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α-LIPOATE CAN PROTECT AGAINST GLYCATION OF SERUM ALBUMIN, BUT NOT LOW DENSITY LIPOPROTEIN

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Summary: Protein glycation may play a role in the pathogenesis of diabetic complications. α-Lipoate (1,2-dithiolane-3-pentanoate) has been reported to prevent glycation and structural modification of bovine serum albumin (BSA). To elucidate the protective mechanism, we tested the effects of enantiomerism, thiol moiety and hydrophobicity of α-lipoate on glycation of BSA and low density lipoprotein (LDL). When BSA (1 mM) was incubated with 500 mM glucose in the presence of α-lipoate homologues or dihydrolipoate (6,8-dimercaptooctanoate, DHLA) at 37°C for 72 h, both α-lipoate (racemic, *R*- and *S*-forms) and DHLA inhibited BSA glycation similarly, but tetranorlipoate (1,2-dithiolane-3-carboxylate) did not. However, under similar conditions, α-lipoate did not inhibit LDL glycation. Scatchard plot analysis demonstrated that 6 mol of α-lipoate bind to 1 mol of BSA with a formation constant of 8.7×10^4 M^{-1}. Therefore, we concluded that α-lipoate protects BSA glycation by hydrophobic binding near the glycation sites of BSA.

Many patients suffer from diabetes mellitus all over the world. Insulin therapy minimizes acute symptoms, but not serious long-term complications such as retinopathy, nephropathy, neuropathy and cardiovascular diseases(1). Many complications arises as a result of diabetic micro- and macroangiopathy(2, 3), where advanced glycation end products deposit under endothelial cells(4). Although several mechanisms have been postulated for the pathogenesis of chronic diabetic complications, protein glycation and oxidation by glucose (glycoxidation) are interesting working hypotheses(5-7). Glucose equilibrates between an open-chain form and a pyranose by forming an intramolecular hemiacetal. The free aldehyde group is thus available to bind covalently to ε amino groups of lysines of proteins to form a ketoimine. This can be rearranged to the more stable ketoamine (Amadori rearrangement)(8, 9). The protein-glucose adduct undergoes further reaction to form advanced glycation end products. In addition, active oxygen species may be formed during the oxidation of glucose; these species could also modify or fragment proteins(7, 10).

α-Lipoate (1,2-dithiolane-3-pentanoate) has recently gained considerable attention for its use in clinical applications. Both α-lipoate and dihydrolipoate (6,8-dimercaptooctanoate) function as antioxidants. The relationships between structure and antioxidant activity of dihydrolipoate and

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its homologues have also been reported(11-13). In addition to its antioxidant activity, Suzuki et al. reported that α-lipoic acid prevents glycation of bovine serum albumin (BSA)(14). Glycation represents both binding of glucose to the protein and modification of proteins as a result of the generation of active oxygen species during glucose oxidation(7-10). Since α-lipoate can work as an antioxidant, it is of interest to study its protective mechanism against protein glycation.

The present study investigated the mechanism of α-lipoate action against protein glycation in relation to α-lipoate's stereospecificity, thiol moiety and hydrophobicity. The results suggest that non-covalent hydrophobic binding to BSA is involved in the protective effects.

MATERIALS AND METHODS

Reagents: *RS*-, *R*- and *S*-α-Lipoate (1,2-dithiolane-3-pentanoate) and tetranorlipoate (1,2-dithiolane-3-carboxylate) were gifts from Asta Medica (Offenbach, Germany) (Fig. 1). Dihydrolipoate (6,8-dimercaptooctanoate, DHLA) was prepared by a previously described method(15). Bovine serum albumin (fraction V, essentially fatty acid free, BSA), ethylenediaminetetraacetate (EDTA, trisodium salt), KBr, and *N*-(2-hydroxyethyl)piperazine-*N'*-(2-methane sulfonic acid) (Hepes) were purchased from Sigma (St. Louis, MO). All other reagents used were of analytical grade.

Preparation of low density lipoprotein (LDL): Fresh plasma from healthy normal humans was obtained from the blood bank. After the addition of EDTA (3 mM), plasma density was adjusted to 1.24 by solid KBr layered under saline and LDL was isolated by a single ultracentrifugation by a vertical rotor (Beckman VTi50)(16). LDL was eluted from Sephadex G25 column equilibrated with 20 mM Hepes-150 mM NaCl (pH 7.4) and used for glycation experiment.

Induction and measurement of glycation: The incubation was carried out in a sterile condition at 37°C in a mixture of BSA (1 mM) or LDL (1.0 mg protein/ml) and glucose (500 mM in BSA and 200 mM in LDL) in 20 mM Hepes-saline buffer (pH 7.4) in the presence of the indicated concentration of α-lipoate homologues. An aliquot was applied to borate affinity column at the indicated time, and glycated and non-glycated protein were separated(17).

Protein binding: BSA (1 mM) was incubated with various concentrations of α-lipoate in 20 mM Hepes-saline buffer (pH 7.4) at 37°C for 18 h, and the sample was centrifuged in a centricon 10 (Amicon, MA). The absorbance of the filtrate was measured at 333 nm and the concentration was calculated by the molar extinction coefficient of 150 $M^{-1} \cdot cm^{-1}$(18).

Others. BSA concentration was calculated using molecular weight of 67 kD (19), and protein concentration of LDL was measured by Lowry method(20). Using Student's *t*-test, $p < 0.05$ were considered significant.

RESULTS

Fig. 1 shows *R*- and *S*-α-lipoate, and tetranorlipoate, the short carbon-chain homologue. In our reaction system, BSA glycation plateaued after 72 h incubation resulting in glycation of about 50% (data not shown). Therefore, the effects of α-lipoate on BSA glycation were studied at 72 h incubation. Irrespective of the stereochemistry of α-Lipoate, all forms (*RS*-, *R*- and *S*-α-lipoates inhibited the glycation equally (Fig. 2). Dihydrolipoate, the reduced form of α-lipoate, also inhibited the glycation of BSA to the same extent as α-lipoate, but tetranorlipoate did not.

Human low density lipoprotein (LDL) is a complex particle with lipids and apo B protein, which is incorporated in a lipid particle, unlike BSA. LDL glycation was followed at 37°C for 7

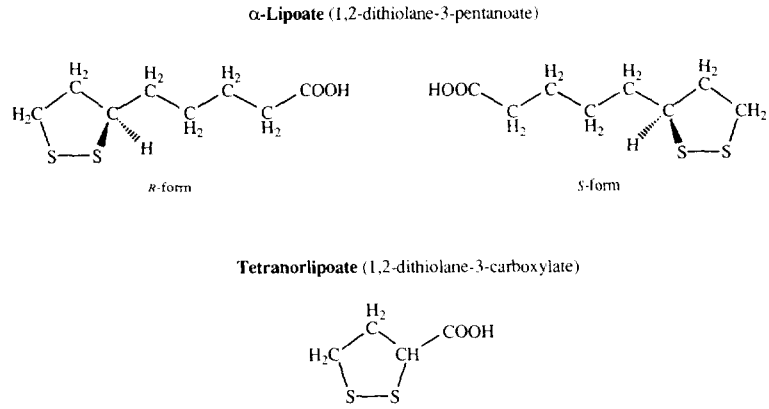

Fig. 1. Enantiomers (*R*- and *S*-forms) of α-lipoate (1,2-dithiolane-3-pentanoate) and tetranorlipoate (1,2-dithiolane-3-carboxylate).

days (Fig. 3). In the positive control (LDL + glucose), about 50 % of LDL was glycated during the week of incubation; α-lipoate did not inhibit LDL glycation.

BSA is well known to bind intrinsic and extrinsic compounds, especially free fatty acid, we hypothesize that α-lipoate could bind to BSA near glycation sites and protect BSA from the glycation. Therefore, the binding of α-lipoate to BSA was analyzed by Scatchard plot (Fig. 4). The high affinity binding was observed with a correlation coefficient of 0.946 (n=29). Six mol of α-lipoate can bind to one mol of BSA with a binding constant of 8.7×10^4 M^{-1}. By contrast, tetranorlipoate did not bind to BSA.

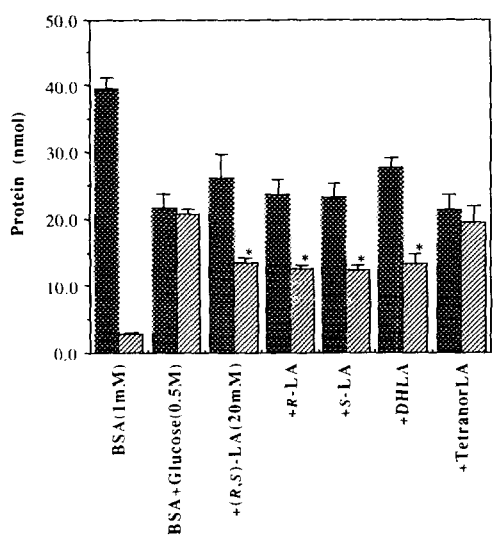

Fig. 2. Effect of α-lipoate, DHLA and tetranorlipoate on BSA glycation.
BSA (1 mM) was incubated in Hepes-saline (pH 7.4) with 500 mM glucose in the presence of α-lipoate (LA), DHLA or tetranorlipoate (tetranorLA) (20 mM) at 37°C for 72 h. Glycated and non-glycated proteins were separated by affinity chromatography and the protein concentrations measured. Closed columns are non-glycated proteins and striped columns are glycated proteins. Values are mean ± SD (n=4). *Significantly different from BSA + glucose.

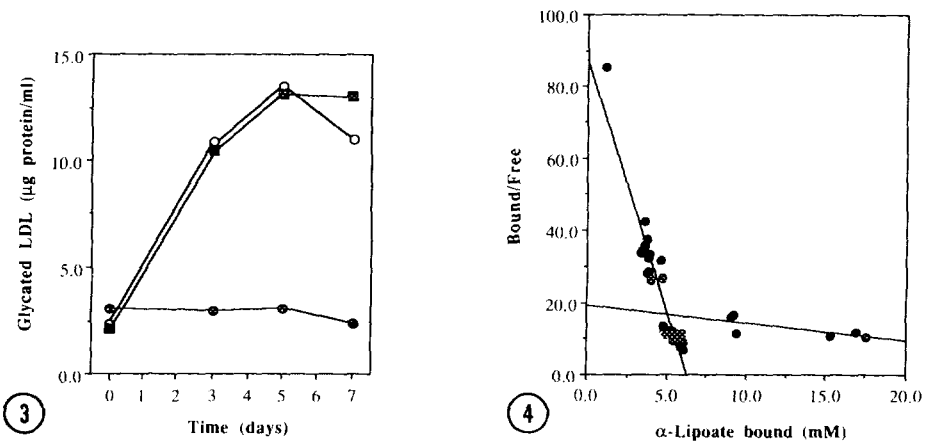

Fig. 3. Effect of α-lipoate on LDL glycation. LDL (1 mg protein/ml) was incubated with 200 mM glucose in the presence of α-lipoate (20 mM) in Hepes-saline (pH 7.4) at 37°C. At the indicated times, glycated LDL were separated by affinity chromatography and protein concentrations measured. Values are averages of duplicate observations.

Fig. 4. The binding of α-lipoate to BSA. BSA (1 mM) was incubated with various concentrations of α-lipoate in Hepes-saline (pH 7.4) at 37°C for 18 h. The sample was centrifuged in a centricon 30 and the α-lipoate concentration in the filtrate was measured by absorption spectroscopy (ε = 150 $M^{-1} \cdot cm^{-1}$).

DISCUSSION

α-Lipoate has a chiral center and shows enantiomerism with *R*- and *S*-forms (Fig. 1). Stereospecificity is often critical in binding as enzyme-substrate complexes are formed. However, α-lipoate did not show any difference in the protection of protein glycation between *R*- and *S*-forms (Fig. 2), suggesting that specific binding does not occur. α-Lipoate has an intramolecular disulfide bond, which participates in oxidation-reduction reactions. Both α-lipoate and its reduced form, DHLA, protected BSA from glycation in the same manner (Fig. 2), suggesting that the preventive effect of α-lipoate against BSA glycation is independent of its redox state. Albumin binds endogenous and exogenous compounds and functions *in vitro* to transport the compounds(21, 22). Since α-lipoate has a hydrophobic carbon chain, it is likely that α-lipoate binds to BSA by hydrophobic interactions, similar to fatty acids. To elucidate the role of the hydrophobic carbon chain, tetranorlipoate was compared with α-lipoate in its protective effect against glycation. Tetranorlipoate did not show a protective effect (Fig. 2), demonstrating the importance of the hydrophobic interaction between BSA and α-lipoate.

LDL is a large particle of more than 1.5 million kD with a cholesterol: phospholipid surface, a core composed primarily of cholesterol ester and a protein (apo B100), which is interdigitated in the surface(23). Protection of LDL against glycation by α-lipoate was also investigated. If α-lipoate inhibits LDL glycation, this could have clinical significance because diabetic patients have a higher frequency of atherosclerosis than non-diabetic individuals(24). Physically, apo B100 has both hydrophilic and hydrophobic domains, the hydrophobic portion of apo B in LDL is submerged in the lipid structure as this differs from BSA. α-Lipoate did not

protect LDL from glycation *in vitro*. The data also emphasize the significance of the hydrophobicity of α-lipoate.

The binding of α-lipoate to BSA was investigated by Scatchard plots, which indicated that six mol of α-lipoate bind to one mole of BSA with a formation constant of 8.7×10^4 M^{-1}. The formation constant is similar to that of octanoate (8.3×10^4 M^{-1})(21), which has the same carbon chain length as α-lipoate. Schepkin et al. also reported that α-lipoate binds to BSA, but not LDL by NMR(25). Glycated human serum albumin binds fewer fatty acid than does non-glycated albumin(26), suggesting that glycation sites are adjacent to fatty acid binding sites. Although covalent interaction with serum albumin has been reported to inhibit the protein glycation, non-covalent interaction of Diclofenac (Voltaren) with human serum albumin was also reported to inhibit glucose attachment to human serum albumin(27). In an early step of BSA glycation, α-lipoate may protect BSA from glycation by masking the glycation site through hydrophobic binding.

Although it has been reported that α-lipoate is effective on diabetes-induced complications in diabetic animals(28) and human(29), the exact mechanisms of the protection have not been understood. The hydrophobic interaction between protein and α-lipoate may emerge as a new mode of pharmacological action of α-lipoate.

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