583.79 Da indicated modifications of Arg-92α, Arg-104β and Arg-31α to hydroimidazolone (MG-H1) adducts, respectively. The modified peptides were absent in control rat hemoglobin. All modifications were confirmed by MSMS fragmentation of the modified peptides (data not shown) using Collision Induced Dissociation [5,20]. Results are summarized in Table 1. The observation was found to be similar to previously published reports on MG-hemoglobin interaction studies, where the reactive α-oxoaldehyde was found to modify arginine residues of the heme protein to hydroimidazolone adducts in vitro [10,21,22].

Among the modified arginines in hemoglobin, Arg-104 in the β chain is most accessible to solvent (103 $Å^2$ in surface exposable area), followed by Arg-92 in α chain (40 $Å^2$ in surface exposable area), as estimated from the web-based program GETAREA [22]. Thus the solvent accessibility and hence MG modification of these residues may be correlated with their exposed surface area as reported earlier. Arg-104 β is located in an alpha-helical region whereas Arg-92 α is located in a non-helical region of the heme protein with no distinct secondary structure (based on the structure of oxyhemoglobin- PDB ID: 1GZX).

On the other hand, Arg-31 in α chain (located in alpha-helix) is buried inside the protein and its surface exposable area is 3.8 $Å^2$. The observation that MG was found to modify Arg-31 in α chain is consistent with previous studies where the reactive α-oxoaldehyde modified arginine residues of the yeast protein enolase located in deep crevice at the dimer interface of the protein structure with reduced surface exposure

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