Papaverine increases human serum albumin glycation

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Abstract Glycation is a non-enzymatic reaction that is initiated by the primary addition of sugars to amino groups of proteins. In the early phase of glycation, the synthesis of intermediates leads to formation of Amadori compounds. In the last phase, advanced glycation end products (AGE) are irreversibly formed following a complex cascade of reactions. It has recently been shown that glycation also affects diabetes-related complications and Alzheimer's disease. In this study, human serum albumin at a concentration of 10 mg/ml was incubated in PBS with 40 mM of glucose and in different concentrations of papaverine (25, 100, 250, 500 μ M) for 42 days at 37 °C. HSA with no additives as well as with glucose 40 mM were incubated as a control and as a glycated sample, respectively. Following the incubation, the samples were prepared for circular dichroism, fluorescence and absorbance techniques. The results showed that in presence of papaverine and glucose, the glycation of HSA increased notably compared with the glycated sample. In conclusion, in this work, we showed that papaverine affects HSA and increases its glycation level.

Keywords Glycation · Human serum albumin · Papaverine · Glucose

1 Introduction

Advanced glycation end products (AGEs), also recognized as glycotoxins, are a group of highly oxidant compounds with pathogenic significance in diabetes and in several other chronic diseases [1]. Glycation of proteins is a complex series of parallel and sequential reactions collectively named the Maillard reaction. In early stages, the non-enzymatic reac-

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tion between reducing sugar and the free amino group of proteins leads to the formation of a glycated protein termed the Amadori product and a later stage results in the formation of AGEs [2]. Protein glycation not only affects the protein function and half-life but also engages signaling cascades transduced by the AGE receptor (RAGE) [3]. Compelling evidence from epidemiological studies indicates that hyperglycemia is an independent risk factor for cardiovascular disease [4]. AGEs can also originate from exogenous sources such as tobacco smoke and diet [5]. Due to their slow turnover rate, extracellular matrix (ECM) proteins are susceptible to AGE modification [6]. AGEs could also affect the composition of the ECM by increased expression of ECM proteins, including fibronectin, collagen types III, IV, VI and laminin [7, 8]. In vitro studies have identified that AGEs can modulate renal tubular growth and migration via an ezrin-dependent pathway [9]. CML and other AGEs have been localized to retinal blood vessels in patients with type 2 diabetes and were found to be correlated with the degree of retinopathy [10]. Administration of AGEs and subsequent RAGE activation replicates the effects of hyperglycemia, whereas RAGE antibody administration suppresses both NF- κ B activation and expression of IL-6 transcripts in sciatic nerve studies [11]. There is increasing evidence that suggests that the antiatherogenic properties of HDL are decreased in patients with diabetes [12, 13]. Intracellular AGE formation also reduces the expression of endothelial NO synthesis (eNOS) by increased eNOS mRNA degradation. Reduced eNOS activity and inactivation of NO by AGEs may play an important role in the defective vasodilatory responses that occur in diabetes [14]. Because of the long half-life and high concentration (40 mg/ml) of human serum albumin in plasma, it is highly sensitive to glycation [15]. Non-enzymatic glycation of serum albumin occurs at multiple sites, in vivo. Glucose specially becomes attached to lys-199, lys-281, lys-439 and lys-525 as well as to some other lysine residues [16]. Quantification of the amount of glycation of human serum albumin and of hemoglobin provides a record of average mid- and long-term blood-sugar concentrations, respectively; this is very useful for the management of diabetes [17]. Also the use of glycated HSA as a short-to-intermediate term marker for glycemic control in diabetes has been examined [18]. Glycated HSA is known to be involved in the pathogenesis of several diseases. Glycated HSA causes autoantibody production and has been related to autoimmune disorder. Beside this, glycated HSA has been associated with nephropathy and HSA glycation has also been linked to changes in the binding of HSA to several drugs such as phenytoin, salicylate, ibuprofen, phenylbutazone and warfarin and small solutes in the body [19, 20]. Glycation of HSA can generate thermodynamically more stable high-molecular weight aggregates having a remarkably increased beta-sheet structure than its non-glycated form. This may interfere with the normal function of HSA, thus contributing to diabetic complications [21]. One of the common beliefs in some societies (especially Eastern communities) is that smoking opium can reduce serum glucose and lipids in diabetes mellitus and is effective in the treatment of diabetes [22]. Opium preparations have long been used in diabetes and there is a wide-spread idea that they are frequently efficient in diminishing the excretion of sugar in the disease [23]. Papaverine is one of the main ingredients in opium that is used primarily in the treatment of visceral spasm, vasospasm and coronary artery bypass surgery; HSA is the main carrier of papaverine in blood streams (Fig. 1) [24]. Papaverine increases renal blood flow, urine output and creatinine clearance, inhibits phosphodiesterase and reduces Na-K-ATPase activity – many other applications have also been identified. [25]. Thus in this study we decided to survey the effect of papaverine on the glycation reaction pathway.

Fig. 1 Papaverine is an alkaloid found in opium [24]

2 Materials and methods

2.1 Chemicals

Human serum albumin ($\geq 96\%$, free fatty acid) and sodium bicinchoninic acid (BCA) were purchased from Sigma. The membrane filters (0.2 μm pore size, 25 mm in diameter) and dialysis tubing (cut-off 10,000 MW) were obtained from Whatman (UK). 2, 4, 6-trinitrobenzene sulfonic acid (TNBSA) was acquired from Fluka. Papaverine was purchased from the pharmacy. D (+) glucose, phosphate-buffered saline (PBS), sodium azide, EDTA and other materials were purchased from Merck (Germany). All solutions were prepared with deionized water.

2.2 In vitro glycation of HSA

HSA 10 mg/ml was incubated in PBS 50 mM (pH 7.4) containing glucose 40 mM and different concentrations of papaverine (25, 100, 250, 500 μM) for 42 days at 37 °C. In addition, HSA was incubated with and without glucose as glycated and control samples, respectively. Furthermore, HSA was incubated with the same papaverine concentrations without any additive under the same conditions. After the 42 days, the samples were dialyzed extensively against PBS at 4 °C and stored at -20 °C. Protein concentration was determined using the BCA assay according to the suppliers' protocol [26, 27].

2.3 Circular Dichroism (CD) experiments

Far-UV CD was used to measure the changes in the secondary structure of HSA (0.2 mg/ml) (Aviv Circular Dichroism Spectropolarimeter model 215) with a path length of 0.1 cm, at a range of 190–260 nm, using ammonium d-10-camporsulfonic acid for calibration. The results were expressed as molar ellipticity $[\theta]$, which is defined as $[\theta] = \theta \text{ millidegrees} / \{10 \times \text{number of amino acid} (lc)\}$, where c is the molar concentration of the sample and l is the length of the light path (cm). The amount of secondary structure associated with the samples was calculated by CDNN software [28].

2.4 Detection of AGE-specific fluorescence

The AGE-fluorescence of all samples (1 mg/ml) was obtained by a Cary Eclipse fluorescence spectrophotometer at Ex/Em (380/390–540 nm). Each point represents the mean of three independent experiments [29].

2.5 Lysine modification

The determination of the level of free lysine residues was carried out using TNBSA, which reacts specifically under mild conditions with free amino groups to form trinitrophenyl

derivatives. In this assay, 4% NaHCO (pH 8.5) and 0.1% (w/v) TNBSA were added to the 0.1 mg/ml protein solution (solubilized with 10% SDS) and were allowed to react at 37 °C for 1 h, followed by the addition of 1N HCL. The absorbance was measured at 335 and the amount of free lysine in each sample was obtained from the mean of three independent experiments. In these experiments, the amino acid valine was used as a standard [30, 31].

2.6 UV-visible experiment

A Shimadzu UV-3100 spectrophotometer equipped with a water bath (± 0.5 °C) was used for recording the spectrum changes of the HSA samples (1 mg/ml in 50 mM sodium phosphate buffer; pH 7.4 at 25 °C) in a wavelength range of 200–550 nm in presence of different papaverine concentrations for investigation of the interaction between papaverine and HSA [28].

2.7 Statistical analysis

The results were expressed as mean $\pm$ SEM. The significance of the positive effect of papaverine was determined by a chi-square test and the results were regarded as significant at $P \leq 0/05$.

3 Results

There was a loss of helical structure as shown by a decrease in the negative ellipticity at 208 and 222 nm following the 42 days incubation of HSA with glucose (H+G or glycated) compared to the control (HSA without any additive or H). However, the CD data for HSA incubated for 42 days with the mixture of glucose and different concentrations of papaverine also showed a larger decrease in the negative ellipticity values compared to the glycated sample (Fig. 2). The AGE fluorescence spectra of the glycated (H+G) sample at Ex/Em (380/390–500 nm) showed an increase compared to the control (H) (Figs. 3 and 4). In the case of HSA that was incubated with the mixture of glucose and papaverine in different concentrations (25, 100, 250 and 500 μ M), the data showed more enhancements in AGE fluorescence spectra compared to the glycated sample.

Fig. 2 Circular Dichroism spectra of all samples that were incubated for 42 days in PBS 50 mM, pH 7.4 at 37 °C. H, G and P indicate HSA, glucose and papaverine respectively

Fig. 3 AGE fluorescence spectra of all samples that were incubated for 42 days in PBS 50 mM, pH 7.4 at 37 °C Ex/Em (380/390–550 nm). H, G and P are the same as in Fig. 1

Fig. 4 AGE fluorescence spectra of all samples at 420 nm. H, G and P are the same as in Fig. 1

Free lysine residues were measured by TNBSA (Fig. 5). The data illustrate the absorbance of TNBSA per milligram protein in each sample. The percentage of free lysine residues in the glycated sample (H+G) was less compared to the control (H). In the presence of different papaverine concentrations, the number of free lysine was less compared to the HSA control (see Table 1).

HSA absorbance was increased in presence of different papaverine concentrations at 280 nm, which is depicted in Fig. 6; it means papaverine has changed HSA structure. Besides this, the data in Fig. 7 show that different papaverine concentrations could not affect

Fig. 5 TNBSA absorbance at 335 nm of all samples that were incubated for 42 days in PBS 50 mM, pH 7.4 at 37 °C. H, G and P are the same as in Fig. 1

Table 1 Free lys number for all samples. H, G and P are the same as in Fig. 1

Samples	Free lys number
H	58
H+G	53
H+G+P25 μM	52
H+G+P100 μM	38
H+G+P250 μM	37
H+G+P500 μM	35

Fig. 6 Effect of papaverine on the HAS structure in PBS 50 mM, pH 7.4 at 37 °C. H and P are the same as in Fig. 1

Fig. 7 Circular dichroism spectra of samples that were incubated for 42 days in PBS 50 mM, pH 7.4 at 37 °C. H and P indicate HSA and papaverine respectively

the HSA secondary structure. Furthermore, the docking analysis by the LigPlot program showed that papaverine interacts with residues in HSA by hydrogen bonds and hydrophobic interactions (Fig. 8). Figure 9 shows the position of lysine residues in the tertiary structure of HSA.

Fig. 8 Docking analysis of HSA with papaverine (Unk583) by the LigPlot program. Papaverine interacts with HSA via hydrogen bonds and hydrophobic interactions

Fig. 9 Three-dimensional structure of HAS. Lysine residues are the dark regions

4 Discussion

Type 2 diabetes is a common disorder that is recognized as a major health problem in Iran and globally. Diabetes is a major cause of morbidity and mortality and is an economic burden to society [22]. AGEs are formed as the result of reducing sugars reaction with proteins in a hyperglycemic condition [32]. Virtually every polypeptide is prone to glycation because of the presence of amino groups at their N-terminus and their lysine residues. Because of the long half-life and high concentration (40 mg/ml) of human serum albumin in plasma, it is highly sensitive to glycation [15]. HSA is a lysine rich protein and as previously mentioned several specific lysine residues, which include lys-199, lys-281, lys-439 and lys-525, are more susceptible to glycation after 2 or 5 weeks of incubation (Fig. 9) [16, 33, 34]. Using glycated HSA as a short-to-intermediate term marker for glycemic control in diabetes has been examined [18]. Some people believe that smoking opium can reduce serum glucose and lipids in diabetes mellitus. This belief is one of the reasons for opium addiction in some parts of Iran and the Middle East and originates from traditional ancient medicine [35]. Papaverine along with morphine, noscapine, codeine and tebain are the main opium ingredients [24]. Thus in this study we examined effects of papaverine on AGE formation. The data were obtained by analysis of AGE-fluorescence, the quantification of modified lysine side chains and the evaluation of the secondary structure of HSA after incubation with glucose in the absence and presence of different concentrations of papaverine. Papaverine was approved to evaluate the functional status of coronary arteries. It is also used to prevent spasmodic pains [36]. Papaverine dosage depends on the type of disease; as a pain killer it can be administered by IV at doses ranging from 30 to 120 mg every 3 h and in this study it was used in the physiological dosage range [37, 38]. Circular dichroism (CD) is an excellent tool for studying secondary structure [39]. An extensive change in the secondary structure of proteins takes place after the glycation process; it means glycation induces α -helix to β -sheet transition that is detectable with circular dichroism (CD) [28, 40]. The far-uv CD spectrum of HSA is characterized by the presence of two strong negative bands at 208 and 222 nm, which represent the helical characteristics of HSA [28]. The far-uv CD spectrum

reveals the glycation of HSA following 42 days of incubation, which induced a loss of α -helix in HSA [40]. However, the presence of papaverine in the mixture of HSA and glucose caused larger decreases in helicity compared to the glycated sample (Fig. 2). Therefore, papaverine plus glucose was reducing more the degree of the secondary structure in HSA. The fluorescence, as an index of advanced glycation, increased linearly over time for HSA incubated with reducing sugar [41]. In order to confirm this, AGE-specific fluorescence was found to be increased in samples that contained papaverine and glucose compared to the glycated sample (Figs. 3 and 4). Glycation is associated with decreased levels of free lysine [42]. The measurement of free lysine residues (Fig. 5 and Table 1) indicates that in the sample with papaverine and glucose, free lysine residues have decreased more compared to the glycated sample. Some progress has been made in identifying factors affecting the glycation of proteins. It has been shown that phosphate can stimulate the rate of glycation of RNase and other proteins such as lysozyme, cytochrome C, albumin and hemoglobin [43]. Binding of papaverine to HSA, as studied by equilibrium dialysis, was more than 90% in all species [44]. Partial denaturation of protein results in the exposure of more lysine residues to glycation. It has been shown that glycation of denatured proteins occurs more readily than glycation of native proteins [45]. Papaverine interacted with HSA with non-covalent forces especially hydrogen and hydrophobic bonds (Fig. 8) and previous studies have shown that papaverine could change the HSA structure (Fig. 6) [46] but it is not in the secondary structure (Fig. 7). Based on our results, we showed that papaverine cannot prevent glycation but, surprisingly, it makes the protein more prone to glycation. This might be due to conformational changes in the structure of proteins after binding of papaverine to the HSA. It seems that papaverine has created ideal conditions for protein glycation.

5 Conclusion

It can be summarized that papaverine, in the presence of glucose, could increase the glycation of HSA and the consequent formation of AGE fluorescence and cause a helicity decrease in the secondary structure of HSA.

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