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Isomeric effects of anti-diabetic α-lipoic acid with γ-cyclodextrin

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ABSTRACT

Aims: Previous studies reported the anti-diabetic effects of α-lipoic acid (αLA) isomers: racemic-αLA, R-αLA, or S-αLA. Previously, we examined the anti-diabetic effects of αLA administered as a food additive, but were unable to demonstrate the differences among different isomers. In this study, αLAs were complexed with γ-cyclodextrin (γCD) for the stability. We then investigated the anti-diabetic effects of racemic-, R-, and S-αLA/γCDs in KKA^{y} mice.
Main methods: Male type 2 diabetic KKA^{y} mice were divided into 5 groups, and fed either a high-fat-diet (HFD), HFD supplemented with γCD, or HFD supplemented with racemic-αLA/γCD, R-αLA/γCD, or S-αLA/γCD for 4 weeks. At the end of the feeding period, HbA1c and adiponectin levels were measured, PPARγ2 mRNA expression levels were assessed in adipose tissues using real-time PCR, and AMP-activated protein kinase (AMPK) phosphorylation levels were evaluated in the liver by Western blotting.
Key findings: The anti-diabetic effects of αLA; the isomeric compounds racemic-, R-, and S-αLA/γCD were investigated using a male type 2 diabetic KKA^{y} mouse model. Significant differences were observed in HbA1c and plasma adiponectin levels between R-αLA/γCD-treated mice and control mice. PPARγ2 mRNA expression levels were slightly higher in racemic- and R-αLA/γCD-treated mice. Moreover, AMPK phosphorylation levels were elevated in racemic-αLA/γCD- and R-αLA/γCD-treated mice, but remained unchanged in S-αLA/γCD-treated mice.
Significance: These results suggested that the stereoisomerism mediates a difference in the anti-diabetic effects of racemic-, R-, and S-αLA/γCDs. Furthermore, the anti-diabetic mechanism of αLA/γCD action may be attributed to the activation of AMPK in the liver.

1. Introduction

Diabetes mellitus (DM) is a common metabolic disorder that is characterized by a relative or absolute lack of insulin. Type 2 DM is more common, accounting for approximately 95% of all DM cases [27]. The incidence of type 2 DM is increasing worldwide [29]; therefore, therapeutic approaches based on novel concepts are urgently required. Our research is focused on α-lipoic acid (αLA), which has been classified as a food additive. In Germany, αLA has been extensively used in medical practice since 1959 and is considered safe and efficient in the treatment of diabetic polyneuropathy symptoms [34–36]. In the SYDNEY trial, intravenous αLA administration rapidly and significantly improved neuropathic sensory symptoms. In the SYDNEY 2 trial, an oral treatment with αLA improved neuropathic symptoms [1,33]. Although αLA is a potent antioxidant and has been widely used as an anti-aging compound in the supplemental foods, it becomes unstable when exposed to low pH, light, or heat [16]. Takahashi et al. demonstrated that racemic-αLA could be stabilized through the complex formation with γ-cyclodextrin (γCD) [23]. We have also recently reported that γCD stabilized R-αLA and yielded an R-αLA/γCD complex [7].

Although the physicochemical properties of the αLA/γCD complex have been examined, the biological activity of αLA/γCD is not clear, and it currently remains unknown whether αLA/γCD has the ability to regulate gene expression in vivo. Therefore, we previously compared the effects of αLA/γCD on DM-related biochemical parameters with those of intact αLA using the type 2 DM model, the KKA^{y} mouse. Our findings showed that the anti-diabetic effects of αLA/γCD were more potent than those without γCD [18], suggesting that γCD may enhance αLA stability in the living body through the complex formation.

αLA has a chiral center at its C_6 carbon, leading to two enantiomers, R- and S-αLA, of which R-αLA is a naturally occurring form [21]. R-αLA is a co-factor for mitochondrial enzymes and plays a central role in energy metabolism. Maitra et al. previously demonstrated that R-αLA decreased the formation of cataracts induced by buthionine sulfoximine in newborn rats, whereas S-αLA did not [15]. Nikolai et al. recently

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investigated the effects of these two enantiomers on energy expenditure in 4-month-old HFD-fed mice [19]. They showed that only the R-αLA/γCD complex significantly increased energy expenditure in mice, whereas the S-αLA/γCD complex had no effect. These findings prompted us to speculate that differences in the isomeric structure of αLA may affect its anti-diabetic effects. Therefore, we prepared αLA/γCD complexes, in which αLA maintained its isomeric structure, and compared the anti-diabetic effects of R- and S-αLA using KKA^y mice.

2. Materials and Methods

2.1. Reagents

R(+)-α-lipoic acid sodium salt (NaRALA) was purchased from Toyo Hakko Co., Ltd. (Japan). S(−)-α-lipoic acid sodium salt (NaSALA) was provided by Changshu Fushilai Medicine & Chemical Co., Ltd. (China). DL-α-Lipoic acid was purchased from Tokyo Chemical Industry Co., Ltd. (Japan). CAVAMAX® W8 FOOD (γCD) was purchased from Wacker Chemie AG (Germany). All reagents used were purchased from Wako Pure Chemical Ind., Ltd. (Japan). An RNeasy Lipid Tissue Mini Kit was purchased from QIAGEN Inc. (Maryland, USA). The 4%–15% Mini-PROTEAN® TGX™ precast gels were purchased from BIO-RAD (California, USA). Specific antibodies against phospho-AMPKα (Thr172) (40H9) and AMPKα (23A3) were purchased from Cell Signaling Technologies (Beverly, USA). The antibody against GAPDH (6c5) was obtained from Santa Cruz (California, USA). Immobilon™ Western Chemiluminescent HRP substrate was purchased from Millipore (Massachusetts, USA).

2.2. Preparation of racemic-, R-, and S-αLA/γCD complexes

The preparation of αLA/γCD complexes was done as previously described [7]. R-, S-, or racemic-αLA was dissolved in deionized water, in which the pH was monitored, and a corresponding molar amount of γCD was added for a 1:1 ratio. The solution was mixed with a mechanical stirrer at 300 rpm for 10 min before adding 1 M HCl for pH adjustments. The suspension was then continuously stirred in the dark for 18 h. All procedures were performed at room temperature, and the suspension temperature did not exceed 25 °C. The freshly prepared suspension was frozen overnight and freeze-dried the next day.

2.3. Component of diets containing isomeric αLAs

All animals had free access to water and semi-synthetic high-fat diets (HFDs) that were high in sugar, and, thus, were hypercaloric [composition of the basal diet (%): sucrose, 33.0, lard fat, 20.0, casein, 20.0; Kobe Women's University Special Diets, Japan]. The diets were prepared for all groups using AIN-93N, which was provided with a mixture of the general diet (Oriental East Co., Ltd., Japan), as shown in supplemental information 1 (a) and (b).

Animals were divided into 5 groups: fed the basal HFD, the basal HFD with added γCD, or the basal HFD supplemented with racemic-, R-, or S-αLA derived from a γCD complex (racemic-αLA/γCD, R-αLA/γCD, and S-αLA/γCD, respectively). Before beginning the study, the stability of αLA contained in the test diets was confirmed by CycloChem Bio. Co., Ltd.

2.4. Animal experiments

Male type 2 diabetic KKA^y mice (4 weeks old and weighing 22–25 g) were purchased from CLEA Japan Inc. (Kyoto, Japan). We used these mice in in vivo experiments when they were 8 weeks old. All animal experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (KPU) and were performed according to the Guidelines for Animal Experimentation of KPU.

2.5. Administration of isomeric αLAs to KKA^y mice

KKA^y mice were kept in the laboratory for 4 weeks. Eight-week-old KKA^y mice with hyperinsulinemia type 2 DM were allowed free access to the diets and tap water. They were housed in an air-conditioned temperature controlled (23 °C ± 2 °C) and humidity controlled (60% ± 10%) room, with lights on from 8:00 to 20:00. Blood samples for the analysis of glucose levels were obtained from the tail vein of each mouse and measured using a Glucocard (Arkray, Kyoto, Japan) every Monday, Wednesday, and Friday. The body weights of KKA^y mice that were allowed free access to the diets and water content were measured after glucose measurements. HbA1c levels in the blood obtained from the tail vein of the mice after the experiments were determined using a DCA 2000 system (Bayer-Sankyo Co., Ltd., Tokyo, Japan). After the experiments, the mice were subjected to a 12-h fast. We then collected blood samples, livers, and adipose tissue. Blood samples for the analysis of triglyceride (TG), and total cholesterol (TCHO) concentrations were obtained from the abdominal inferior vena cava under anesthesia with light ether. Plasma was obtained by centrifuging the whole blood for 10 min at 650 *g*. The plasma concentrations of TG, and TCHO were measured using a Fuji Dry-Chem System (Fuji Medical Co., Tokyo, Japan). The HbA1c values were measured by an immunoassay method (DCA 2000 system, Bayer-medical Co., Ltd., Tokyo, Japan). Adiponectin plasma levels were determined using the adiponectin immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA).

2.6. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was obtained from mouse adipose tissue using a QIAzol (QIAGEN) and RNeasy Lipid Tissue Mini Kit. RNA concentration was determined by NanoDrop. Samples were further purified using a DNase I Amplification Grade kit (Invitrogen, California, USA). Isolated RNA (0.5 μg) was reverse transcribed to produce first strand cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, USA). Real-time PCR was performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystem, Tokyo, Japan) and SYBR-Green. The primers used for target gene detection and expression quantification are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. Relative mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

2.7. Immunoblotting analysis

Isolated liver tissues (150 mg) from mice were homogenized in 1.5 mL of buffer I (20 mM Tris–HCl, 150 mM NaCl, 1 mM Na_3VO_4, 5 mM NaF, 1 mM PMSF, and 5 μg/mL leupeptin; pH 7.4). The homogenate was centrifuged at 14,500 *g* for 20 min at 4 °C, the supernatant was collected, and the protein concentration was determined using the BCA protein assay kit (Thermo Scientific, Illinois, USA) with BSA as a standard. The lysates (10 μg of total protein) were loaded per lane and electrophoresed on a 4%–15% Mini-PROTEAN® TGX™ precast gel. The resolved proteins were transferred onto a PVDF membrane. The membrane was blocked with 5% BSA in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS/T). The blocked membrane was incubated overnight at 4 °C with primary antibodies in 5% BSA–TBS/T. The following antibodies were used: phospho-AMPKα (Thr172) (40H9), AMPKα (23A3), and GAPDH (6c5) at 1:1000 dilutions. The membrane was then incubated with a HRP-conjugated secondary antibody at room temperature for 1 h and visualized using an Immobilon™ Western Chemiluminescent HRP substrate and by exposure to Amersham Hyperfilm™ ECL (GE Healthcare UK Ltd., Buckinghamshire, UK). For the detection of total AMPKα, the membrane was stripped using Stripping Solution (Wako, Osaka, Japan) and reprobed with AMPKα (23A3) antibody