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Detection of Orally Administered Inositol Stereoisomers in Mouse Blood Plasma and Their Effects on Translocation of Glucose Transporter 4 in Skeletal Muscle Cells

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ABSTRACT: Simple pharmacological studies on inositol stereoisomers are presented in this study. Male ICR mice were orally administered 1 g/kg BW of three inositol stereoisomers, *myo*-inositol (MI), D-*chiro*-inositol (DCI), and *scyllo*-inositol (SI), and blood plasma samples and skeletal muscle fractions were prepared after an hour. The plasma samples were subjected to gas chromatography−coupled time-of-flight mass spectrometry (GC-TOF-MS) analysis. None of the three stereoisomers was seen in untreated samples, but substantial amounts ranging from 2.5 to 6.5 mM were detected only after administration, indicating that orally administered inositol stereoisomers were readily absorbed and their levels elevated in the bloodstream. In addition, plasma of SI-administered animals contained substantial MI, suggesting a possible metabolic conversion of SI to MI. In the skeletal muscle fractions, glucose transporter type 4 (GLUT4) content in the plasma membrane increased, indicating that inositol stereoisomers stimulated GLUT4 translocation.

KEYWORDS: *myo-inositol, D-chiro-inositol, scyllo-inositol, GLUT4, GC-TOF-MS*

INTRODUCTION

Glucose transporter type 4 (GLUT4) is one of the glucose transporters generally found in skeletal and cardiac muscles and adipose tissues, which are the major insulin-responsive tissues in the body.[1] Whereas blood glucose levels are basal, insulin concentrations are kept at low levels and GLUT4 is sequestered in intracellular vesicles. Once blood glucose levels rise, insulin concentrations are elevated, stimulating the GLUT4-containing vesicles to translocate to the plasma membrane.[2] Muscle contraction and exercise also stimulate GLUT4 translocation. As the vesicles fuse with the plasma membrane, GLUT4 exerts its function of absorbing glucose into the cell. Within the cell, glucose is rapidly consumed via glucokinase-catalyzed phosphorylation to glucose-6-phosphate, which then enters glycolysis or is polymerized into glycogen. In this manner, glucose absorption into the cells lowers blood glucose levels.[3]

Inositol or 1,2,3,4,5,6-cyclohexanehexol has nine possible stereoisomers, epimerizing the six hydroxyl groups. The U.S. FDA lists inositol as generally recognized as safe (GRAS) for human consumption under 21 CFR 184.1370. *myo*-Inositol (MI) is known as the most abundant natural stereoisomer and is the structural basis of several secondary messengers in eukaryotic cells.[4] The other inositol stereoisomers are relatively rare in nature, but some have specific and useful health-promoting activities. For instance, D-*chiro*-inositol (DCI) has been shown to be beneficial for patients with hyperglycemia by restoring insulin sensitivity[5] and with polycystic ovary syndrome by restoring normal ovulation.[6] Accumulating evidence indicates that another isomer, *scyllo*-inositol (SI), has exceptional potential as a therapeutic agent for Alzheimer's disease.[7]

Our previous studies demonstrated that glucose uptake in rat L6 myotubes, involving translocation of GLUT4 to the plasma membrane, was stimulated by some inositol stereoisomers and derivatives including MI, SI, and DCI.[8] We also reported that orally administered MI and D-pinitol, 3-*O*-methyl-DCI, increased GLUT4 translocation in skeletal muscle cells, resulting in lowered levels of not only glucose but also insulin in blood.[9] These observations suggest that some inositol stereoisomers are able to perform an insulin-like function in vivo, which is possibly promising for diabetes, hyperglycemia, and other related diseases. However, there are no reports describing the in vivo effects of orally administered inositol stereoisomers. We focused on DCI and SI in pharmacological comparisons with MI, given their potential use as orally administered therapeutic agents. The present study quantified the oral absorption of the inositol stereoisomers into the bloodstream and evaluated their efficiency in stimulating GLUT4 translocation in skeletal muscles in ICR mice.

MATERIALS AND METHODS

Chemicals. The three inositol stereoisomers, MI, SI, and DCI, used in this study are products of Wako Pure Chemical Industries (Osaka, Japan). Their structures are shown in Figure 1. Anti-GLUT4 goat IgG, anti-insulin receptor β-subunit (IRβ) rabbit IgG, anti-goat IgG, and anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Blocking-One was obtained from Nacalai Tesque (Kyoto, Japan), and Can Get Signal immunoreaction enhancer solution was purchased from Toyobo (Osaka, Japan). The polyvinylidene difluoride (PVDF) membrane and ECL Plus used for immunoblotting were purchased from Pall Gelman Laboratory (Tokyo, Japan) and GE Healthcare Bio-Science (Piscataway, NJ, USA), respectively.

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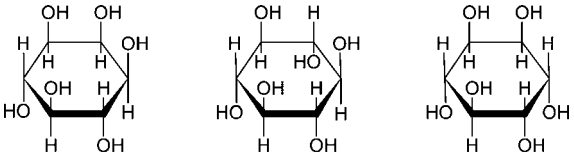

Figure 1. Structures of MI, SI, and DCI.

Animal Treatments. Animal experiments were conducted according to the *Guidelines for the Care and Use of Experimental Animals* at Kobe University Rokkodai Campus (Permission 21-07-02). Male ICR mice (7 weeks old, 22−25 g) were obtained from Japan SLC (Shizuoka, Japan) and acclimatized for 1 week in an air-conditioned room with a controlled environment of 25 ± 2 °C under a 12 h light/dark cycle with free access to an AIN-93 M purified diet and tap water.

The acclimatized mice (8 weeks old, 30−35 g) were randomly assigned to groups of six animals each: MI, SI, DCI, and control (C). After they were starved for 16 h, mice were orally administered a dose of 1 g/kg BW of respective inositol stereoisomers in water (for SI, two additional dosages of 0.5 and 2 g/kg BW were employed). The dosages were determined from the results of preliminary experiments (data not shown) and a previous study.[9] The mice assigned to group C were given water as a vehicle control (1 mL/kg BW). One hour after the administration, the mice were sacrificed under anesthesia using sodium pentobarbital. Blood was collected by cardiac puncture from each mouse into a heparinized microcentrifuge tube, and plasma samples were collected as supernatants following centrifugation at 1000*g* for 15 min at 4 °C. The plasma samples were stored at −20 °C until analysis. The soleus muscle was removed from the hind legs of the mice for use in immunoblot analysis as described below.[10]

Detection of Inositol Stereoisomers in Blood Plasma. The plasma samples were subjected to gas chromatography−coupled time-of-flight mass spectrometry (GC-TOF-MS) as previously described.[11−13] An aliquot (15 μL) of the plasma samples was mixed with 7.5 μL of 100 mM adipic acid as the internal standard for GC-TOF-MS (it is known that no adipic acid is found in plasma). The mixture was evaporated completely in a test tube under vacuum, and the dried pellet was dissolved in an extraction mixture of water, chloroform, and methanol (2:2:5) and incubated at 15 °C for 30 min with vigorous shaking. After centrifugation at 40 °C for 3 min at 16000*g*, the supernatant was transferred to another tube and diluted appropriately with pure water. After another centrifugation at 4 °C, part of the supernatant was dried completely in another tube. The dried pellet was dissolved with 50 μL of 20 mg/mL methoxyamine hydrochloride in pyridine to perform derivatization at 30 °C for 90 min with vigorous shaking. After the addition of 0.1 mL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (GL Science, Tokyo, Japan), the derivatized substances were further incubated at 37 °C for 30 min, and aliquots (1 μL) were injected via an Agilent 7683B autosampler into an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a 30 m × 0.25 mm i.d. fused silica capillary column coated with 0.25 μm CP-Sil 8 CB low bleed (Varian Inc., Palo Alto, CA, USA) and coupled to a Pegasus HT time-of-flight mass spectrometer (TOFMS) (Leco Corp., St. Joseph, MI, USA). The injector temperature was 230 °C, and the helium gas flow rate through the column was 1 mL/min. The column temperature was held at 80 °C for 2 min, then raised by 15 °C/min to 330 °C, and held there for 10 min. The transfer line and the ion source temperature were set at 250 and 200 °C, respectively. The ion source was generated by a 70 eV electron beam; 20 scans per second were recorded in the mass range of m/z 85−500. The acceleration voltage was turned on after a solvent delay of 200 s. Peak deconvolution identification and quantification were performed using the Pegasus ChromaTOF software package ver. 4.21 (Leco Corp.). Commercially available authentic inositol stereoisomers were derivatized and analyzed in parallel with the experimental samples. The mass spectra and retention time obtained were used to identify the stereoisomers. Of the various fragment ions produced from each of inositol stereoisomers (data not shown), the one at m/z 147 was the most prominent and specific and, thus, chosen as the unique ion for inositol, the intensities of which were used for the peak area calculation. The concentrations of stereoisomers were calculated on the basis of the ratio of inositol peak area to the internal standard peak area. As previously reported, our GC-TOF-MS system supported quantitative liner rage of MI from 0.1 to 100 μM,[13] and the measurement was performed within the range with accuracy.

Detection of GLUT4 in Skeletal Muscle Cells. Plasma membrane fraction and tissue lysate were prepared from skeletal muscle as described previously.[14] Skeletal muscle was chopped and homogenized in buffer A [10 mM Tris-HCl at pH 7.8, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), 5 μg/mL of aprotinin, and 10 μg/mL of leupeptin] containing 0.1% Nonidet P-40, passed through a 22 gauge needle three times, and then spun at 1000*g* for 10 min at 4 °C. The recovered pellet was suspended in buffer A and spun at 1000*g* for another 10 min at 4 °C. Once again, the pellet was suspended in buffer A containing 0.1% Nonidet P-40 and then centrifuged at 10000*g* for 20 min at 4 °C. The pellet was solubilized into buffer A containing 1% Nonidet P-40 and then centrifuged under the same conditions to obtain plasma membrane fraction. To obtain the tissue lysate, part of the homogenized skeletal muscle in buffer A was lysed with buffer B [10 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5 mM DTT, 1 mM PMSF, 5 μg/mL of aprotinin, and 10 μg/mL of leupeptin] and centrifuged at 16000*g* for 20 min at 4 °C

Aliquots of the plasma membrane fraction were used for detecting GLUT4 translocation and IRβ, and the tissue lysate was used for detecting GLUT4 expression.[15] After the proteins had been separated by 10% SDS−polyacrylamide gel electrophoresis, they were transferred to a PVDF membrane. The membrane was blocked for 1 h with Blocking One solution diluted with TBST (10 mM Tris-HCl at pH 8.0, 150 mM NaCl, and 0.05% Tween-20) and then incubated for 1 h at room temperature with the primary antibody for GLUT4 (anti-GLUT4, 1:5000) and IRβ as the internal control of the plasma membrane fraction (anti-IRβ, 1:1500). The PVDF membrane was further incubated at room temperature with an appropriate secondary antibody conjugated with horseradish peroxidase, and specific immune complexes were detected with ImmunoStar LD (Wako Pure Chemical Industries, Ltd.) and Light-Capture II (ATTO Corp., Tokyo, Japan).

Statistical Analysis. Data were subjected to one-way analysis of variance (ANOVA) followed by the Tukey−Kramer multiple-comparison test.

RESULTS AND DISCUSSION

Oral Absorption of Inositol Stereoisomers Elevated Their Levels in Bloodstream. We previously examined GLUT4 translocation after the mice were orally given D-pinitol at 2 g/kg BW.[9] As a result, D-pinitol significantly increased the GLUT4 translocation in the plasma membrane at 60 and 120 min compared with that at 0 min. On the other hand, it did not affect the translocation at 30 min. Thus, we selected the time point as 60 min after the administration of inositol derivatives in the present study. The blood plasma samples prepared from mice administered 1 g/kg BW of inositol stereoisomers were subjected to GC-TOF-MS analysis as described above, and representative chromatograms are shown in Figure 2. Our GC-TOF-MS system was originally designed to perform metabolome analysis, but was also found to distinguish inositol stereoisomers efficiently and precisely.[12,13] To the best of our knowledge, this is the first study in which the system has been applied to analyze plasma from animals. The DCI-, MI-, and SI-administered plasma samples gave specific ion peaks for the respective inositol stereoisomers (Figure 2M,S,D), whereas the vehicle control (Figure 2C) sample gave no significant peak. The SI-administered plasma gave an additional peak corre-

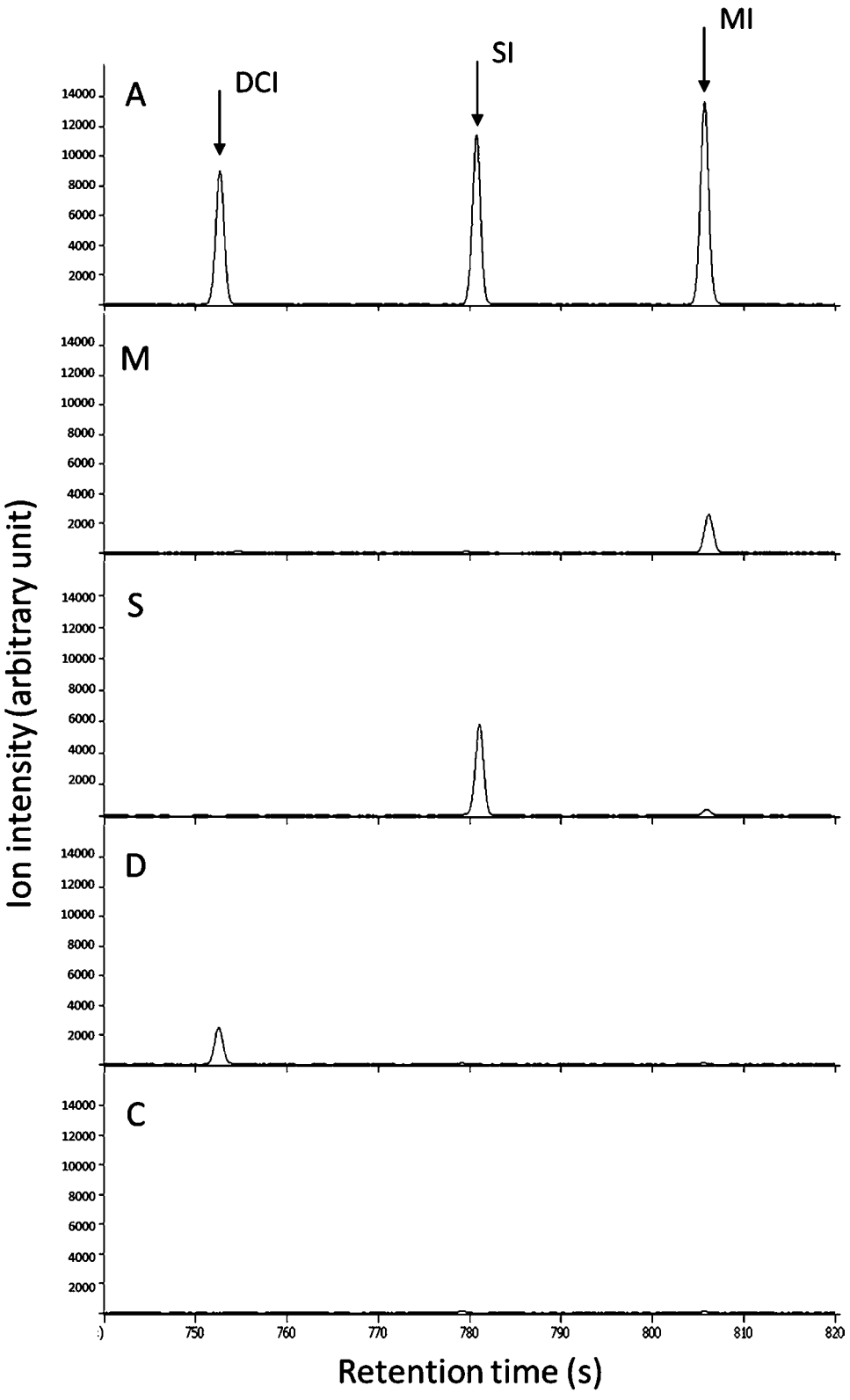

Figure 2. GC-TOF-MS analysis of blood plasma prepared from mice administered 1 g/kg BW of MI (M), SI (S), DCI (D), and vehicle control (C). Representative results are shown from each of the animal groups with standard substances (A).

sponding to MI (Figure 2S). On the basis of the intensities of the specific ion peaks, the plasma concentrations of the respective inositol stereoisomers were calculated (Table 1). The inositol stereoisomers in individual plasma were found at concentrations ranging from 2.5 to 6.5 mM. Levels of MI in plasma appeared lower than those of SI and DCI, so a statistical analysis was performed and revealed that levels of only SI were significantly higher than those of the others (Table 1). These results suggested that MI and DCI might be absorbed less efficiently or might be incorporated and/or excreted, thus

Table 1. Blood Plasma Levels of MI, SI, and DCI in Mice Administered Each of the Inositol Stereoisomers

animal group	MI concn[a] (mM)	SI concn[a] (mM)	DCI concn[a] (mM)
MI	2.67 ± 0.72 a	ND[b]	ND
SI	0.94 ± 0.06 b	6.44 ± 1.28 c	ND
DCI	ND	ND	4.00 ± 0.94 a
C	ND	ND	ND

[a]Mean ± SD, n = 6. Values followed by the same letter are not significantly different at the 95% confidence level. [b]ND, not detected (<0.1 mM).

disappearing more quickly from the bloodstream than SI. Although we cannot distinguish these possibilities at present, the results clearly show that each of the three inositol stereoisomers was readily absorbed, leading to elevated blood levels that were maintained for 1 h.

The SI-administered plasma also contained amounts of MI corresponding to nearly one-third of the amounts found in MI-administered plasma (Table 1). The results indicated the unexpected possibility that part of the SI might be converted to MI efficiently in vivo. When mice were administered two additional doses of 0.5 and 2 g/kg BW of SI, plasma SI levels rose significantly with increasing doses, whereas MI levels appeared to remain constant (Table 2). These results suggested

Table 2. Blood Plasma Levels of MI and SI in Mice Administered Various SI Doses

SI dose (g/kg BW)	MI concn[a] (mM)	SI concn[a] (mM)
0.5	0.94 ± 0.06 a	2.89 ± 0.78 b
1.0	0.94 ± 0.06 a	6.44 ± 1.28 c
2.0	0.94 ± 0.11 a	14.44 ± 2.83 d

[a]Mean value ± SD, n = 6. Values followed by the same letter are not significantly different at 95% confidence level.

that more SI could be absorbed orally with increasing doses, whereas the capacity for in vivo conversion of SI to MI might be limited and/or controlled by unknown mechanisms.

Effects of Three Inositol Stereoisomers on GLUT4 Translocation. A plasma membrane fraction and whole tissue lysate were prepared from the skeletal muscle of mice administered 1 g/kg BW of inositol stereoisomers and subjected to immunoblot analysis (Figure 3). The three animal groups that received orally administered inositol stereoisomers tended to show increased GLUT4 contents in plasma membrane compared with the vehicle control, whereas neither expression levels of GLUT4 in tissue lysate nor IRβ contents in plasma membrane were affected (Figure 3A). The results agreed with the previously reported stimulation of GLUT4 translocation by orally administered MI[9] and also indicated the new finding that both DCI and SI were able to induce GLUT4 translocation to the plasma membrane in vivo immediately after single-dose administration (Figure 3B). Indeed, DCI is known to be beneficial for treatment of hyperglycemia,[5] possibly owing to its ability to induce GLUT4 translocation in skeletal muscle, enabling rapid glucose disposal. Previously, SI was shown to induce GLUT4 translocation in tissue culture of L6 myotubes.[8] The present study revealed that single-dose administration of SI elevated its plasma levels enough to achieve GLUT4 translocation most efficiently among the three inositol isomers (Figure 3B). This result suggests that SI would potentially be effective for treatment not only of Alzheimer's disease[7] but also

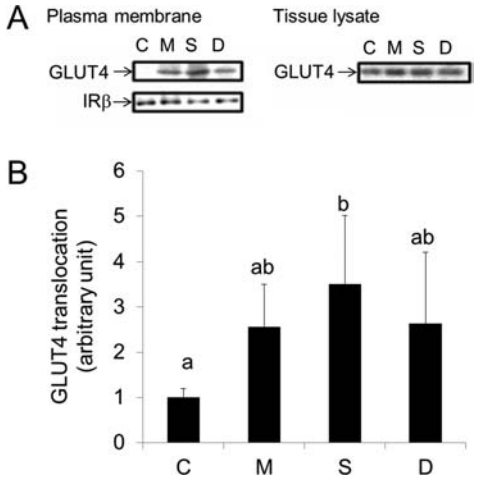

Figure 3. Effects of inositol stereoisomers on GLUT4 translocation in skeletal muscle of mice. (A) GLUT4 and IRβ in the plasma membrane fraction and GLUT4 in the tissue lysate. The protein levels were detected by immunoblot analysis as described in the text. Representative results are shown from each of the animal groups administered 1 g/kg BW of inositol stereoisomers MI (M), SI (S), DCI (D), and vehicle control (C). (B) GLUT4 translocation estimated by measuring the density of the GLUT4 band in the plasma membrane fraction from each mouse. Values (mean ± SD, n = 6), presented with the same letters are not significantly different at the 95% confidence level.

of hyperglycemia and its related diseases. It is known that the expression of GLUT4 on the plasma membrane depends on translocation of GLUT4-containing vesicles, involving a cascade of successive activation of protein kinases.[1−3] Further in-depth studies are required to elucidate how those inositol stereoisomers are involved in translocation of GLUT4-containing vesicles.

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Notes

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ABBREVIATIONS USED

BW, body weight; DCI, D-*chiro*-inositol; DTT, dithiothreitol; FDA, U.S. Food and Drug Administration; GC-TOF-MS, gas chromatography−coupled time-of-flight mass spectrometry; GLUT4, glucose transporter type 4; GRAS, generally recognized as safe; IRβ, anti-insulin receptor β-subunit; MI, *myo*-inositol; PMSF, phenylmethanesulfonyl fluoride; PVDF,

polyvinylidene difluoride; SD, standard deviation; SDS, sodium dodecyl sulfate; SI, *scyllo*-inositol

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