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LIPOATE PREVENTS GLUCOSE-INDUCED PROTEIN MODIFICATIONS

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Nonenzymatic glycation has been found to increase in a variety of proteins in diabetic patients. The present study examined a possibility of preventing glycation and subsequent structural modifications of proteins by α-lipoic acid (thioctic acid) as lipoate, a substance which has gained attention as a potential therapeutic agent for diabetes-induced complications. Incubation of bovine serum albumin (BSA) at 2 mg/ml with glucose (500 mM) in a sterile condition at 37°C for seven days caused glycation and structural modifications of BSA observed by SDS-PAGE, near UV absorption, tryptophan and nontryptophan fluorescence, and fluorescence of an extrinsic probe, TNS (6-(*p*-toluidinyl)naphthalene-2-sulfonate). When BSA and glucose were incubated in the presence of lipoate (20 mM), glycation and structural modifications of BSA were significantly prevented. Glycation and inactivation of lysozyme were also prevented by lipoate. These results suggest a potential for the therapeutic use of lipoic acid against diabetes-induced complications.

KEY WORDS: Albumin, diabetes, glycation, lipoate, lipoic acid, lysozyme, protein structure, thioctic acid.

INTRODUCTION

Post-translational protein modification is an important regulator of cellular functions. Nonenzymatic glycation has been studied in proteins such as the lens crystallins,[1] serum albumin,[2] red cell membrane proteins,[3] collagen,[4] and ferritin.[5] ε-Amino groups of lysine has been identified as the major site of nonenzymatic glycation.[6,7] Increasing evidence suggests a role of glucose-induced protein modifications in the complications of diabetes.[8]

α-Lipoic acid (thioctic acid), as lipoate, is a cofactor for α-ketodehydrogenase complex, i.e. the decarboxylation reaction of pyruvate. It is usually bound to the enzyme complex at lysine as lipoyl lysine. This vitamin like substance has recently gained some attention for the use in treatment of diabetes-induced complications.[9–12] Natraj *et al.*[9] reported that administration of lipoate in alloxan diabetic rats resulted in reductions in blood sugar, serum pyruvate and acetoacetate levels and increases in liver glycogen and fat synthesis. In addition, lipoate content in diabetic rat was significantly lower than that of normal rats.

The present investigation further extends information on the effectiveness of lipoate on diabetes-induced biochemical alterations by demonstrating that lipoate prevents glycation of proteins induced by a long-term incubation with glucose. Prevention of the structural and functional modifications of proteins subsequent to glycation were

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also observed in the presence of lipoate providing biophysical means of ascertaining such a phenomenon.

MATERIALS AND METHODS

Bovine serum albumin (BSA, Sigma; 2.0 mg/ml) or lysozyme (EC 3.2.1.17, Sigma; 2.0 mg/ml) was incubated under sterile conditions in 100 mM KH_2PO_4–KOH (pH 7.4) for 7 days at 37°C with a gentle shaking. Some contained D-glucose (Sigma; 500 mM) and/or α-lipoate (3–30 mM). Incubated media were dialyzed against phosphate buffer overnight and the protein concentration was determined by Bio-Rad Coomassie blue assay.[13] *R,S*-α-Lipoic acid (Asta Medica, Frankfurt, Germany) was dissolved in 100 mM KH_2PO_4–KOH (pH 9.0) and neutralized to pH 7.4 with HCl at room temperature.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Daiichi 10–20% Mini Gel (Integrated Separation Systems, Hyde Park, MA), stained by coomasie blue, and analyzed using a gel scanning program, NIH Image 1.37.

Glycation was estimated using the thiobarbituric acid (TBA) methods described by Flückiger and Winterhalter.[14]

Ultraviolet-visible absorption spectra were obtained using a Shimadzu UV 160U Spectrophotometer.

Fluorescence measurements were performed with a Perkin-Elmer MPF-44A spectrofluorometer. Tryptophan and nontryptophan fluorescence were obtained by excitation at 295 nm and 340 nm, respectively. Fluorescence probe, 6-(*p*-toluidinyl)naphthalene-2-sulfonate (TNS) was purchased from Molecular Probes Inc (Junction City, OR), and TNS fluorescence was measured with excitation 365 nm as described by Andley *et al.*[15] using a Perkin-Elmer MPF-44A Spectrofluorometer.

Lysozyme activity was determined by monitoring the rate of lysis of *Micrococcus lysodeikticus* at 450 nm as described by Shugar[16] at 25°C and at pH 7.0 on a Shimadzu UV 160U Spectrophotometer.

Statistical differences were obtained using student's *t*-test at $P < 0.05$.

RESULTS

A seven-day incubation of BSA (2 mg/ml) in a sterile condition with glucose (500 mM) caused glycation as estimated by TBA assay (Table I). The extent of glycation was 12.2 nmoles glucose per mg of protein. The glycation decreased to 5.8 nmoles when BSA plus glucose was incubated with 20 mM lipoate. With various concentrations of lipoate (3–30 mM), a concentration dependence of the effect of lipoate on glycation was observed (Figure 1). Lipoate did not have any effects on TBA assay.

Alterations of SDS-PAGE band characteristics were observed in glycated BSA, suggesting an occurrence of structural modifications of the protein. Glucose incubated BSA had a wider band around 68 kDa and also a band at 90–95 kDa appeared (Figure 2). Addition of the same concentration of glucose into the medium of BSA at the time of performing SDS-PAGE did not show this effect, confirming that the observed changes in band characteristics were not due to the effect of glucose on the

TABLE I
Effect of lipoate on glycation of bovine serum albumin

	nmol/mg protein
control	3.5 ± 0.3
+ glucose	12.2 ± 1.0*
+ glucose + lipoate	5.8 ± 1.5*
+ lipoate	3.9 ± 0.7

[glucose] = 500 mM; [lipoate] = 20 mM.
Values represent mean ± S.E. of 6 replicates.
*Significantly different from each other at $P < 0.05$.

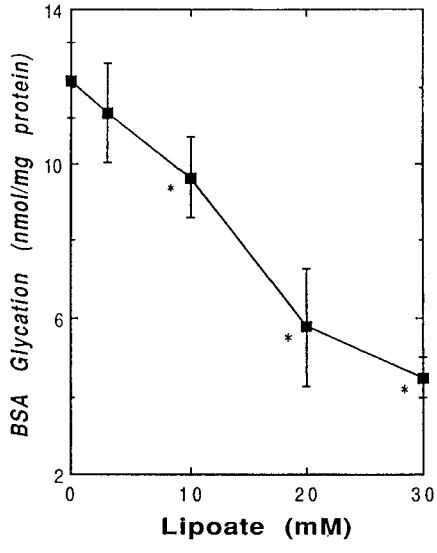

FIGURE 1 Concentration dependence of the effect of lipoate on glycation of bovine serum albumin. Incubation conditions: [BSA] = 2 mg/ml; [glucose] = 500 mM; pH 7.4; 37°C. *Significantly different from control value at $P < 0.05$.

gel system. Such changes in the band characteristics of BSA were not observed when BSA and glucose were incubated with 20 mM lipoate. Lipoate alone did not cause any changes in the band characteristics. The same results were obtained when the media were incubated with sodium azide (1 mM) confirming that the growth of microorganisms due to failure of sterile conditions during the incubation was not the cause of the observed results.

Other structural parameters which are known to be affected by glycation were presented by the incubation with lipoate. The absorption spectrum of glycated BSA

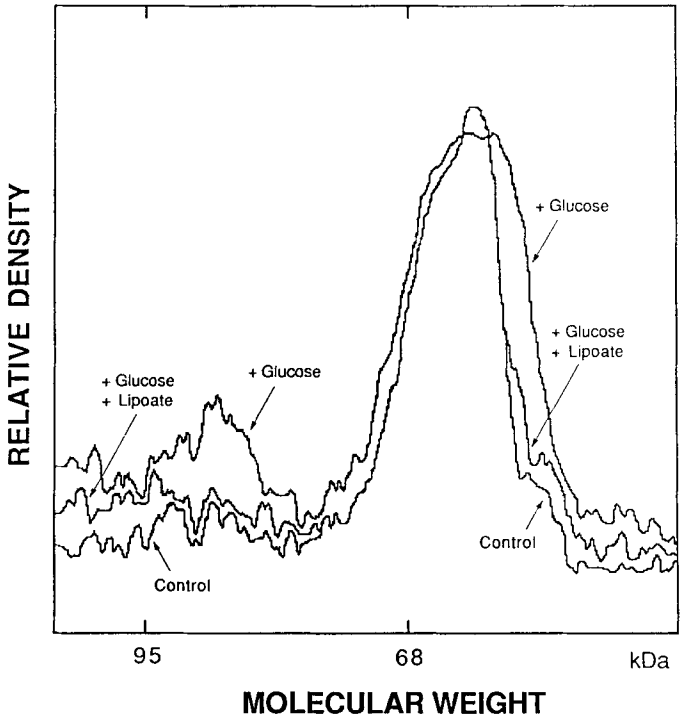

FIGURE 2 Effect of lipoate on glucose-induced modification of SDS-PAGE of bovine serum albumin. Incubation conditions: [BSA] = 2 mg/ml; [glucose] = 500 mM; [lipoate] = 20 mM; pH 7.4; 37°C.

was higher than that of nonglycated BSA in the near-UV region at 270–280 nm and 310–360 nm. Incubation with lipoate (20 mM) partially prevented this glycation-induced absorption spectrum changes (data not shown). Changes in intrinsic and extrinsic fluorescence induced by glycation was also prevented by lipoate. Tryptophan fluorescence intensity decreased 50% at emission wavelength of 340 nm (Figure 3A) and nontryptophan fluorescence intensity increased from 10 to 95 arbitrary units at emission wavelength of 420 nm (Figure 3B) in glycated BSA. These changes were partially prevented by incubation with lipoate (60% in tryptophan fluorescence and 80% in nontryptophan fluorescence). A decrease in the extrinsic fluorescence of TNS induced by glycation was prevented (70%) by lipoate (Figure 3C). Lipoate alone did not cause any changes.

The protective effect of lipoate on glycation of BSA was also observed in another model protein, lysozyme. Incubation of lysozyme (2 mg/ml) with 500 mM glucose caused glycation, and this was partially prevented by incubation with 20 mM lipoate (Table II). Inactivated enzymatic activity of lysozyme was partially preserved by lipoate (Table II).

DISCUSSION

The present study demonstrated that lipoate exhibited a clear protective effect against protein structural modifications induced by a long-term incubation with glucose.

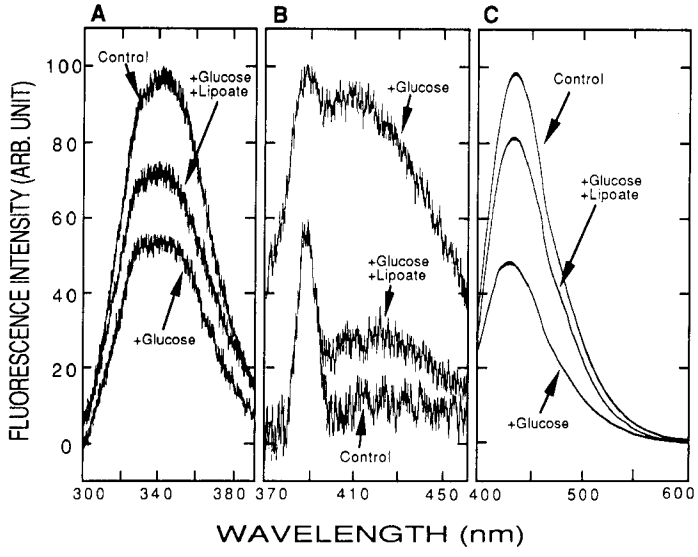

FIGURE 3 Effect of lipoate on glucose-induced alterations of intrinsic and extrinsic fluorescence of bovine serum albumin. (A) Tryptophan fluorescence spectra were obtained at excitation wavelength of 295 nm and emission wavelength as indicated with 5 nm slit. (B) Nontryptophan fluorescence spectra were obtained at excitation wavelength of 340 nm and emission wavelength as indicated with 5 nm slit. (C) TNS fluorescence spectra were obtained at excitation wavelength of 365 nm and emission wavelength as indicated with 5 nm slit. [TNS] = 25 nmol/mg protein. Incubation conditions: [BSA] = 2 mg/ml; [glucose] = 500 mM; [lipoate] = 20 mM; pH 7.4; 37°C.

TABLE II
Effect of lipoate on glycation and glucose-induced inactivation of lysozyme

	Glycation (nmol/mg protein)	Enzyme activity (% control)
control	0.1 ± 0.2	100
+ glucose	8.1 ± 1.4*	64 ± 9**
+ glucose + lipoate	4.7 ± 1.1*	82 ± 9
+ lipoate	0.3 ± 0.0	100 ± 0

[glucose] = 500 mM; [lipoate] = 20 mM.
Values represent mean ± S.E. of 3 replicates.
*Significantly different from each other and from control value at $P < 0.05$.
**Significantly different from control value at $P < 0.05$.

Long term incubation of proteins with glucose has been known to add glucose molecules onto proteins particularly at lysine residues. Such glycation causes changes in macromolecular protein structure. Our results show glycation caused a shift in absorption spectrum in the near-UV region which may be a result of the formation of kynurenine products[17] or 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazol,[18] tryptophan oxidation, and conformational changes decreased in the hydrophobic protein domain as indicated by the TNS fluorescence. SDS-PAGE also demonstrated the

formation of larger molecular weight species possibly a product of dimerization as a result of glycation.

The effect seems less likely due to the direct effect of lipoate on glucose which more likely to occur at 1:1 mole to mole basis because 20 mM lipoate was able to decrease 50% of the effect of 500 mM glucose. This could be interpreted as the direct binding of lipoate to the protein, probably at the same site as glucose binds, thus, protecting potential glycation sites. The predominant site of nonenzymatic glycation on human[19] and rat[20] serum albumin has been identified to be lysine-199 *in vitro*, although bovine serum albumin does not contain lysine residue at this portion of the amino acid sequence.[21] On the other hand, Garlick and Mazer[22] identified the predominant site of glycation *in vivo* to be lysine-525, which is conserved in all three serum albumins. Since the carboxylic acid group of lipoate can react with ε-amino group of lysine to form a lipoyl lysine linkage, it is a conceivable notion that lipoate preferentially competes with glycose for the glycation site. Aspirin has been reported to compete with the lysine-199 glycation site with glucose in human serum albumin.[23] This hypothesis, however, needs to be further tested particularly because the direct effects of lipoate on protein structure were not observed in any of the parameters measured in the present study. Also our results on the protection of lysozyme suggest this phenomenon may not be specific to a particular protein.

Although some evidence suggest usefulness of lipoate as a therapeutic agent for the complication of diabetes, exact mechanisms of how this compound would exert such protective effects are not well understood. Glycation of proteins has been known to result in alterations of biochemical structures and functions.[8] Thus, preventing protein glycation seems to be very beneficial for diabetic patients. In our laboratory, it was recently found that dietary supplementation of *R*-α-lipoic acid reduces the glycohemoglobin content in the streptozotocin-induced diabetic rat model (unpublished data). We have recently reported that the α-lipoate redox couple is an effective antioxidant.[24] The involvement of oxygen free radicals in both diabetes and glycation is well documented.[25,26] Thus, the protective effects of lipoate against diabetes-induced complications may relate to its antioxidant properties. Lipoate is a naturally occurring compound which is less likely to cause life-threatening side effects. Further investigations seem warranted on the potential clinical use of lipoic acid for diabetic patients.

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