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Protective ability and binding affinity of captopril towards serum albumin in an in vitro glycation model of diabetes mellitus

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Abstract

One-quarter of adult population in different world regions are reported to live with hypertension, of whom, a high percentage had a diabetes mellitus (DM). This co-morbid state is believed to act synergistically on accelerating the long-term diabetic complications. Therefore, adequate treatment of high blood pressure is essential for diabetic patients, and should be always directed to their benefits. Albumin glycation is still the most important explanation for the pathogenesis of chronic diabetic complications. Our in vitro experiments induce non-enzymatic glycation of bovine serum albumin (BSA) under physiological conditions. The levels of advanced glycation end products (AGEs) were measured by their characteristic intrinsic fluorescence. Additions of captopril at concentrations from 5 to 50 μM caused 10–47% reduction in the formation of AGEs. Captopril binding properties of native and glycosylated BSA were characterized, and its affinity towards the native albumin was unaltered by the in vitro glycation. Therefore, we concluded that captopril could protect against albumin glycation, and it has a similar binding affinities towards native and glcosylated protein. Anti-glycation effect may help to attenuate the serious long-term diabetes related complications. Besides, the unchanged pharmacokinetic parameters provide an essential extra beneficial effect for diabetic hypertensive patients.

Keywords: Binding; Bovine serum albumin; Captopril; Fluorescence; Glycation

1. Introduction

The prevalence of hypertension is rising rapidly worldwide. Evidence suggests that the current world percentage of the hypertensive patients (25%) is predicted to increase by about 60% in 2025 [1]. Hypertension coexists in as many as two-third of patients with diabetes mellitus (DM). This association resulted in an accelerated development of diabetic complications such as retinopathy, nephropathy, neuropathy and cardiovascular diseases [2–4]. Diabetic complications usually arise as a result of non-enzymatic protein glycation which leads to formation of heterogeneous, toxic, and antigenic advanced glycation end products (AGEs) [5–7]. AGEs contribute to diabetic complications through a series of pathological changes such as increased atherogenicity of LDL [8], increased basement membrane permeability [9] and decreased insulin binding to insulin receptors [10]. AGEs play, as well, an important role in diabetic micro- and macroangiopathy where it deposits under endothelial cells [11]. The initial step of AGEs formation start up through nucleophilic additive reaction of free amino groups of proteins with the aldehyde group of glucose that leads to an intermediate product (Schiff bases), which in turn convert to stable ketoamines by an Amadori rearrangement. AGEs are highly reactive irreversibly bound complex structures that continue to react with nearby amino groups to produce intra- and intermolecular crosslinks [12,13]. Glycosylated albumin accounts for about 80% of the circulating glycated protein, and it is one of the most important pathological conditions associated with structural alteration of albumin, that may results in modification of its characteristic biological activities and functional properties [14–17]. Insulin therapy minimize acute symptoms of diabetes, but not serious long-term complications, and it has been proposed that once the progress of excessive albumin glycation has begun, subsequent remediation

Abbreviations: AGEs, advanced glycation end products; BSA, bovine serum albumin; DM, diabetes mellitus; G6P, glucose-6-phosphate; HAS, human serum albumin; RAGE, receptor for advanced glycation end products

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of hyperglycemia would not prevent diabetic complications [18].

The previously reported structural and functional features of the albumin molecule have been proposed to explain the benefits of being in high concentration, as epidemiological studies have established that reduced level of serum albumin are associated with an increased mortality risk. In diseased population as well as in the general population, it has been estimated that the odds of death increase by about 50% for each 2.5% g/l decrement in the initial albumin level. Besides, binding of drugs to serum albumin is one of the most important pharmacokinetic determinants. The reversible binding of drugs to albumin is thought to be beneficial as long as it extends the length of time at which the free therapeutically active molecules remains above its efficacious concentration. Alternatively, too tight a binding and too slow a release will abolish, or restrict the action of the drug. The design of new drugs and determination of its therapeutic dosage, together with studying all of the possible interference reactions, should take advantage of this property [19–21]. Consequently, it is important to know the affinity of a drug towards infirm and/or healthy forms of serum albumin. Human serum albumin (HSA) and bovine serum albumin (BSA) have an extensive structural similarity [22], and thus, BSA, instead of HSA, was used in this study because of its low cost and easy availability. Fluorescence spectroscopy is a valuable technique to study the drug binding to plasma albumin because of its sensitivity, accuracy and ease of use [23].

The angiotensin converting enzyme inhibitors (ACE inhibitors) have demonstrated a powerful ability to reduce proteinuria and attenuate the rate of progression of renal disease in diabetic nephropathy [24]. The aim of this study is to evaluate the ability of captopril, representing ACE inhibitors to provide an essential extra protection for diabetic patients through maintaining of structural and functional parameters of the albumin molecule. These consequently prevent all of the pathologies associated with any changes in the integrity of this unique molecule. The present aim could be achieved through investigating the ability of captopril to reduce in vitro glycation of albumin. The glycation-mediated structural alterations of albumin molecule may have an impact on its functional properties, and thus it becomes important to correlate them with binding affinity towards captopril.

2. Materials and methods

Commercial preparation of bovine serum albumin which is purified from the blood of non-hyperglycaemic bovines, and thus considered to be minimally glycosylated (fraction V, essentially fatty acid free) was obtained from BDH, Poole, UK. Captopril, aminguanidine HCl and Tris were obtained from Sigma Chemical Company (St. Louis, USA). All other chemicals used were of the highest analytical grade.

2.1. In vitro glycation of BSA

BSA was dissolved in 100 mM Tris–HCl buffer (pH 7.4), containing 0.1% sodium azide (NaN_3) to yield a stock solution of 40 mg/ml. This solution was subsequently diluted with concentrated solutions of glucose-6-phosphate (G6P), Captopril and aminoguanidine made in Tris–HCl buffer (pH 7.4) to get a final concentration of 10 mg/ml BSA and 150 mM glucose-6-phosphate with or without the indicated concentration of captopril (5–50 μM), and aminoguanidine (2 mM). Reaction mixtures of 5 ml were incubated at 37 °C, for 14 days [25]. Incubations were carried out in sterile capped polystyrene test tubes (Falcon, Becton Dickinson, NJ, USA). All incubations were carried out in triplicate parallel tubes. Control sample of BSA (10 mg/ml) was prepared and treated similarly, except that either sugar or inhibitors were not added.

Following incubation, specific fluorescence of the incubated samples were monitored using a spectrofluorimeter (JASCO FP-550, JASCO Corporation, Tokyo, Japan) at the excitation and emission maxima of 350 and 450 nm, respectively, versus an un-incubated blank containing the protein, sugar and inhibitor [26], and used to measure the formation of advanced glycation end products [27].

Prior to binding experiments, the incubated sample mixtures of BSA/G6P and solitary BSA are dialyzed against 100 mM Tris buffer, pH 7.4, in order to remove extra-unbound glucose, and any other impurities. Protein concentrations of the dialyzed two samples were assayed according to Biuret method [28], using bovine serum albumin as standard. Diluted solutions (2 μM) of the two albumin forms are then prepared. The effect of glycation on BSA molecular conformation was evaluated by assaying the fluorescence intensity of its solutions. Fluorescence measurements of incubated dialyzed native and glycosylated BSA solutions (2 μM each) were monitored using an excitation and emission wavelength of 295 and 340 nm, respectively [29].

2.2. Binding studies

Binding studies were performed with two different incubated and dialyzed BSA preparations: native BSA preparation and in vitro induced highly glycosylated BSA preparation, as previously described.

BSA preparations (2 μM each) were titrated with increasing concentrations of captopril (50–500 μM), and the fluorescence quenching was measured at excitation wavelength of 295 nm, and emission wavelength of 340 nm. Both excitation and emission slit widths were 5 nm.

The declined fluorescence intensity was plotted versus increasing captopril concentration, and non-linear least-squares analysis (Microcal Origin, Microcal Software Inc., Northampton, USA) was applied to calculate the dissociation constants for the two different BSA preparations using the model described by [29]:

$$F = 1 - \left(\frac{Q_{\max} \times [\text{captopril}]}{K_d + [\text{captopril}]} \right)$$

where F is the fluorescence intensity, $Q_{\max}$ the maximum fluorescence that can be quenched and K_d is the dissociation constant for the binding of captopril to albumin. The data are presented as theoretical best-fit lines superimposed on experimental data.

3. Results

The formation of AGEs was assessed by monitoring the production of fluorescent products at excitation and emission maxima of 350 and 450 nm, respectively, as previously explained [26,27]. The fluorescence intensity of this glycophore which is characteristic of AGEs, was highly increased through incubation of BSA with glucose-6-phosphate, as described in Section 2. Captopril, in the different applied concentrations (5–50 μM) significantly inhibited this specific fluorescence increase, which was most strongly suppressed by 2 mM aminoguanidine, the known inhibitor of glycation process (Fig. 1).

In this study, our principal interest was to determine the relative binding affinities of captopril towards unmodified (native) and glycosylated BSA. Therefore, the binding experiments were performed under identical conditions for the two albumin forms. Dialyzed sample mixtures (previously incubated with or without glucose-6-phosphate) were used to prepare diluted solutions of native and glycosylated BSA (2 μM each), as formerly described in Section 2, in order to study captopril–BSA binding parameters.

Prior to binding studies, intrinsic fluorescence intensity of the prepared diluted solutions of the two albumin forms was estimated to determine whether or not; glycation of albumin changed its conformation. The fluorescence intensity of the couple of tryptophan residues (Trp213 and Trp134) has been used as an index of the conformational changes in the BSA molecule. Fig. 2 shows that glycation of albumin resulted in remarkable decrease (59%) in tryptophan fluorescence compared with native or unmodified BSA.

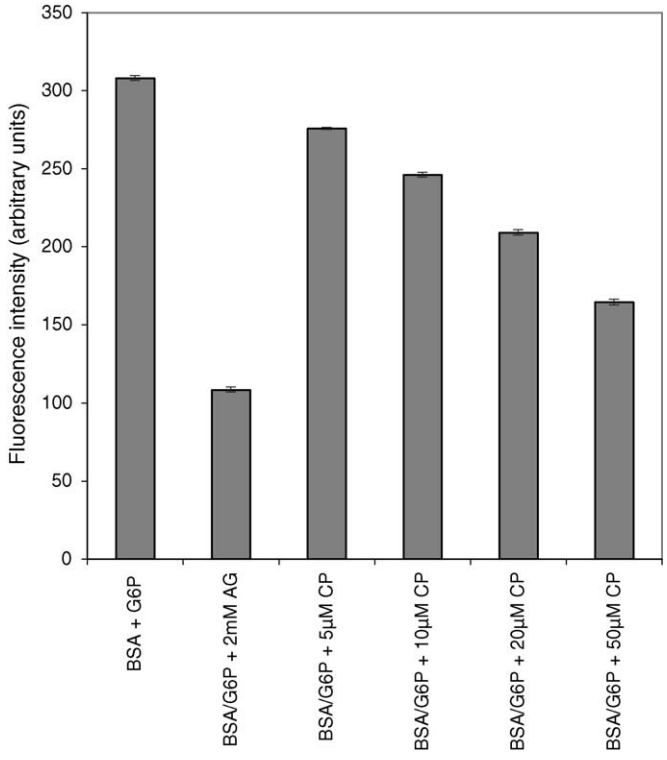

Fig. 1. Inhibitory effect of captopril on AGEs formation. Effect of captopril on AGEs formation assessed by fluorescence intensity. BSA (10 mg/ml) was incubated with G6P (150 mM) for 14 days at 37 °C in the presence and absence of aminoguanidine (AG), and different concentrations of captopril (CP). Advanced albumin glycation was determined by measurement of fluorescence intensity vs. un-incubated blank containing the protein, sugar and inhibitor, as described in Section 2. Values are the means ± standard error of three experiments.

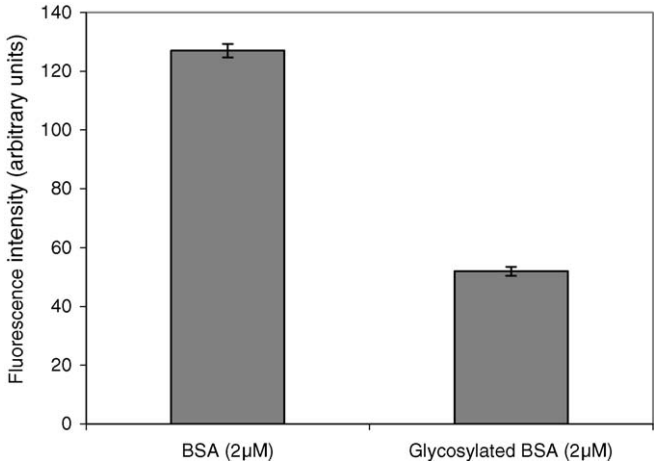

Fig. 2. Intrinsic fluorescence intensity of native and glycosylated BSA. Intrinsic fluorescence intensity of native and glycosylated BSA solutions (2 μM each) were evaluated using an excitation and emission wavelength of 295 and 340 nm, respectively, as described in Section 2. Fluorescence intensity of the glycosylated BSA was 0.41 of the unmodified form. Values are presented as mean ± standard error of three experiments.

Fig. 3 shows that binding of captopril with native and glycosylated BSA at pH 7.4 causes a concentration-dependant decrease in the protein intrinsic fluorescence. The total amount of fluorescence intensity to be quenched was considered 100%, and from the fractional quenched, the dissociation constant of captopril bound to albumin was estimated considering one binding

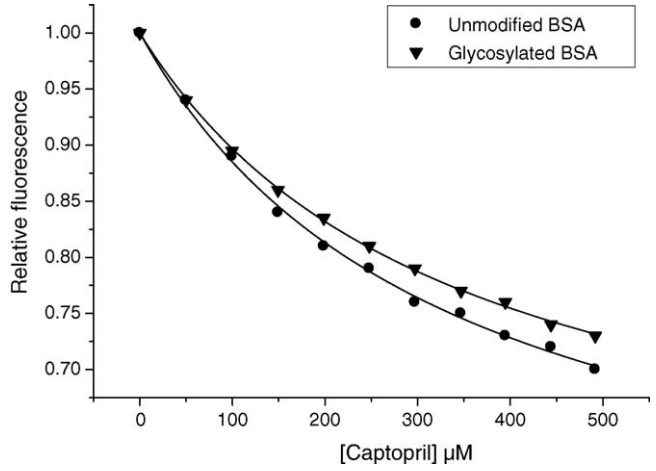

Fig. 3. Binding of captopril to native and glycosylated BSA. A fixed concentration of each BSA form (2 μM) in Tris–HCl buffer, pH 7.4, was titrated with increasing concentrations of captopril (0–500 μM), and the quenching of BSA tryptophan fluorescence was recorded. Excitation was held at 295 nm, whilst the fluorescence emission was monitored at 340 nm. Values are averages of tiplicate observations. Captopril binding was analysed by non-linear curve fitting to calculate the dissociation constant for each BSA form, using the model described by Johansson [29]. The data are presented as theoretical best-fit lines superimposed on experimental data.

site only, the highest affinity site [30]. The line through the data points is a best-fit curve derived using Eq. (1) [29]. The dissociation constants (± standard error), calculated in the same manner, were 332 ± 17 μM for the native BSA, and 340 ± 20 μM for glycosylated BSA with Q_{max} (± standard error) = 0.5 ± 0.013 and 0.4 ± 0.014, respectively. There was no significant difference in the binding parameters of captopril towards the two forms of this unique protein.

4. Discussion

The advanced glycation end products, and not the early glycation products are thought to play a role in the generation of many complications seen in diabetes mellitus [25]. It has been reported that AGEs contribute to the development of those complications through binding to cell surface receptors, including the receptor for advanced glycation end products (RAGE). RAGE is present on the cell surface of a variety of cells, including endothelial cells, mononuclear phagocytes and hepatocytes. RAGE is a multiligand receptor that has been found to bind to BSA glycated with several reducing sugars including glucose, fructose and ribose. The specific biochemical characteristics of AGEs responsible for RAGE binding yet to be elucidated [31]. The ability of AGEs, produced in the present study through G6P-mediated glycosylation of BSA, to bind with RAGE is currently unknown. Fluorescence has been used to investigate the influence of captopril on the formation of AGEs in the BSA/G6P system because of the specific fluorescent glycophore that increases during the process of glycation [13,27]. G6P was previously reported to be more reactive than glucose in the glycation process, and it has been used to allow limitation of the samples incubation time [32]. The phosphate group of G6P facilitates the intramolecular rearrangement of Schiff base to form Amadori products, probably through the changes in surface negative charges due to the phosphate group of G6P [33,34].

Fig. 1 clearly reveals that captopril was capable of inhibiting AGEs formation when assessed by fluorometry. This finding is in harmony with previous report consuming HSA/glucose system [35] instead of BSA/G6Psystem used in this study. Moreover, this figure exhibits comparative sight of the inhibitory effect of captopril to the aminoguanidine, the distinguished AGEs inhibitor [27,35,36].

Free radicals are identified to stimulate AGEs production by autooxidation of sugars [7], thus the antioxidant function would decrease the concentration of free radicals. It has been observed that a combination of two different antioxidants effectively inhibited glycation more than a single antioxidant, which is evidence for the antioxidant mechanism of inhibition [26]. Free radicals scavenging ability of captopril has been previously reported [37,38], and this could explain at least in part, the observed protective effect of captopril against AGEs formation, however, the exact mechanism remains to be elucidated.

The intrinsic fluorescence of BSA is primarily due to tyrosine and tryptophan residues. Their quantum yields of fluorescence are sensitive to fluorophore environments within the three-dimensional protein structure [39], and this used in search of possible conformational changes of the protein imposed by non-enzymatic glycation. Although the direct contribution of tyrosine residues in BSA to its intrinsic fluorescence is very small [40]. BSA was excited at 295 nm, where tryptophan mainly absorbs, in order to minimize tyrosine residue interference. In glycosylated albumin (Fig. 2), the quantum yield of the tryptophan fluorescence has been reduced by about 59% that reflects changes in the tryptophan microenvironment. Earlier studies of Bourdon et al. [19], Coussons et al. [21] and Shaklai et al. [30] confirmed that the in vitro glycation of BSA associated with structural changes, without destruction of this molecule.

Binding studies were performed with two different BSA preparations: native BSA preparation, which considered being minimally glycated (6–10%) [19], and highly glycosylated BSA preparation, as previously described. Binding of captopril to the two forms of albumin molecule is formed by its tertiary structure, which involved different part than the major glycation sites, proved by the binding parameter of captopril towards BSA that unaltered by the in vitro glycation of the protein. Q_{max} of the obtained dissociation constants were found to be near half of unity. Q_{max} values could imply that the fluorescence of only one of the two tryptophan residues of BSA molecule is effectively quenched [29], suggesting that only one of them, at least, is not involved in captopril binding to BSA.

The similar binding constants of captopril towards native and glycosylated BSA, together with its ability to protect the BSA structural integrity may help to maintain the functional and biological properties of this unique protein. In choosing an antihypertensive agent for a diabetic patient, effectiveness needs to be accompanied with favourable properties for those categorized hypertensive patients. It would be beneficial to select a drug whose pharmacokinetics is not going to be highly affected, and its mechanism of action includes protection of serum albumin, the important circulating protein. Nevertheless, it is still not clear whether or not the similar binding of captopril to the native and modified BSA is also manifest as a similar affinity for native and glycosylated HSA. Further investigation are greatly needed to attain this target by purifying HSA from healthy and diabetic patients rather than using commercially available HSA preparations [41] in order to come closer to the real in vivo situation.

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