Kinetics of fatty acid binding ability of glycated human serum albumin

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Kinetics of fatty acid binding ability of glycated human serum albumin (HSA) were investigated by fluorescent displacement technique with 1-anilino-8-naphtharene sulphonic acid (ANS method), and photometric detection of nonesterified-fatty-acid (NEFA method). Changing of binding affinities of glycated HSA toward oleic acid, linoleic acid, lauric acid, and caproic acid, were not observed by the ANS method. However, decreases of binding capacities after 55 days glycation were confirmed by the NEFA method in comparison to control HSA. The decrease in binding affinities was: oleic acid (84%), linoleic acid (84%), lauric acid (87%), and caproic acid (90%), respectively. The decreases were consistent with decrease of the intact lysine residues in glycated HSA. The present observation indicates that HSA promptly loses its binding ability to fatty acid as soon as the lysine residues at fatty acid binding sites are glycated.

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1. Introduction

Glycation is a reaction between *a*- and/or *e*-amino groups in proteins and carbonyl groups of reducing sugars involving the reversible formation of Schiff base followed by the aldimine rearrangement to form the Maillard reaction products (Brownlee and Cerami 1981). The products, so called advanced glycation end products (AGEs), are thought to play an important role in the complications of diabetes (Brownlee *et al* 1988). Glycation of various kinds of proteins occurs under physiological conditions, and glycated proteins are widely detected on the proteins having relatively slow turnover rates, such as haemoglobin A (Shapiro *et al* 1980), membrane collagen (Bailey *et al* 1976), human serum albumin (HAS) (Iberg and Flückiger 1986), and lens crystallin (Lyons *et al* 1991) etc. Because glycation is a post-translated modification, glycated proteins are inevitably affected in their functions. Even in cases of the proteins having short turn over rates, such as RNase (Watkins *et al* 1985) and human erythrocyte Cu-Zn-superoxide dismutase (Arai *et al* 1987), their catalytic activities were found to decrease by glycation.

HSA is the most abundant protein that comprises 60% of human plasma protein and has 19 days for a half-life time (Waldmann 1977). HSA has a single polypeptide chain comprised of 585 amino acid residues. There is no site for enzymatic glycosylation. One of the main contributions of HSA is to maintain osmotic pressure and pH of blood (Figge *et al* 1991). One of the most striking functions of HSA is its ability to bind reversibly with many kinds of compounds in blood plasma and show particularly high affinity to variety of saturated and unsaturated fatty acids of various chain lengths (Lee and MCMenamy 1980). Hence, HSA is recognized as the primal transporting protein of fatty acids that would be practically insoluble in blood plasma, and also as a major

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glycated protein in blood plasma of such patients. The aim of the present study is to examine the detail of kinetics of fatty acid binding ability of HSA by glycation: i.e. binding of HSA with fatty acid was quantitatively monitored during glycation over 50 days.

2. Materials and methods

2.1 *Chemicals*

HSA fraction V and 1-anilino-8-naphtharene sulphonic acid (ANS) were purchased from Sigma (St. Louis, MO, USA). Unless otherwise indicated, all chemical reagents were the guaranteed reagent grade of Nacalai Tesque (Kyoto, Japan).

2.2 *Removal of fatty acid from HSA*

The lyophilized HSA was dissolved in distilled water and defatted by passing though an activated charcoal column as described by Chen (1967). The defatted HSA was lyophilized again after thorough dialysis against distilled water.

2.3 In vitro *glycation of HSA*

HSA (40 mg/ml) in PBS was poured into sterilized test tubes with 100 mM glucose after passage through a 0·2 µm membrane filter and incubated at 37°C. Aliquots were drawn periodically and used directly for the following fatty acids binding assay. As a control, HSA was also incubated under the same condition without glucose.

2.4 *ANS fluorescence displacement technique (ANS method)*

Fatty acid binding affinity of HSA was estimated by the ANS method with oleic acid, linoleic acid, lauric acid, and caproic acid according to the reported procedures with some modifications (Takikawa and Kaplowitz 1986). To an HSA solution (2·5 µg in 100 µl of PBS) placed in 96 flat-bottom wells (Nunc Micro Well Plates, Nalge Nunc International, Rochester, NY, USA), was added an ANS solution in 50 µl PBS to be final concentration of 2·5, 7·5, 12·5, and 17·5 µM. A suspension of fatty acid of various concentrations (0–10 µM) were added to the above HSA-ANS complex and kept at room temperature in the dark for 15 min. Fluorescent quenching [$\Delta F(\%)$] caused by the displacement of ANS with fatty acid was measured (l_{ex} 360 ± 40 nm, l_{em} 460 ± 40 nm) using a cyto fluor 4000 plate reader (Applied Biosystems, Foster City, CA, USA).

2.5 *Determination of fatty acid binding capacity of HSA*

Experiment for binding of fatty acid to HSA was performed by the method of Spector and Hoak (1969) and fatty acid binding capacity of HSA was determined photometrically using bathocuproine and $CuSO_4$ as colouring reagents (Duncombe 1964). Fatty acid (1 µmol) and Celite (1·0 g) were suspended in 10 ml of *n*-hexane, and the suspension was dried *in vacuo*. The dried Celite was added to 4 ml of aqueous HSA (40 mg/ml in distilled water). The suspension was stirred for 15 min at room temperature and centrifuged at 10,000 *g* for 10 min. The supernatant was applied to the quantification of fatty acid by using a NEFA Test Wako (Wako Pure Chemical Industries, Osaka, Japan). One ml of $CuSO_4$ (0·13 M in distilled water) and 3 ml of heptane containing chloroform (745 mg/ml) were added to 0·2 ml of the supernatant. The mixture was stirred for 2 min and centrifuged at 10,000 *g* for 10 min to separate the lower organic phase. To 2 ml of the organic phase was added 2 ml of colouring solution [2·1 mM bathocuproine in heptane containing 2(3)- *t*-butyl-4-methoxyphenol (380 µg/ml) and chloroform (600 mg/ml)]. Increase in the intensity of yellow colour was measured at 480 nm with a JASCO V-560 colorimeter (Japan Spectroscopic Corp., Tokyo, Japan).

2.6 *Amino acid analysis of HSA*

Glycated and control HSA were reduced by $NaBH_4$ prior to the acid hydrolysis. A 50-folds excess of $NaBH_4$ was slowly added and the mixture was stirred for 1 h on an ice bath. To decompose unreacted $NaBH_4$, pH was lowered below 3·0 with acetic acid and the reduced HSA was lyophilized. The lyophilized HSA was redissolved in 6 N HCl containing 1% wt/v phenol and heated at 110°C under N_2 atmosphere for 21 h. Residual lysine residues were determined by amino acid analysis (Bidlingmeyer *et al* 1984) with a Pico Tag system (Waters Corp., Millford, MA, USA).

3. Results

3.1 *Effect of glycation on fatty acid binding ability of HSA*

Fatty acid binding affinities of glycated HSA were monitored over 50 days by ANS method. ANS, a hydrophobic fluorescent probe, can bind to hydrophobic sites of proteins and ANS-protein complex produces drastic increase of fluorescent intensity, and then addition of a ligand to the complex results in quenching of fluorescence due

to the displacement of ANS. When fatty acid was added to the ANS-HSA complex, binding of fatty acid to HSA were detected as quenching of fluorescence. Oleic acid (18 : 1), linoleic acid (18 : 2), lauric acid (12 : 0), and caproic acid (6 : 0) were examined for taking the difference of their chain length and saturation into account.

The binding assay of oleic acid with HSA on 55th day is shown in figure 1 as a typical example. HSA incubated with glucose [glycated HSA (figure 1A)] and without glucose [control HSA (figure 1B)] were compared. Four lines show each result of the displacement of ANS-HSA complex by various concentrations of oleic acid (0–7 µM) in case of different concentrations of ANS (from up to bottom: 17·5, 12·5, 7·5, and 2·5 µM). Fluorescent intensity of ANS-HSA complex decreased dose-dependently and drew downward saturation curves. Glycated and control HSA were very similar curves in shape. In the presence of 2·5 µM ANS, decrease of fluorescence [$\Delta F(\%)$], reached below 25% by addition of up to 7·0 µM of oleic acid. Magnitude of $\Delta F(\%)$ became the smaller in case of the higher concentrations of ANS presented.

To confirm a type of displacement (inhibition) reaction, [oleic acid]/$\Delta F(\%)$ were plotted against [oleic acid] (figure 2). Those plots showed linear lines in both cases of glycated and control HSA. The X-axis interceptions of every linear line in figure 2 represented the apparent dissociation constants (K_{dapp}) of oleic acid in the presence of the given concentration of ANS. The values of K_{dapp} and the maximum change in fluorescence [$\Delta F(\%)_{max}$], which should be observed when all the ANS-HSA complex would be displaced by oleic acid, were calculated by least square fitting using eq. 1.

$$\frac{[\text{Fatty acid}]}{\Delta F(\%)} = \frac{[\text{Fatty acid}]}{\Delta F(\%)_{max}} + \frac{K_{dapp}}{\Delta F(\%)_{max}}. \quad (1)$$

To calculate inhibition constant of ANS (K_i) for oleic acid binding site in HSA, the values of Y-axis interceptions of each line in figure 2, which meant $K_{dapp}/\Delta F(\%)_{max}$, were plotted against [ANS] (figure 3). Those plots exhibited linear line again. Since ANS bound to some extent non-specifically to the hydrophobic site of protein, therefore the number of ANS molecules bound to HSA would be greater than that of oleic acid did. Because ANS were displaced competitively by oleic acid, the displaced ANS molecules were those that had been in the same sites where oleic acid attached. Therefore, the X-axis interceptions of each line in figure 3 represented the real inhibition constant K_i of ANS at the oleic acid binding site of HSA. K_i values of ANS were calculated by least square fitting to eq. 2 to be 6·8 ± 0·5 µM for glycated, and 7·5 ± 0·5 µM for control HSA.

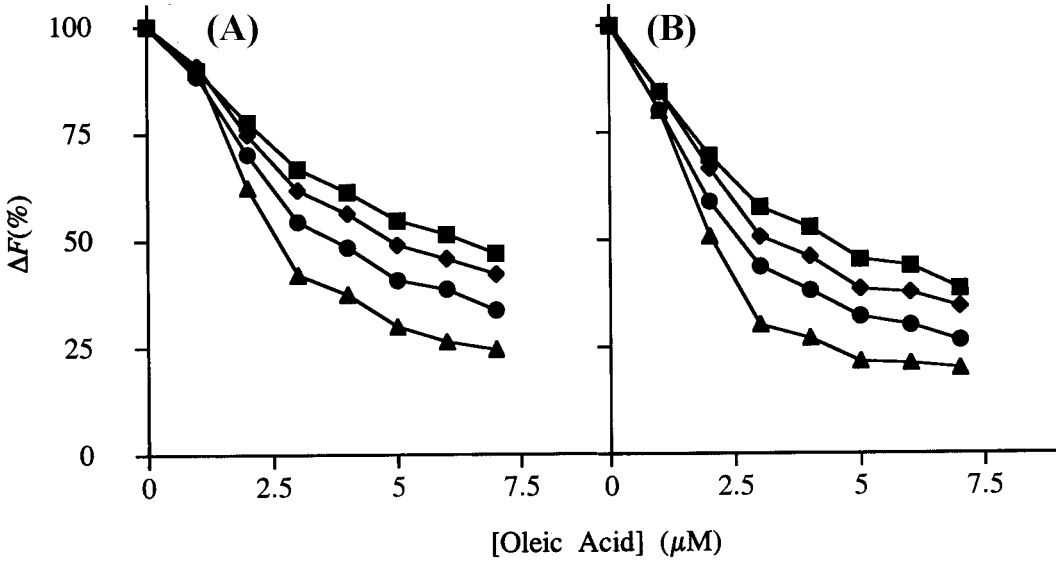

Figure 1. ANS fluorescence displacement assay for oleic acid binding to HSA. HSA (40 mg/ml) in 67 mM sodium phosphate buffer pH 7·4 (PBS) was incubated at 37°C in the presence or absence of 100 mM glucose for 55 days. Glycated HSA (**A**) and control HSA (**B**) were compared for oleic acid binding. HSA (2·5 µg in 100 µl PBS) was mixed with ANS to be final concentrations of 17·5 (■), 12·5 (◆), 7·5 (●), and 2·5 (▲) µM. The extinction of fluorescence of ANS-HSA complex [$\Delta F(\%)$] by addition of various concentrations of oleic acid (0–7 µM) was measured at l_{ex} = 360 ± 40 nm, l_{em} = 360 ± 40 nm at room temperature. Data are means ± SD values for three independent experiments.

$$\frac{K_{dapp}}{\Delta F(\%)_{max}} = \frac{K_d}{\Delta F(\%)_{max} * K_i}[ANS] + \frac{K_d}{\Delta F(\%)_{max}}. \quad (2)$$

There was no apparent difference in K_i between glycated and control HSA. The relationship among real dissociation constant (K_d), apparent dissociation constant (K_{dapp}), and inhibition constant (K_i) is given as eq. 3 when the situation for competitive displacement of ANS by fatty acid is established (above mentioned).

$$K_{dapp} = K_d\,(1 + [ANS]/K_i). \quad (3)$$

The values of K_{dapp} from figure 2 and K_i from figure 3 were applied to the eq. 3, and the real K_d of oleic acid was now calculated to be $1{\cdot}7 \pm 0{\cdot}1$ µM for glycated HSA and $0{\cdot}9 \pm 0{\cdot}1$ µM for control HSA. K_d values for four kinds of fatty acids at various glycation times were calculated in the same manner and summarized in table 1. No significant change in K_d of HSA toward four kinds of fatty acid was unexpectedly observed during glycation. However, the Y-axis interception in figure 3 for glycated HSA, which represented $K_d/\Delta F(\%)_{max}$, was larger than that of control HSA. This difference resulted in the decrease of $\Delta F(\%)_{max}$, since the real K_d values for both the glycated HSA and control HSA were almost the same. The slope in figure 2, which represents $[\Delta F(\%)_{max}]^{-1}$, was indeed steeper in glycated HSA (figure 2A) than that in control HSA (figure 2B). There was a decreasing tendency of $\Delta F(\%)_{max}$ during glycation when relative ratio of the maximum change in fluorescence $[R\Delta F_{max}(\%)]$ was calculated by dividing $\Delta F(\%)_{max}$ of glycated HSA with that of control HSA. Such a decreasing tendency was confirmed (figure 4) by not only using oleic acid but also other fatty acids were used

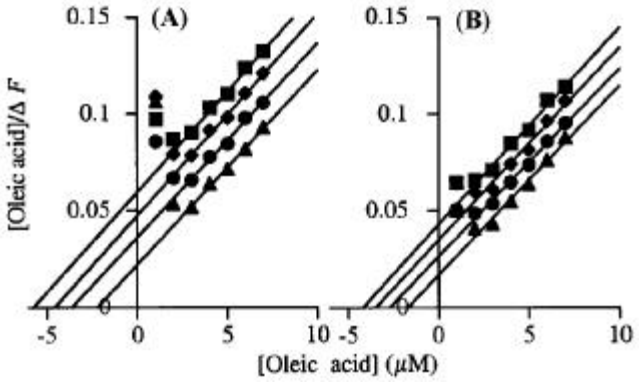

Figure 2. Linear plotting for [Oleic acid]/ΔF(%) vs [oleic acid] for oleic acid binding to glycated (**A**) and control HSA (**B**) in the presence of ANS [17·5 (■), 12·5 (◆), 7·5 (●), and 2·5 (▲) µM]. The values of K_{dapp} and $\Delta F(\%)_{max}$ were calculated by least square fitting to eq.1. Lines were the theoretical lines drawn according to eq. 1 using the calculated values, and then Y-axis interception of each line indicating $\Delta F(\%)_{max}/K_{dapp}$ was determined.

3.2 *Determination of fatty acid binding capacity of glycated HSA*

As shown in table 1 and figure 4, dissociation constants of four fatty acids to HSA (K_d) did not change, but $R\Delta F_{max}(\%)$ values decreased during glycation. This indicated that fatty acid binding capacity of glycated HSA was weakened. To confirm this, the NEFA method was done. Fatty acid coated on Celite was added to the aqueous solution of HSA and stirred. Fatty acid can move into aqueous phase by formation of the complex with HSA. The fatty acid-HSA complex was extracted as salt with Cu^{2+} into heptane, and the salt was quantified using bathocuproine to form a yellow chelate compound. The concentration of the fatty acid was determined by photometrically at 480 nm. Stoichiometry of each fatty acid binding to the intact HSA was calculated as between 1 and 2 molecules per HSA molecule, which was determined at the time = 0 of glycation. The calculated stoichiometry in the NEFA method was in good accordance with the reported value determined under physiological condition (Peters 1985).

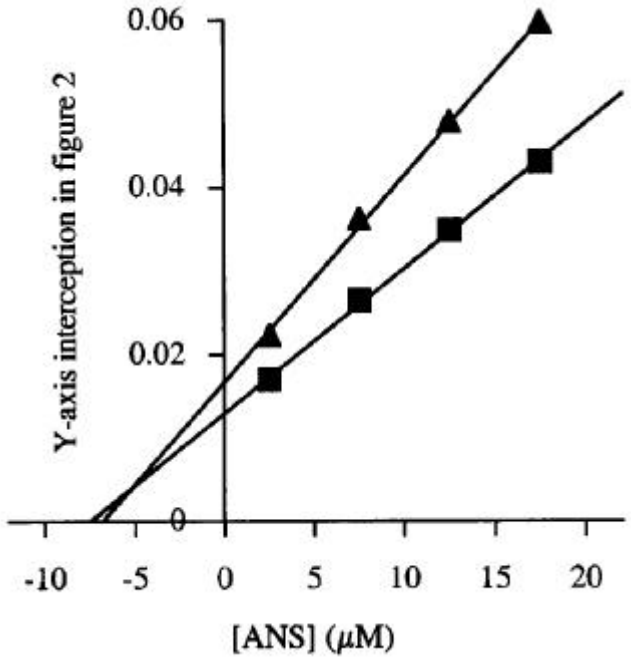

Figure 3. Linear replotting of Y-axis interception of figure 2 vs [ANS] for glycated (▲) and control HSA (■). The X-axis interception means minus inhibition constant ($-K_i$) of ANS at the oleic acid binding site of HSA.

Table 1. Effect of glycation on dissociation constants of fatty acids binding to HAS.

		Glycation time (days)						
Fatty acid	HSA	0	3	13	16	33	44	55
Oleic acid (μM)	Glycated	0·9 ± 0·1	1·9 ± 0·1	1·2 ± 0·2	1·3 ± 0·1	2·2 ± 0·1	1·8 ± 0·3	1·7 ± 0·1
(18 : 1)	Control	0·9 ± 0·1	1·6 ± 0·2	1·4 ± 0·2	1·1 ± 0·0	2·7 ± 0·2	1·1 ± 0·1	1·3 ± 0·0
Linoleic acid (μM)	Glycated	3·5 ± 0·3	1·7 ± 0·1	2·0 ± 0·1	2·3 ± 0·2	1·9 ± 0·4	2·1 ± 0·2	2·9 ± 0·2
(18 : 2)	Control	3·5 ± 0·3	1·5 ± 0·1	2·1 ± 0·1	2·1 ± 0·2	1·6 ± 0·1	2·0 ± 0·2	1·4 ± 0·4
Lauric acid (μM)	Glycated	0·07 ± 0·0	0·12 ± 0·1	0·11 ± 0·2	0·12 ± 0·2	0·35 ± 0·3	0·26 ± 0·4	0·48 ± 0·5
(12 : 0)	Control	0·07 ± 0·0	0·15 ± 0·1	0·08 ± 0·1	0·19 ± 0·1	0·11 ± 0·1	0·11 ± 0·2	0·28 ± 0·2
Caproic acid (mM)	Glycated	2·6 ± 0·8	2·8 ± 0·3	2·9 ± 0·2	2·2 ± 0·5	1·4 ± 0·1	3·3 ± 0·5	2·4 ± 0·6
(6 : 0)	Control	2·6 ± 0·8	3·3 ± 0·0	3·7 ± 0·3	3·7 ± 0·3	3·7 ± 0·3	3·6 ± 0·3	3·3 ± 0·3

Values are means ± SD at three independent experiments.

Decreases of fatty acid binding capacities of glycated HSA are shown in figure 5 based on the capacity of control HSA as 100%. Fatty acid binding capacities of HSA toward four fatty acids tested were found to decrease along with glycation time. The curves decreased slowly in the first 10–15 days, dropped in the next 15–30 days, and decreased slowly again in the last 20 days. The magnitudes of decreasing was oleic acid > linoleic acid > lauric acid > caproic acid. The capacities for the fatty acids having long alkyl chains seemed to be affected seriously by glycation.

3.3 *Amino acid analysis of glycated HSA*

Amino acid analysis of glycated HSA was performed to confirm the amount of residual lysine residues. The result showed that about 25% of total lysine residues of HSA were modified during 55 days glycation (figure 6). These were correspond to 15 lysine residues among 59. Figure 6 also shows that the rate of modification of lysine residues was slow in the first 10 days. A considerable decrease was observed in 10–20 days, and then the rate was slow down after 20 days. Such multi-phasic curve was well consistent with that of fatty acid binding capacities shown in figure 5. It was therefore confirmed that the glycation of lysine residues paralelled to the decrease of capacity of fatty acid binding of HSA.

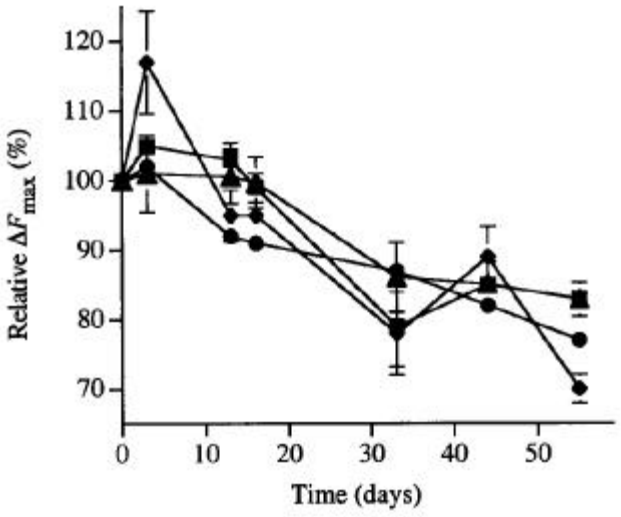

Figure 4. Change of $\Delta F(\%)_{max}$ of glycated HSA during glycation. $\Delta F(\%)_{max}$ of glycated and control HSA toward various fatty acid were calculated from Y-axis interception of linear lines in figure 3. These $\Delta F(\%)_{max}$ values were compared and represented as relative change of $R\Delta F_{max}(\%)$ for oleic acid (◆), linoleic acid (■), lauric acid (●), and caproic acid (▲) based on the $\Delta F_{max}(\%)$ values of control HSA as 100%.

4. Discussion

Values of dissociation constants (K_d) determined in the present study employing ANS method were different when equilibrium dialysis was carried out (Ashbrook *et al* 1975) with radio isotope labelled fatty acids between aqueous HSA solution and *n*-heptane: their dialysis experiment needed incubation of HSA in a two-phasic system containing buffer and hydrophobic solvent. Such condition was quite different from that in the blood stream where HSA is normally localized. On the other hand, present ANS method need not expose HSA to such hydrophobic solvents and requires only for measurement.

In this study, no significant change has been observed in K_i for ANS and K_d for fatty acids in glycated HSA compared with control HSA. However, fatty acid binding capacity was decreased by glycation along with the accumulation of modified lysine residues. This observation means that fatty acid could no longer bind to the fatty

acid-binding site when the lysine residue located in the site was once glycated. The ANS method detected change in fluorescence at only the sites that have not been glycated. Consequently, we could detect no difference in the binding affinity of fatty acid between glycated HSA and control HSA. These indicated that fatty acid-binding ability would be lost by glycation, as if its switch is turned off.

Positively charged amino acid residues located in fatty acid-binding site contributed to a static interaction with the anionic carboxylate of fatty acid, and *e*-amino group of lysine residue was quite important (Reed 1986). Glycation of HSA occur predominantly at lysine residue (Garlick and Mazer 1983). The primal glycation site was Lys-525 and this lysine residue occupied about 30% of total glycated amino acid residues in HSA (Iberg and Flückiger 1986). Glycation at Lys-525 drastically decreased (approximately 1/20) the affinity to *cis*-parinaric acid, an analogue of long chain fatty acid (Shaklai *et al* 1984). X-ray crystallographic study (Bhattacharya *et al* 2000) revealed that HSA combined with six molecules of long-chain fatty acid such as myristic acid, palmitic acid, and stearic acid confirming that the Lys-525 in subdomain IIIb interacted with carbonyl group of myristic acid. On the other hand, Murtiashaw and Winterhalter (1986) could not find any depression for palmitate binding and palmitate transport to HL-60 cells in glycated HSA after 4 days glycation. Reed (1986) asserts that three lysine-residues are primarily associated with the carboxyls of long-chain fatty acid, namely Lys-116, Lys-349, and Lys-473. It was thought that reactiveness of these lysine residues with glucose would be lower than of Lys-525. Based on above considerations, the fatty acid-binding ability might not be necessarily interrupted by glycation of Lys-525, and furthermore that it would be difficult to estimate the effect of glycation on fatty acid binding ability of HSA in short glycation time. As a result, it might be taken for 10–30 days to be glycated these lysine residues and affect the binding ability of HSA in our conditions (figures 4, 5 and 6).

The glycation of HSA might not be so fast reactions, but approximately more than 10% population of HSA was reported to be glycated even in normal human serum (Shaklai *et al* 1984). In the patients of type 2 diabetes, an elevation of serum glucose level would result in more significant glycation of HSA and affect its functions. In

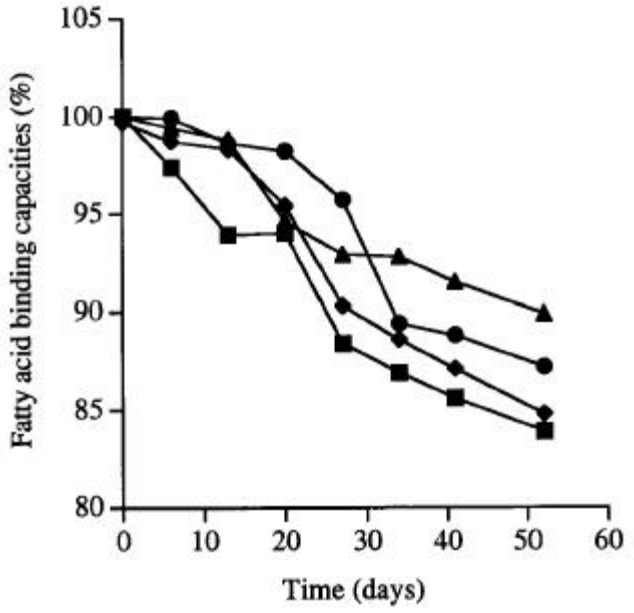

Figure 5. Fatty acid binding capacities of glycated HSA toward oleic acid (◆), linoleic acid (■), lauric acid (●), and caproic acid (▲) during glycation. Glycated and control HSA were dialyzed against distilled water and lyophilized. The lyophilized of HSA was redissolved in distilled water and mixed with Celite carrying fatty acid. After centrifuging, the supernatant HSA-fatty acid complex would be suspended was applied to a NEFA Test Wako using bathocuproine and $CuSO_4$ as colouring reagents for quantification of fatty acid binding. The yellow colour formed was measured at 480 nm. Relative fatty acid binding capacity of glycated HSA were represented based on that of control HSA as 100%. Data are means ± SD values for three independent experiments.

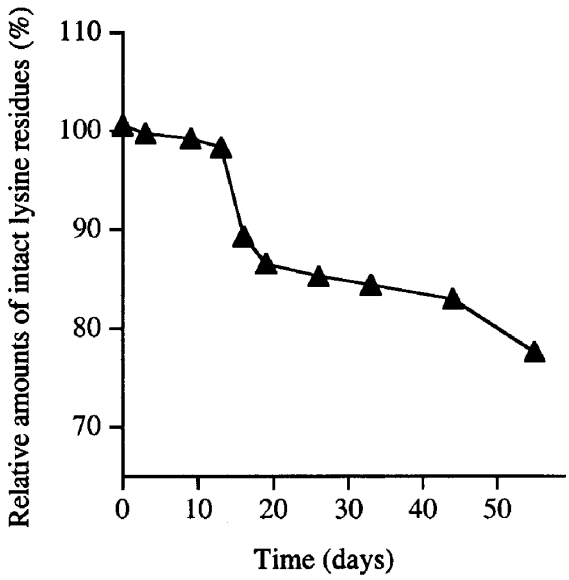

Figure 6. Decrease of intact lysine residues during glycation monitored by amino acid analysis. Glycated and control HSA were reduced by $NaBH_4$ and hydrolyzed by 6 N HCl containing 1% wt/v phenol. Relative amount of residual lysine residues (%) in glycated HSA were expressed based on that of control HSA. Data are means ± SD values for three independent experiments.

this study, it was confirmed that fatty acid-binding ability of HSA has decreased during *in vitro* glycation.

Type 2 diabetic patients tend to rely on fatty acids as one of the fuels for tissues such as heart or skeletal muscle instead of glucose because of insufficient glucose metabolism (Lopaschuk 2002). On the other hand, raised circulating free fatty acid concentration have been strongly implicated in the insulin resistance (McGarry 2002; Kashyap *et al* 2004; Chaves *et al* 2005) and in an impairment of *b*-cell response to glucose (Unger 2002). Several findings have been proposed about rise of the concentration of serum fatty acid level in type 2 diabetes, but our finding also might be one of the major factors. Thus binding, transport, and metabolism of fatty acids through HSA would suffer by glycation in such patients.

Acknowledgements

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